

Application of Metabolomics in Inflammatory Demyelinating Diseases of the Central Nervous System

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Abstract

The prototypical central nervous system (CNS) inflammatory demyelinating disease (IDD) is multiple sclerosis (MS), which is a complex, immune-mediated disease involving genetic and environmental factors, both contributing and interacting, to produce the underlying immunopathogenesis. The inherent pathogenic heterogeneity within MS makes it challenging for a single candidate biofluid marker to reflect all the underlying disease processes, both neuroinflammatory and neurodegenerative, at the same time. Thus, the combination of biofluid markers into a composite biomarker, i.e. a biomarker signature, is a feasible approach to provide a summative representation of various disease processes.

Metabolomics is well-placed for this type of summative approach as a single metabolomics experiment provides hundreds (if not thousands) of metabolites at one go, i.e. a metabolic snapshot, which represents the downstream products of all neuroinflammatory and degenerative processes. Against this background, this thesis explores whether the combination of ^1H nuclear magnetic resonance (NMR) spectroscopy-based metabolomics with multivariate dimensionality reduction modelling can be used to construct discriminatory models to inform on the diagnosis, disease activity and progression of CNS IDD, in particular MS, with inference to the underlying pathophysiological processes.

The results in this thesis demonstrated that it is possible to use blood metabolomics to provide molecular validation to phenotypic clusters, derived from pattern recognition modelling, within antibody-negative CNS IDD patients, and in so doing, identified individuals who likely have MS and those with antibody-mediated CNS pathology. In a focal rat model of MS, both the blood and CSF metabolome were significantly perturbed during acute neuroinflammation and these changes evolved over time. As the model does not produce overt clinical signs, these metabolic signatures, and indeed individual metabolites, provide pathogenic insights that are solely attributable to MS-like

lesion formation. Drawing from these observations, the same methodology was applied to MS patients who are in relapse. Using serum metabolomics, it is possible to differentiate patients in relapse from patients who have no evidence of clinical inflammatory activity. Similar to that observed in rodents, the metabolic differences decreased with time away from relapse and specific metabolite biomarkers could be identified. These were then found to be applicable in an individualised manner, differentiating between relapse and remission status. The results reported in this thesis also illustrate that serum metabolomics was able to distinguish between relapsing-remitting MS (RRMS) from its secondary progressive (SPMS) phase, validating the previous work done by the Oxford group. In addition to the patient-based studies, this thesis also describes studies performed to validate the methodologies used in relation to sample-handling variations that are commonly encountered in the clinic. It was found that the accuracy of this RRMS vs. SPMS metabolomics test was maintained despite these variations, supporting its clinical applicability.

In summary, these results show that metabolomics has substantial potential in precision medicine in CNS IDD by: (1) aiding in CNS IDD diagnosis, (2) providing early detection of active neuroinflammation, and (3) allowing for the objective definition of SPMS.

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List of abbreviations

^1H	Hydrogen-1
1D	One-dimensional
2D	Two-dimensional
ABC	Avidin-biotin complex
Ab	Antibody
ABCG1	ATP-binding cassette transporter G1
Ab-NMOSD	Antibody-mediated NMOSD
ADMA	Asymmetric dimethylarginine
ANLS	Astrocyte-neuron lactate shuttle
ANOVA	Analysis of variance
APC	Antigen presenting cells
AQP4	Aquaporin-4
AU	Arbitrary units
AUC	Area under the curve
BBB	Blood brain barrier
BCAA	Branched chain amino acids
BCG	Bacillus Calmette-Guerin
BMI	Body mass index
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CDMS	Clinically definite multiple sclerosis
CDP	Cytidine diphosphate
CFA	Complete Freud's adjuvant
CHI3L1	Chitinase 3-like 1
CI	Confidence interval

CIS	Clinically isolated syndrome
CNS	Central nervous system
COSY	Correlation spectroscopy
CPMG	Carr-Purcell-Meiboom-Gill
CSF	Cerebrospinal fluid
CTP	Cytidine triphosphate
CXCL	Chemokine (C-X-C motif) ligand
DAB	Diaminobenzidine
DIS	Dissemination in space
DIT	Dissemination in time
DMF	Dimethyl fumarate
DMT	Disease modifying therapies
DTH	Delayed-type hypersensitivity
EAAT	Excitatory amino acid transporters
EAE	Experimental autoimmune encephalomyelitis
ECL	Electrochemiluminescence immunoassay
EDSS	Expanded disability status scale
ELISA	Enzyme-linked immunosorbent assay
FDR	False discovery rate
GABA	γ -amino butyric acid
GAD	Gadolinium
GC-MS	Gas chromatography-mass spectrometry
GFAP	Glial fibrillary acidic protein
GLS	Glutaminase
GLUT	Glucose transporter
GPx	Glutathione peroxidase
GR	Glutathione reductase

GS	Glutamine synthetase
GSH	Glutathione
GSSH	Oxidised glutathione GSSH
HC	Healthy controls
HDL	High-density lipoprotein
HMDB	Human metabolome database
HRP	Horseradish peroxidase
IDD	Inflammatory demyelinating diseases (IDD)
IDL	Intermediate-density lipoprotein
IFN	Interferon
IL	Interleukin
IND	Inflammatory neurological diseases
iNOS	Inducible nitric oxide synthase
IPND	International Panel for NMO diagnosis
KA	Kynurenic acid
KEGG	Kyoto encyclopaedia of genes and genomes
LBL	Low brain lesion
LC-MS	Liquid chromatography-mass spectrometry
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LETM	Longitudinally extensive transverse myelitis
LOD	Limit of detection
LysoPC	Lysophosphatidylcholine
LysoPE	Lysophosphatidylethanolamine
MAGNIMS	Magnetic resonance Imaging in multiple sclerosis
MBP	Myelin basic protein
MCT	Monocarboxylate transporters

min	Minutes
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NAWM	Normal appearing white matter
NfL	Neurofilament-light
NMDA	N-methyl-D-aspartate
NMOSD	Neuromyelitis optica spectrum disorders
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
OCB	Oligoclonal bands
OPLS-DA	Orthogonal partial-least squares discriminant analysis
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCA	Principal component analysis
PE	Phosphatidylethanolamine
PLP	Proteolipid protein
ppm	Parts per million
PPMS	Primary progressive multiple sclerosis
PPP	Pentose phosphate pathway
QA	Quinolinic acid
QC	Quality control
RCT	Randomised controlled trial
RIS	Radiologically isolated syndrome

RNS	Reactive nitrogen species
ROC	Receiver operating curve
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis
s	seconds
SIP	Sphingosine-1-phosphate
SD	Standard deviation
SEM	Standard error of mean
SNAT	Sodium-coupled neutral amino acid transporter
SNC	Subjective neurological complaints
SPMS	Secondary progressive multiple sclerosis
TB	Tuberculosis
TCA	Tricarboxylic acid
TNF	Tumour necrosis factor
TOCSY	Total correlation spectroscopy
VIP	Variable importance in projection
VLCFA	Very long chain fatty acids
VLDL	Very-low-density lipoprotein
vs.	Versus

Chapter 1: Introduction

1.1 General introduction

Multiple sclerosis (MS) is the most common cause of non-traumatic neurological disability in young adults with evidence that its incidence is increasing (Koch-Henriksen et al., 2010). The diagnosis of MS has undergone tremendous change within the last two decades, firstly with the introduction of widespread magnetic resonance imaging (MRI) to identify individuals who have high likelihood of developing MS (Thompson et al., 2018), and secondly, the discovery of antibodies to aquaporin-4 (AQP4-Ab) and myelin oligodendrocyte glycoprotein (MOG-Ab) which allowed distinct clinical entities previously under the umbrella of MS to be identified (Lennon et al., 2005; Mader et al., 2011). Given the clinical and pathogenic heterogeneity of MS, it is highly likely that there are still distinct pathogenic subtypes (with varying clinical courses) within the disease neurologists call MS, and there is still a need to better characterise patients who are antibody-negative and yet have overlapping MS and neuromyelitis optica spectrum disorders (NMOSD) phenotypes.

The structural information offered by conventional MRI has unquestionable utility in MS diagnosis, however, there is still a huge need for molecular biofluid markers that can reflect the dynamic pathophysiological processes that occur in MS, both neuroinflammatory and neurodegenerative, the clinically observable correlates of which are disease activity and progression (Lassmann, 2018). Such biofluid markers could provide valuable information to improve MS diagnosis, reflect disease activity, monitor progression and also assess response to disease modifying therapies (DMT). The presence of cerebrospinal fluid (CSF) specific oligoclonal bands (OCB) has been shown to aid diagnosis and has prognostic value in that it predicts individuals with clinically isolated syndrome (CIS) who will go on to develop MS (Kuhle et al., 2015; Tintore et al., 2015). Recently, promising biomarkers like neurofilament-light (NfL) and chitinase 3-like 1 (CHI3L1) have been shown to perform the same role (Comabella et al., 2010; Mane-Martinez et al., 2016), with NfL being

associated with baseline disease activity, as well as future relapses and disability worsening (Disanto et al., 2017; Kuhle et al., 2019), although its utility in an individualised manner remains to be ascertained. As the treatment algorithm in MS shifts from merely preventing relapses to the reduction of disability worsening and progression (indeed, this is what matters most to patients), there is also an urgent need to identify biomarkers that can inform on progression, and, specifically the separation of secondary progressive MS (SPMS) from relapsing-remitting MS (RRMS).

The overall aim of this thesis is to identify specific metabolic signatures and metabolites that can be utilised as biomarkers to improve the diagnosis and prognostication of central nervous system (CNS) inflammatory demyelinating diseases (IDD), in particular MS, and to explore the metabolic signatures present in rodents, given that all other conditions are controlled, to provide pathological inference to the metabolites observed. Metabolomics experiments were conducted using ^1H nuclear magnetic resonance (NMR) spectroscopy and spectral data were analysed by dimension reduction multivariate statistical analyses. In human studies, blood metabolomics was performed which represents a non-invasive way and pragmatic way to allow for serial monitoring that is required in a chronic disease such as MS. Both CSF and blood metabolomics were performed in rodents to be able to explore, in a reductionist approach, how a focal MS-like brain lesion affects the metabolome in these biofluids.

1.1.1 Aims of this thesis

Against this background, this thesis investigated the following hypotheses:

1. That blood metabolomics can distinguish between patients with MS from those with antibody-positive NMOSD, and provide discriminatory metabolites, which in combination with clinico-MRI data can classify antibody-negative patients who have overlapping MS and NMOSD phenotypes.

2. That CSF and blood metabolomics can distinguish between rodents with focal delayed-type hypersensitivity (DTH) brain lesion from control animals at different experimental time points, and identify discriminatory metabolites that correlate with neuroinflammatory and neurodegenerative pathological changes.
3. That blood metabolomics can distinguish between patients in relapse from those away from a relapse, and give discriminatory metabolite biomarkers that can be applicable in an individualised manner.
4. That blood metabolomics can distinguish between the relapse-remitting phase of MS from the secondary progressive phase, and this distinction is resistant to sample-handling variations commonly encountered in the clinics.

1.2 The unmet need for diagnostic and prognostic biomarkers in CNS IDD

1.2.1 Multiple sclerosis – the prototypical inflammatory disease of the central nervous system

MS is the most common CNS IDD, with an estimated prevalence of 2.2 million people, with a higher prevalence seen in countries with higher socioeconomic status (GBD 2016 Multiple Sclerosis Collaborators, 2019). Females tend to be affected more (~75%), and the young age of onset (20 – 30 years old) as well as its progressive nature mean that the economic cost is relatively high compared to other chronic diseases (Adelman et al., 2013; Reich et al., 2018; Svendsen et al., 2018).

MS is also more prevalent in regions with temperate climates, providing indirect evidence that vitamin D (or a lack of) and seasonal infections may have a role to play in its pathogenesis (Levin et al., 2010; Orton et al., 2011; Reich et al., 2018), although recent clinical trials investigating the effect of vitamin D supplementation did not achieve their primary efficacy outcomes (Camu et al., 2019; Hupperts et al., 2019). Whilst genetic contribution is also present as evidenced by the robust association with HLA DRB1*1501 haplotype as well as the discovery of risk alleles associated with immune pathways, the genetic contribution (in a polygenic manner) accounts for only ~30% of the

overall disease risk (Dendrou et al., 2015). Indeed, monozygotic twins show 30 – 50% concordance while the risk for first degree relatives range from 2 – 4%, juxtaposed against a 0.1% risk in the general population (Reich et al., 2018). These observations suggest that it is unlikely that MS is a result of a single causative factor, rather, its pathogenesis is likely triggered by environmental factors in a genetically susceptible individual.

In the majority of patients (~80 – 85%), the disease follows a relapsing-remitting course initially (Compston et al., 2008; Confavreux et al., 2006). RRMS is characterised by transient clinical episodes of neurological worsening – termed relapses, which patients usually recover from although not always completely. Relapses are interspersed by periods of clinical stability (i.e. remission), in which there is little evidence of neurological worsening (at least not clinically observable by neurological examination). However, after about 20 years from disease onset, although the rate of relapses is reduced or has even come to a halt, approximately 50 – 60% of RRMS patients develop a gradual accumulation of disability particularly in the domains of ambulation, cognition, and sphincter control (Tremlett et al., 2008; Vukusic et al., 2003). This secondary progressive course, termed – SPMS, although gradual, continues unabated in the absence of relapses resulting in marked disability ranging from requiring ambulatory assistance to a total loss of self-care in advanced cases. In a minority of patients, progression occurs straight from the disease outset, usually without the presence of relapses – a disease course known as primary progressive MS (PPMS) (Lublin et al., 2014). Clinically and radiologically, PPMS and SPMS are comparable and have similar disease trajectories (Kuchling et al., 2014; Leray et al., 2010), albeit the onset of PPMS is a decade earlier (Tremlett et al., 2017). It still remains an open debate as to whether PPMS is the same disease as SPMS, but without the initial relapsing-remitting phase, as suggested by the presence of gene regions associated with neurodegeneration in a subset of PPMS patients (Jia et al., 2018). Pathologically, the fundamental features of diffuse inflammation in the white and grey matter are essentially the same in both PPMS and SPMS, however, quantitatively, there are conflicting reports

regarding the number of active white matter lesions, remyelinated plaques and tertiary lymphoid follicles (Bramow et al., 2010; Choi et al., 2012; Kutzelnigg et al., 2005; Luchetti et al., 2018).

1.2.2 Heterogeneity in the MS disease course

What have been described above are conclusions that have been made at group level observation. Clinically, it is clear that MS is a heterogeneous disease with significant inter-individual differences. Some patients develop numerous relapses while others have a few, although it is uncertain how relapses affect future long-term disability. In addition, within individuals, it is still challenging to predict future clinical inflammatory activity, response to a given DMT or the onset of secondary progression (as well as its trajectory), despite being given all possible clinical and ancillary information. This heterogeneity also extends to MRI features, with significant inter-individual differences observed in terms of lesion load, number of gadolinium (GAD)-enhancing lesions, lesion locations, and rates of brain atrophy, despite apparent similarities in age, gender and disease onset.

The root of this heterogeneity must lie in the pathogenic heterogeneity of MS and the resultant pathological differences observed between individuals. However, the current tools at a neurologist's disposal, namely, detailed clinical examination and conventional MRI, are unable to fully capture the entirety of pathogenic/pathological processes occurring within patients. Although clinical examination and MRI overlap considerably with regards to the pathological substrate that they measure (e.g. relapses and GAD-enhancing lesions reflecting acute lesion formation, and progression and brain atrophy likely representing ongoing axonal loss and demyelination), there are occasions when these two approaches disagree, i.e. the clinico-radiological paradox, precisely because each is detecting processes that the other cannot. These concepts are explained in the sections below.

1.2.2.1 Pathogenic and pathological heterogeneity

MS white matter lesions are characterised by an inflammatory (macrophage/microglial) and immune (CD8 positive T cells) cellular infiltrate, perivascular cuffing with immune cells (T cells, B cells and

occasionally plasma cells), blood brain barrier (BBB) leakage, reactive astrogliosis, demyelination, and axonal loss (Lassmann, 2018). Within a given individual, many of these lesions are at different stages of lesion evolution, which are suitably termed – active, chronic active, or inactive remyelinated/demyelinated lesions, according to the inflammatory cellular content/pattern as well as the degree of demyelination or remyelination within lesions. Cortical lesions also represent a major pathological substrate in MS, and in particular, an abundance of subpial lesions. These lesions have an intriguing relationship (though not always associated) with meningeal inflammation (Howell et al., 2011), which, in advanced cases aggregates into tertiary lymphoid structures consisting of T cells, B cells, and plasma cells (Serafini et al., 2004). RRMS patients have a higher proportion of active white matter lesions while in progressive (both SPMS and PPMS) patients, slow expansion of pre-existing focal white matter lesions, diffuse damage of the normal appearing white and grey matter, meningeal inflammation and subpial lesions predominate (Lucchinetti et al., 2000).

Indeed, four distinct pathological patterns of white matter lesions have been described, and the same pattern has been reported to be conserved across lesions within an individual (Lucchinetti et al., 2000). The commonest patterns are type I and type II, both of which show macrophage as well as T cell infiltration. The distinction between these two patterns lies in the presence of immunoglobulin and complement components, which is exclusive to pattern II. In pattern III and IV lesions, oligodendrogliopathy predominates. These are typified by a loss of oligodendrocytes at the active border while the inactive centre contains no oligodendrocytes, with pattern III specifically showing apoptosis of oligodendrocytes. Although there is controversy regarding the above concepts, with some investigators proposing that these patterns represent different stages of lesion evolution rather than distinct pathological types, this still illustrates that there is considerable pathological heterogeneity within an individual, more so across individuals. These pathological insights also infer that diverse immunopathological mechanisms are present in MS, at different stages of the disease within individuals as well as between individuals.

1.2.2.2 The clinico-radiological paradox

As discussed above, clinical and radiological assessments individually do not have the sensitivity to detect all the neuroinflammatory and neurodegenerative pathogenic processes that occur in the CNS of an MS patient, and when they are at odds with one another, the clinico-radiological paradox arises. This paradox is best explained in the context of disease 'activity', where 'activity' in this case refers to the presence of active MS lesions characterised by inflammatory/immune cellular infiltration, demyelination and axonal loss.

The clinical correlate of new waves of T cell and macrophage infiltration with acute lesion formation is an episode of new or worsening neurological symptoms/signs – a relapse. To date, patient history and neurological examination, as compared to previous examinations, are the principal methods used to establish relapses. This is often supplemented by MRI whereby the presence of GAD-enhancing or new/enlarging T₂ lesion/s within a CNS locale consistent with the neurological deficits offers unequivocal support for a clinical relapse. However, in clinical practice, a clinico–radiological discordance at the time of relapse is not uncommon. The presence of new clinical deficits without MRI correlates could be due to the poor sensitivity of conventional MRI to detect small lesions, particularly in the spinal cord, optic nerve and cortical grey matter (Chard et al., 2017; Hickman, 2007; Kearney et al., 2015). Indeed, only approximately 10 – 15% of cortical lesions can be detected in patients, even when using a seven Tesla (7 T) MRI (Hametner et al., 2018). Newer MRI detection techniques such as double inversion recovery, phase-sensitive inversion recovery, magnetisation transfer imaging as well as using even higher field MRI could help to improve detection of such lesions (Chard et al., 2017; Filippi et al., 2012), but these are currently limited to research settings and not widely available. Returning to the clinico-radiological paradox, the converse is also well-recognised, i.e. the presence of radiological activity without clinical correlates. These subclinical inflammatory events could be due to: (1) the imperfect sensitivity of routine neurological examination and disability measures such as the Expanded Disability Status Scale (EDSS) (Kurtzke,

1983), which largely disregards the contributions of upper limb function and cognitive impairment in addition to the scale being non-linear (Uitdehaag, 2018), and (2) the fact that clinical manifestation is dependent on lesion topography and size, i.e. only when clinically eloquent regions are involved (Chard et al., 2017; Kalincik, 2015).

Regardless of which side the paradox is being viewed from, what ultimately matters to the patient is long-term disability (and its prevention). Although it has been shown that there is an robust association of relapses and disability worsening in the short-term (from incomplete recovery) (Hirst et al., 2008; Keegan et al., 2002), the relationship between relapses and long-term disability is controversial with some studies reporting an association of high relapse rates early in the disease and long-term disability (Goodin et al., 2016; Jokubaitis et al., 2016; Scalfari et al., 2010), while a recent study on a large prospective cohort found no such relationship (Cree et al., 2019). The same can be said of the development of new MRI lesions, with its impact on long-term disability remaining contentious (Cree et al., 2019; Fisniku et al., 2008). Brain atrophy, particularly grey matter atrophy, has been reported to correlate with disability progression (including cognitive measures) (Eijlers et al., 2018; Ghione et al., 2018). However, atrophy occurs relatively slowly such that an interval change between annual scans may not be observable, e.g. the in the INFORMS study, the mean brain volume change from baseline in PPMS patients given placebo was only -0.55% at year 1 (Miller et al., 2018). This is compounded by the lack of standardisation for atrophy measurement and is influenced by technical, biological (e.g. dehydration) and treatment (i.e. pseudoatrophy after DMT commencement) factors (Rocca et al., 2017), thus it is not as yet clinically applicable in individual patients.

1.2.3 The current biomarker landscape in MS

As illustrated above, the heterogeneity observed in MS is largely due to the pathogenic/pathological heterogeneity and, in part, contributed by the limitations of current tools (i.e. biomarkers) used to

reflect these neuroinflammatory and neurodegenerative processes. The National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Definitions Working, 2001). The use of clinically useful biomarkers would thus allow specific features of MS to be defined which could facilitate MS diagnosis and prognosis, as well as the assessment of response to DMT and the side effects from DMT.

However, it has to be noted that few biomarkers, especially biofluid markers, have been found to be clinically applicable in the management of MS patients. A few reasons could account for this observation. The first is that there is no ‘gold standard’ with regards to clinical MS diagnosis, apart from perhaps typical MS histopathology with consistent clinical and radiological features. However, very few patients would have had a brain biopsy and certainly would not have warranted one if the clinical and radiological features were already typical for MS. Despite the seemingly similar clinical and radiological features, it is recognised that there is heterogeneity present with regards to immunopathology and immunopathogenesis between patients, as evidenced by well-conducted pathological studies in patients that were clinically diagnosed to have ‘MS’ (see Section 1.2.2.1). MS, as with many neurological diseases, is quite unlike other disciplines such as oncology whereby an unequivocal gold standard diagnosis (i.e. histology) is readily available for living patients. The presence of such a ‘goal post’/‘anchor’ makes subsequent biomarker discovery, be it prognostic or assessing therapeutic response, easier, as the initial pathological substrate is more conserved across patients. The second reason, which is in part related to the first reason, is because of the differential levels of neuroinflammation and neurodegeneration across patients, and indeed within patients at various time points in their disease course. Many biomarkers have been investigated to serve as a surrogate for specific process occurring within the CNS of an MS patient, e.g. NfL reflecting axonal loss, myelin basic protein as a marker for demyelination and gadolinium enhancement on MRI for

acute neuroinflammation. However, neuroinflammation and neurodegeneration are occurring at the same time within patients, therefore it is unlikely that a single candidate biomarker can holistically capture these highly dynamic processes and indeed the interplay between these processes.

The most widely used biomarker in clinical practice is MRI, which has indeed revolutionised MS diagnosis and aided in the prognostication of MS. This is complemented by the use molecular biofluid-based markers, of which only CSF OCB is in routine clinical use, although others like NfL and CHI3L1 appear promising. A concise appraisal of their diagnostic and prognostic utility in MS, as well as their limitations, is provided below. AQP4-Ab and MOG-Ab are no longer considered as biomarkers of MS because CNS IDD associated with them are now classified as separate clinical entities, therefore they will be described later on in the Introduction of Chapter 3 instead.

1.2.3.1 The utility of conventional MRI in MS

The advent of widespread MRI use in the 1990's offers the MS neurologist, for the first time, a glimpse into the CNS of an MS patient, and, with it, the opportunity to study the characteristics of MS lesions as well as the regions that are involved. This also represented a paradigm shift in the diagnosis of MS, from using clinical relapses and examination to establish dissemination in time and space, to the recognition that MRI lesions can also provide evidence of such concepts. Over the last two decades, iterations of the McDonald criteria have encompassed these observations such that MRI dissemination in time (DIT) (as evidenced by the appearance of a new T₂ lesion compared to a baseline scan and/or the simultaneous appearance of GAD-enhancing and non-enhancing lesions) and MRI dissemination in space (DIS) (as defined by the presence of at least 1 T₂ lesion characteristic of MS in ≥ 2 of 4 regions of the CNS: periventricular, cortical/juxtacortical, infratentorial, spinal cord) can replace clinically determined DIT and DIS (Thompson et al., 2018). Further refinements to the diagnostic criteria has led to an increase in sensitivity (referenced to the development of clinically definite MS [CDMS]), resulting in a larger proportion of patients presenting with CIS to be diagnosed

with MS, however with a trade-off of lower specificity (Hyun et al., 2019; van der Vuurst de Vries et al., 2018).

Besides helping to establish MS in patient presenting with CIS, MRI also has the potential to distinguish MS from other conditions (i.e. MS mimics) such as NMOSD, cerebrovascular disease, leukodystrophies, migraine, CNS vasculitis as well as other toxic/metabolic, infective and neoplastic causes, based on lesion distribution and morphology although considerable overlap exists (Geraldes et al., 2018). In addition, the presence of the central vein sign within MS lesions (using either threshold proportions or absolute counts) appears to have high specificity for MS as compared to non-MS controls (Maggi et al., 2018; Sinnecker et al., 2019). However, this may prove to be time consuming in routine clinical practice especially in patients with high lesion load.

As mentioned, MRI is particularly useful in the context of relapses, with the demonstration of GAD-enhancing or new/enlarging T₂ lesion/s at a neuroanatomical location expected for the clinical deficits providing robust support for a relapse. Clinicians also rely heavily on MRI as a surrogate marker of disease activity during routine visits, with the presence of new lesions (as compared to the previous scan) providing evidence of radiologically defined disease activity, to justify treatment escalation particularly in the context of new clinical symptoms/signs. Indeed, drug agencies and expert centres incorporate MRI activity as a key component in their definition of aggressive/highly active/severe MS (NICE, 2020; Rush et al., 2015).

While MRI is invaluable in the diagnosis of MS, its prognostic value, in particular with regards to white matter lesion load, is still an open debate. Although it has been reported that the baseline MRI disease 'burden' is associated with long-term disability (Fisniku et al., 2008), the relationship between new lesions and long-term disability is unclear, especially at an individual level (Goodin et al., 2016), and treatment escalation for these subclinical events is less certain. Indeed, a recent large prospective study showed that the development of new T₂ lesions did not correlate with EDSS

worsening measured at the next annual visit or with long-term disability worsening (Cree et al., 2019).

In addition, conventional MRI has limitations, especially with regards to the detection of small lesions and inference into pathogenic processes. It does not offer the ability to discriminate between RRMS and SPMS, although in SPMS there is usually (but not always) higher lesion load and greater reduction in brain volume, while PPMS is often associated with fewer brain lesions. In other words, MRI cannot distinguish between RRMS and progressive MS. As mentioned, only 10 – 15% of cortical lesions are radiologically demonstrable even when using high field 7 T MRI (Hametner et al., 2018), in particular subpial lesions, which is in fact the most abundant type of cortical lesion on histopathology. Thus, clinical 1.5 T or even 3 T MRI would potentially miss even more of this major pathological substrate, its importance being that it is now increasingly recognised that cortical lesions correlate with measures of cognitive impairment (Calabrese et al., 2009; Curti et al., 2018). Similarly, ‘normal appearing’ white matter (NAWM) on conventional MRI has obvious demonstrable abnormalities on applying advanced MRI techniques (De Santis et al., 2019; Liu et al., 2015), with histopathology shows perivascular cuffing, mild diffuse parenchymal infiltration of CD8 positive T cells, diffuse axonal injury with secondary demyelination, reactive astrogliosis and widespread microglial activation (Lassmann, 2018). Logistical considerations also impose a challenge to frequent MRI examinations, as it is not practicable to perform an MRI each time a patient has a new symptom due to the high time and cost expenditure. In addition, there has been safety concerns pertaining to GAD accumulation leading to the judicious use of GAD-enhanced MRI, which precludes the assessment of the ‘acuity’ of the imaged lesions (Chard et al., 2017; Ramalho et al., 2016).

1.2.3.2 The utility of CSF oligoclonal bands in MS

CSF OCB can be thought of as the pioneering biomarker in MS, being first introduced in the Poser criteria in 1983 (Poser et al., 1983), then discarded, and now reincorporated back into the 2017

revisions to the McDonald criteria (Thompson et al., 2018). The presence of CSF specific OCB, measured by isoelectric focusing, indicates intrathecal immunoglobulin production by plasma cells residing within the CNS. It was this observation that long supported the belief (though not widely shared by the MS community previously) that B cells partake in MS pathogenesis, but it was not till recently that this was well-recognised and with it, the introduction of B cell depleting therapies for MS. Nearly all (~90%) patients with CDMS have CSF OCB, i.e. a very high sensitivity (Dobson et al., 2013). Intuitively, this means that if a patient does not have CSF OCB, she or he is very unlikely to have MS – a recognised red flag in the diagnostic workup of MS. With regards to its specificity, it is well-known that CSF OCB can occur in a variety of neuroinflammatory diseases such as NMOSD, neurosarcoidosis, autoimmune encephalitis, neuroborreliosis, CNS tuberculosis and CNS lymphoma, amongst others. Indeed, a meta-analysis which included comparative inflammatory aetiologies showed that the specificity of CSF OCB is only 61% (Petzold, 2013). These observations illustrate that CSF OCB alone has high sensitivity, but modest specificity. Thus, as a standalone test, it is most useful to ‘rule out’ MS, but less useful to ‘rule in’ MS. For this reason, the use of OCB in the evaluation of individuals suspected of having MS may be leading to over diagnosis.

The value of CSF OCB in MS lies in its prognostic utility whereby its presence at CIS time point independently predicts the development of a second attack, i.e. CDMS, shown by various studies across different patient cohorts (Arrambide et al., 2018; Kuhle et al., 2015; Tintore et al., 2015). The reintroduction of CSF OCB into the latest diagnostic criteria has cause some amount of confusion, where it can be used as a replacement for the requirement for the demonstration of DIT. In other words, it can be used a proxy to substitute the need for DIT criterion, within the context of the McDonald criteria (Thompson et al., 2018). However, some has failed to see (or read the paper’s footnote) that this does not demonstrate DIT per se in a temporal sense. The key study that led to the incorporation of CSF OCB into the 2017 criteria showed that by combining the (fulfilment of) DIS criterion and CSF OCB, high specificity (88.1%) could be achieved as referenced to the 2010

McDonald criteria (Arrambide et al., 2018). Thus, its (CSF OCB) inclusion was to increase the specificity of the criteria in the absence of DIT. However, the rationale for this was not explicitly stated in the text of the 2017 criteria paper, whereas a separate rationale, in that CSF OCB is a prognostic biomarker (which is also correct but unfortunately resulting in confusion that the presence of CSF OCB means DIT in the temporal sense of the word), was used to justify the inclusion of CSF OCB into the 2017 criteria. However, taking a pathological point of view, the presence of CSF OCB may indeed suggest a temporally dependent process, as it is postulated that CNS plasma cell populations increase with disease duration (Pryce et al., 2018), and a recent study in radiologically isolated syndrome (RIS) patients showed that CSF OCB was an independent predictor for clinical conversion to CIS as well as to CDMS (Matute-Blanch et al., 2018).

1.2.3.3 The potential utility of NfL in MS

To date, of the several emerging biofluid markers, NfL appears to be the most promising. Traditional detection techniques like conventional enzyme-linked immunosorbent assay (ELISA) and electrochemiluminescence immunoassay (ECL) could reliably detect the relatively higher concentrations of NfL present in the CSF, however, these lack sensitivity to accurately measure blood NfL due to the very low levels in blood. The arrival of the Simoa® technology, which has greatly enhanced sensitivity, allowed for accurate blood NfL measurements (Kuhle et al., 2016). This has led to blood NfL being explored as a practicable non-invasive biomarker by eliminating the need for a lumbar puncture (and indeed repeated lumbar punctures) for serial CSF NfL measurements.

NfL is a cytoskeletal protein that is expressed by both central and peripheral neurons. Its exact function is not fully understood, but is currently thought to be involved in preserving axonal cytoskeletal architecture, axonal transport and radial growth (Rao et al., 2003). Given its exclusivity in neurons, NfL is believed to be a specific biomarker of neuroaxonal damage, first being released into the extracellular matrix, then into the CSF, and finally into the blood (Khalil et al., 2018),

although the exact mechanisms of NfL efflux is not well-described. Furthermore, it has been shown that NfL is highly stable and measurements remain robust despite variations in sample-handling (freeze-thaw cycles or delayed blood centrifugation) (Koel-Simmelink et al., 2014). Therefore, NfL represents an attractive approach to quantitate and monitor the level of neuroaxonal injury in MS.

A recent meta-analysis on the diagnostic value of CSF NfL (measured by a standard commercial ELISA) showed that patients with CIS, RRMS and SPMS had higher levels of CSF NfL compared to patients with subjective neurological complaints (SNC) (Bridel et al., 2019). However, CSF NfL levels were not statistically different between SNC and PPMS patients as well as treated RRMS against untreated RRMS. Within MS subtypes, RRMS had the highest level of CSF NfL, followed by SPMS and then PPMS. Compared to other inflammatory neurological diseases (IND), there were no differences in CSF NfL between IND with CIS or with RRMS (regardless of treatment status). In a separate meta-analysis investigating CSF NfL levels (measured by ELISA) specifically within MS patients, it was demonstrated that CSF NfL level was higher in: (1) CDMS compared to controls, regardless of relapse status or stage of MS, (2) RRMS in relapse compared to RRMS in remission, and (3) RRMS compared to progressive MS (Martin et al., 2019). These observations illustrate that within the context of a known MS diagnosis, CSF NfL could distinguish between different stages of MS and also between relapse and remission.

With regards to blood NfL, it has been reported that within the context of clinical trials: (1) baseline NfL correlated with baseline T₂ lesion load and number of GAD-enhancing lesions, (2) high baseline NfL level was associated with on-study new/enlarging T₂ lesions, relapses, brain volume loss, confirmed disability worsening and (3) DMT treatment led to a reduction in NfL levels that was observable by six months (Kuhle et al., 2019). In large observational cohorts, higher blood NfL level was reported to be associated with: (1) an increased risk of relapses, (2) EDSS worsening, (3) GAD-enhancing lesions, while DMT treatment was associated with lower levels of blood NfL (Disanto et al., 2017; Novakova et al., 2017; Varhaug et al., 2018). These reports demonstrate that serum NfL

reflects inflammation driven neuroaxonal damage and has prognostic value particularly with respect to future relapses, disability worsening and therapeutic response. However, with regards to differentiating RRMS from progressive MS, it has been shown the blood NfL is higher in progressive MS (SPMS and PPMS combined) compared to RRMS patients (Disanto et al., 2017), which is contrary to those observations made using CSF NfL.

A few considerations also remain that precludes the implementation of NfL measurements in routine clinical practice. Firstly, it is observed that there is significant overlap of NfL levels between controls and MS (Bridel et al., 2019; Kuhle et al., 2019), and to date there is no proposed threshold at which MS patients can be accurately distinguished from controls, therefore implying that its applicability is highly contextual. This is perhaps not unexpected as NfL is not specific to MS and elevated levels are observed in a variety of neurodegenerative conditions (e.g. Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis) as well as with advancing age (Bridel et al., 2019; Disanto et al., 2017; Lin et al., 2019; Mattsson et al., 2019; Verde et al., 2019). There has also been a recent study reporting that associations between blood NfL and disease outcomes could be confounded by body mass index (BMI) and blood volume (Manouchehrinia et al., 2020). Secondly, although within-individual increases in blood NfL longitudinally have been reported in Alzheimer's disease (Preische et al., 2019), such intra-individual data has been scarce in MS (Akgun et al., 2019). It is hugely important to demonstrate this, as differences observed at group level may not be seen at an individual level. Furthermore, while levels appear to be elevated during relapses at group level analysis, there is little data on how long this increase persists within patients, and how responsive it is to further axonal injury from inflammatory events, especially given the long half-lives of neurofilaments (in neuronal cultures) and reports of persistently elevated NfL months even after mild traumatic head injury (e.g. boxing, soccer heading) (Shahim et al., 2017; Wallace et al., 2018; Yuan et al., 2009). This gradual decrease in NfL level could be due to the phenomenon of Wallerian degeneration where there is a slow 'die-back' of axons with a correspondingly slow release of NfL. It

is also uncertain if the released NfL is further degraded into constituent peptides, which may alter the outcome of quantitative assays. This could also account for the seemingly slow decline post-injury. As mentioned, it is postulated that NfL efflux first occurs into the CSF and then into the blood, therefore NfL levels in these 2 biofluid compartments should be tightly correlated. However, it should be pointed out that the correlation between blood and CSF NfL in MS studies, although statistically significant, is only moderate with correlation coefficients ranging from 0.589 to 0.62 (Disanto et al., 2017; Novakova et al., 2017), and the discordance in CSF and blood NfL with respect to MS subtype (RRMS vs. progressive) further illustrates this point.

1.2.3.4 The potential utility of CSF chitinase 3-like 1 in MS

Chitinase 3-like 1 (CHI3L1) is a glycoprotein secreted by a variety of cell types that include activated macrophages, astrocytes, chondrocytes, neutrophils, vascular smooth muscle cells and tumour cells, thus it is not specific for multiple sclerosis (Comabella et al., 2010; Llorens et al., 2017; Querol-Vilaseca et al., 2017). It is unsurprising that within neurological conditions, elevated CSF CHI3L1 has not only been reported in MS, but also in sporadic Creutzfeldt Jacob Disease, Alzheimer's disease and tauopathies, and is likely to reflect the neuroinflammatory component (activated macrophages and astrocytes) within these dementias (Llorens et al., 2017; Querol-Vilaseca et al., 2017). To date, its utility in MS has been mainly prognostic, and, restricted to the separation of CIS individuals who later go on to develop CDMS against those who remained as CIS.

The first study that identified its potential utility in CIS used a proteomics screen on CSF, followed by candidate biomarker selection, and found that CSF CHI3L1 (measured by ELISA) was elevated in CIS patients who later converted to CDMS (Comabella et al., 2010). The study also reported that high CSF CHI3L1 correlated with the baseline number of GAD-enhancing lesions as well as T₂ lesions, and was associated with disability progression and shorter time to CDMS upon follow-up. A follow-up study validated the observation of higher CSF CHI3L1 in CIS patients who converted to CDMS, and in

a Cox regression model corrected for co-variables, again demonstrated that high CSF CHI3L1 level was associated with faster disability worsening and earlier development of CDMS (Canto et al., 2015). These findings were supported by several subsequent studies (Gil-Perotin et al., 2019; Hinsinger et al., 2015; Modvig et al., 2015). Its prognostic utility however was not demonstrated in an RIS study, which found that CSF CHI3L1 was not an independent predictor for the risk of conversion from RIS to RRMS (Thouvenot et al., 2019). As expected, the main impediment to routine CSF CHI3L1 is the need for serial lumbar punctures and further studies should be performed to determine if blood CHI3L1 could be useful in the above context, as well as to explore the dynamics of CHI3L1 levels prospectively in relation to clinical and MRI activity as well as with long-term disability worsening and progression.

1.3 Metabolomics as a tool for biomarker discovery

1.3.1 Metabolomics for biomarker discovery in biomedical research

Metabolomics is an emerging approach for biomarker discovery in precision medicine and for identifying disease pathways underpinning clinical phenotypes (Patti et al., 2012; Wishart, 2016). It involves the comprehensive study of low molecular weight (<1,500 Da) metabolites, i.e. the metabolome, which include amino acids, carbohydrates, biogenic amines, lipids and organic acids (Wishart, 2016), and it is predicted that there are around 150,000 human metabolites (Markley et al., 2017). However, technical limitations in the past have prevented the identification and quantification of a vast number of these metabolites, limiting the use of metabolomics in biomedical research. This is rapidly changing with the advent of advanced chemistry-based detection techniques like NMR spectroscopy and mass spectrometry, coupled with the availability of powerful statistical software allowing complex multivariate analysis to be performed (Wishart, 2016). Indeed, supervised multivariate analysis techniques like orthogonal partial-least squares discriminant analysis (OPLS-DA) now enables investigators to construct class-based mathematical models defined by the clinical question of interest (Worley et al., 2013). This approach enables metabolic signatures

characteristic of specific diseases to be defined, instead of requiring identification of a unique (or a few) candidate-led biomarker (Lindon et al., 2003).

Endogenous metabolites are produced during metabolism and are essential for energy production, cellular signalling, and physiological control (Johnson et al., 2016). They represent the end products of upstream biological processes that involve the genome, epigenome, transcriptome, and proteome (**Figure 1.1**) (Patti et al., 2012). A change in the expression level of a gene or protein from a genomics or proteomics study is not necessarily correlated with a change in protein activity or directly linked to a disease state as a result of epigenetic regulation, post-transcriptional and post-translational modifications, whereas metabolic profiling provides a 'snapshot' what is actually happening at a biochemical level. Indeed, all cellular processes are mediated by metabolites and their measurements provide a direct functional readout of the cellular state. As the metabolome is 'downstream' of these processes, it is hence 'closest' to the clinical phenotype. Metabolites can also be derived exogenously (e.g. medications, diet, lifestyle) and metabolomics permits the study of these exogenous metabolites as well as the metabolic products of host-exposome interactions (**Figure 1.1**). These characteristics enable metabolomics to be well-placed as a biomarker discovery platform, as it takes into account both host and environmental contributions through the use of comprehensive biochemical profiling.

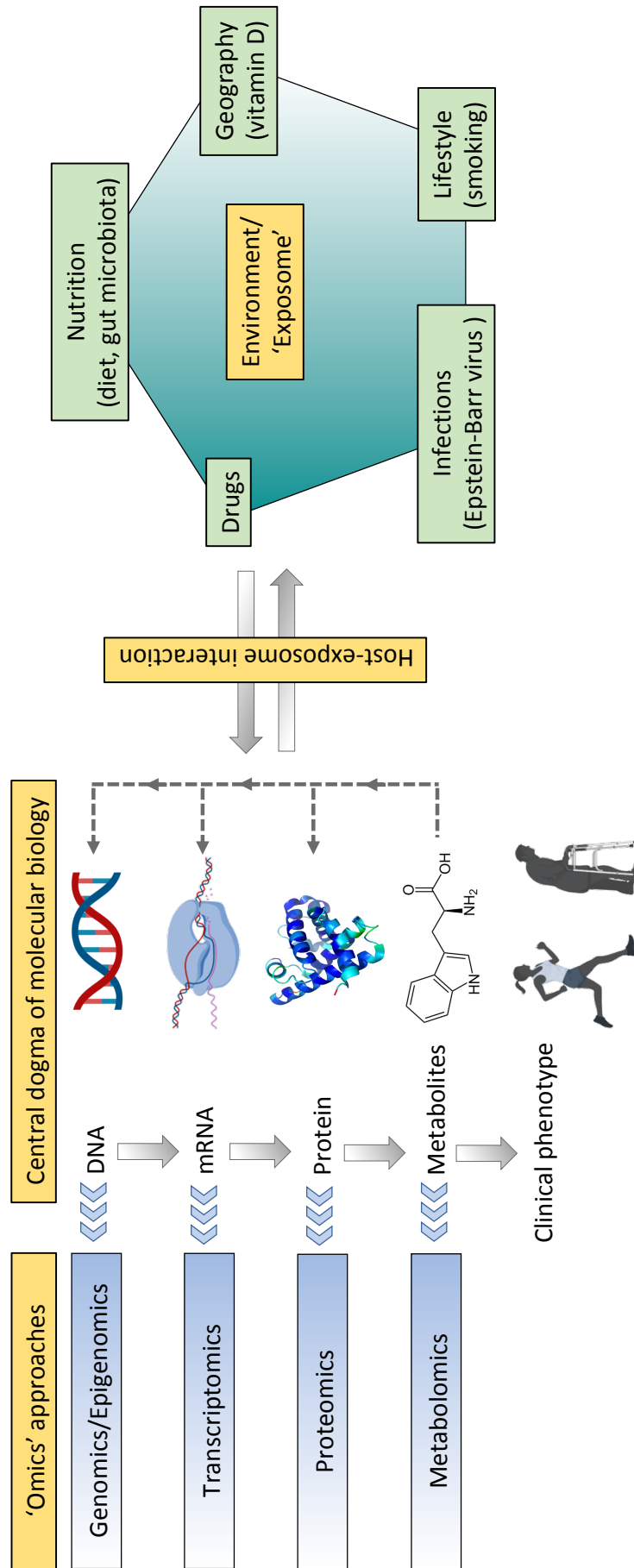


Figure 1.1: Overview of metabolomics as a platform for biomarker discovery. The metabolome best reflects the clinical phenotype, as metabolites are the biological end products of upstream processes involving gene and protein expression. Metabolites can also alter gene and protein expression, via feedback mechanisms, e.g. acting as substrates that regulate post-translational modifications that affect protein function (Wellen et al., 2009). Metabolomics also take into account the metabolic contributions from the environment/exposome as well as the host-exposome interaction – an important consideration given that the exposome contributes significantly to the pathogenesis of MS, likely via the mechanisms of molecular mimicry, novel autoantigen presentation, recognition of sequestered CNS antigen released into the periphery or bystander activation (Dendrou et al., 2015). The known environmental contributors to MS pathophysiology are shown above.

1.3.2 Analytical platforms in metabolomics

Currently, there are three main analytical technologies available to perform metabolic profiling – NMR spectroscopy, gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS). Each technology has its own intrinsic advantages and disadvantages, which have already been extensively reviewed (Dunn et al., 2011; Emwas, 2015; Wishart, 2016).

Table 1.1 provides a comparative summary of these three technologies. Despite their differences, there is now an emerging trend to use both NMR spectroscopy and mass spectrometry in a complementary manner to broaden the coverage of the metabolome with studies showing that some metabolites are uniquely identified by one technique while other metabolites are detected by both platforms (Bhinderwala et al., 2018; Marshall et al., 2017; Psychogios et al., 2011). Indeed, it has been shown that a combined NMR/mass spectrometry approach leads to enhanced metabolite identification in complex biological mixtures (e.g. human urine) (Bingol, Bruschweiler-Li, et al., 2015; Bingol & Bruschweiler, 2015).

Two strategies are used in metabolomics studies – global (untargeted) and targeted approaches. Global metabolomics comprehensively analyses all measurable metabolites in a sample, generating an extensive dataset that must be decomposed into fewer components with the use of advanced multivariate statistical analysis. This approach is ideal for comprehensive biochemical profiling for biomarker discovery, and can generate novel targets and hypotheses, however, it also means the chemical unknowns are also detected, especially with a mass spectrometry platform. In contrast,

targeted metabolomics measures pre-defined chemical groups and known metabolites, arising from the understanding of the enzymes and metabolic pathways involved, thus allowing for a focused and detailed study of these metabolic processes. The global metabolomics strategy was adopted for this thesis as it is the best-suited approach to answer the hypotheses set out. A combined global and targeted approach was undertaken for Chapter 4, which aimed to explore biomarkers for clinical relapses.

Table 1.1: Comparative summary of NMR, GC-MS and LC-MS. Modified from (Wishart, 2016) with kind permission from the journal.

Analytical technology	Advantages	Disadvantages
NMR	• Excellent reproducibility • Intrinsically quantitative • Short experimentation time (few minutes per sample) • Minimal sample preparation (no sample adulteration) • Non-destructive • Identity of most spectral signals known • Allows identification of novel compounds • Robust technology with long instrument lifespan • Full automation possible	• Cannot detect non-protonated compounds, inorganic ions • Less sensitive (Limit of detection, LOD $\geq 1 \mu\text{M}$) • Require larger sample volumes (200 – 500 μL) • Larger instrument footprint
GC-MS	• Good sensitivity (LOD 0.5 μM) • Many spectral features are known • Quantitative with calibration • Detects most organic and some inorganic molecules • Robust technology • Can be partially automated • Lesser sample volume needed (100 – 200 μL)	• Difficult to identify novel compound • Requires separation and sample derivatisation to become volatile • Destructive • Long experimentation time (20 – 40 minutes)
LC-MS	• Excellent sensitivity (LOD 0.5 nM) • Detects most organic and some inorganic molecules	• Most spectral features are unknown • Difficult to identify novel compound

	• Can be mostly automated • Minimal sample volume needed (100 – 200 μL)	• Not quantitative • Requires separation • Destructive • Long experimentation time (10 – 40 minutes) • Less robust technology and short instrument lifespan
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In this thesis, NMR spectroscopy was used as the analytical platform in view of its high reproducibility, quantifiable nature, and that most NMR spectral features are readily identifiable including unknown signals, through the use of correlation NMR pulse sequences/2D NMR experiments (see section **1.6.6.5**). In addition, sample preparation is straightforward and each NMR experimental takes an average of 5 – 8 minutes (for the pulse sequences used in this thesis). These advantages added to the fact the whole system could potentially be fully automated mean its applicability and scalability for clinical use is a distinct possibility. Indeed, some commercial NMR companies have developed fully automated systems and have introduced clinical tests measuring lipoproteins (with in-depth subpopulation measurements) for coronary heart disease risk stratification, as well as metabolite biomarkers for improved glomerular filtration rate estimation and identification of kidney graft rejections (Nightingale, 2020; numares, 2020a).

1.3.3 Metabolomics study workflow for clinical research

The workflow of clinical metabolomics studies largely similar to studies exploring biomarkers for diagnostic, prognostic, or predictive purposes, with a few exceptions. There is a need for strict sample (especially blood) processing following established protocols because ongoing metabolic processes within the sample can alter the concentrations of time-sensitive metabolites. This is elaborated in section **2.1.1**, Material and Methods, and in the Introduction of Chapter 6. Another feature of metabolomics (and indeed with other ‘omics’ technologies) is the huge amount of data that is generated. NMR spectroscopy experiments typically produce around 200 – 300 variables and

this could be in the thousands for mass spectrometry. Univariate analysis, whilst intuitive for interpretation, is inappropriate for this purpose because of the existing correlations between metabolites (which is expected since metabolic pathways are by and large interconnected), and corrections for multiple comparisons (e.g. Bonferroni correction, false discovery rate [FDR] correction using Benjamini-Hochberg method) may give rise to false negatives and yet still does not consider the metabolome as a whole. As such, multivariate dimensionality reduction methods are required as these take into consideration all the metabolites at the same time (and their associated correlations), to identify metabolic patterns/signatures as well as differences between patient groups (in the case of supervised methods). These multivariate techniques also allow the top metabolites accounting for group differences to be identified. The details of these multivariate techniques are detailed in section **2.2.1**, Materials and Methods. **Figure 1.2** illustrates the typical workflow of a clinical metabolomics study.

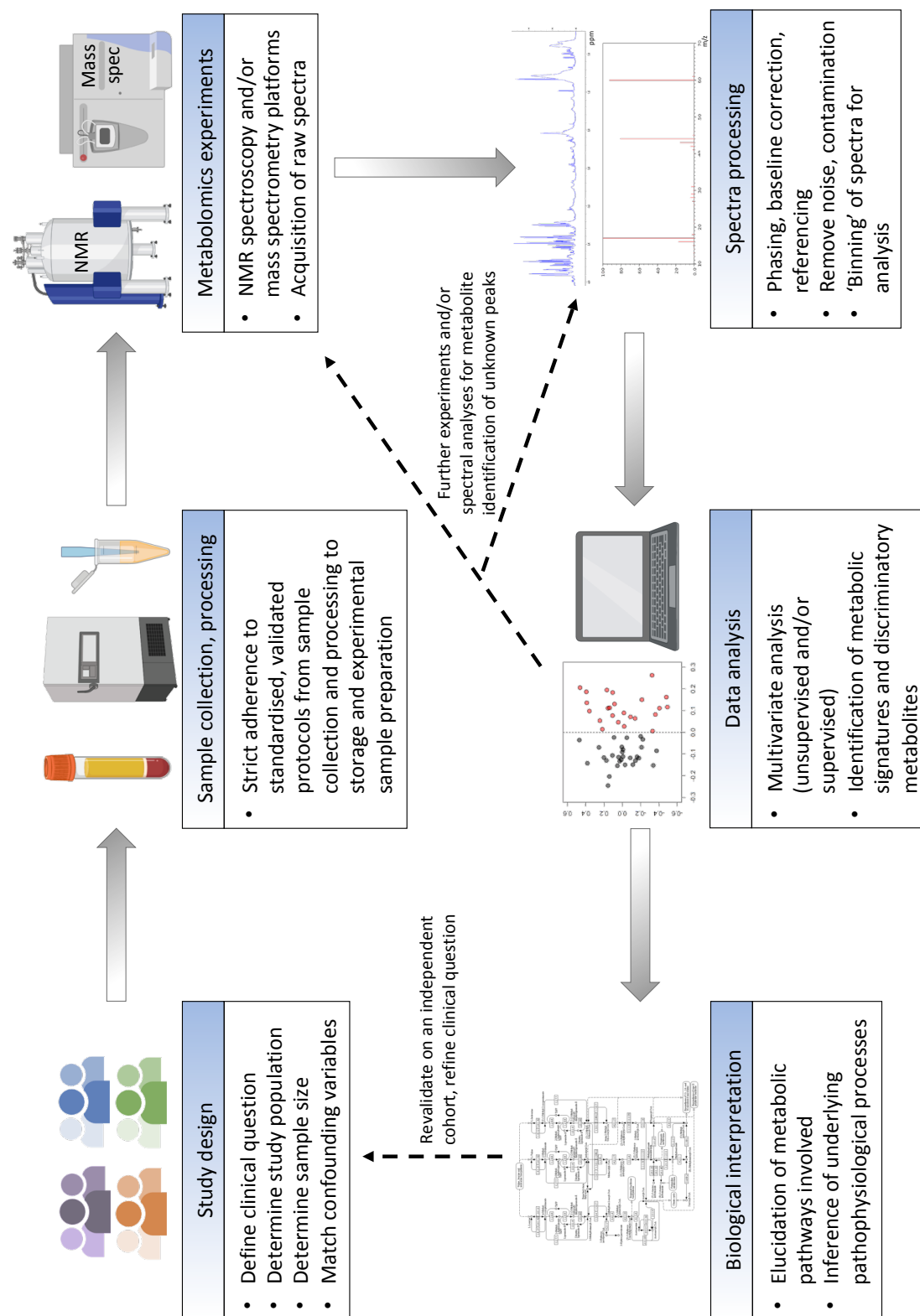


Figure 1.2: Workflow of a clinical metabolomics study.

1.4 The rationale for a metabolomics approach for biomarker discovery in CNS IDD

The archetypal CNS IDD is MS which is a complex, immune-mediated disease involving genetic and environmental factors, both contributing and interacting, to produce the underlying immunopathogenesis (Dendrou et al., 2015). The complexity and heterogeneity of MS makes it challenging for a single candidate biofluid marker to reflect all the underlying disease processes, both neuroinflammatory and neurodegenerative, at the same time within an individual MS patient. Indeed, currently available biomarkers are suited to provide a measure of distinct pathophysiological processes. For example, NfL (both in blood and in CSF) is a good marker to infer upon acute neuroaxonal loss from neuroinflammation, while CSF OCB is indicative of intrathecal immunoglobulin production, and CSF CHI3L1 likely informs on the degree of neuroinflammation (Lassmann, 2018). Rather than to analyse these proteins as standalone biomarkers as it is usually done, perhaps a more appropriate approach would be to combine these markers into a composite to provide a summative interpretation of various disease processes, however, this has rarely been done (Gil-Perotin et al., 2019).

Metabolomics is well placed for this type of summative approach. A single metabolomics experiment provides hundreds (if not thousands) of metabolites at one go, i.e. a metabolic snapshot, which are the downstream products of all neuroinflammatory and degenerative processes that is happening within the CNS of a MS patient, whilst accounting for contributions from the exposome and the host-exposome interaction. Combined with multivariate dimensionality reduction methods, a constellation of metabolic biomarkers, i.e. a biomarker signature **Figure 1.3**, can be generated to recapitulate the complex interplay between endogenous and exogenous disease factors, as well as the global pathophysiological process which is ever changing. It is also apparent that metabolomics is intrinsically responsive to metabolic perturbations resulting from fluctuating disease states which makes it well-positioned for dynamic disease monitoring, whereas a genetic-based biomarker is likely to remain static over time.

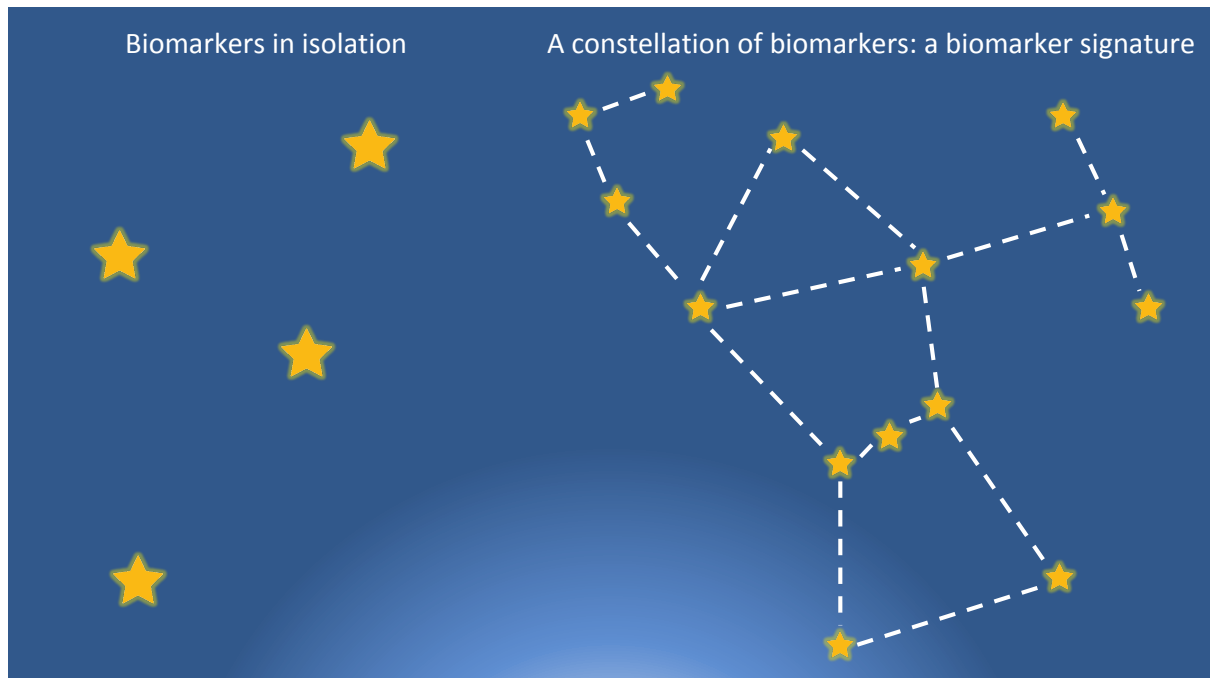


Figure 1.3: Approaches in biomarker discovery. Conventional biomarker discovery strategy involves using a single candidate biomarker, akin to looking at a single star in the night sky. In contrast, metabolomics combined with multivariate statistical analysis enables a disease specific (and indeed a disease-state specific) metabolic signature, made up of a combination of metabolites, to be identified, just like a constellation of stars. The metabolic signature (constellation) is thus the biomarker.

1.4.1 The biological basis for metabolic perturbations arising from MS pathogenesis

Recent evidence has demonstrated the production, intensity, and duration of immune responses are tightly controlled by immune cells by regulating their intracellular metabolic profile, which in turn is affected by environmental factors such as infections and nutritional status (Norata et al., 2015; O'Neill et al., 2016). The biological plausibility of the metabolomics approach to identify MS-specific metabolic signatures/biomarkers can be explained within the context of MS pathogenesis, as detailed in the following sections. An overview of these processes is illustrated in **Figure 1.4**.

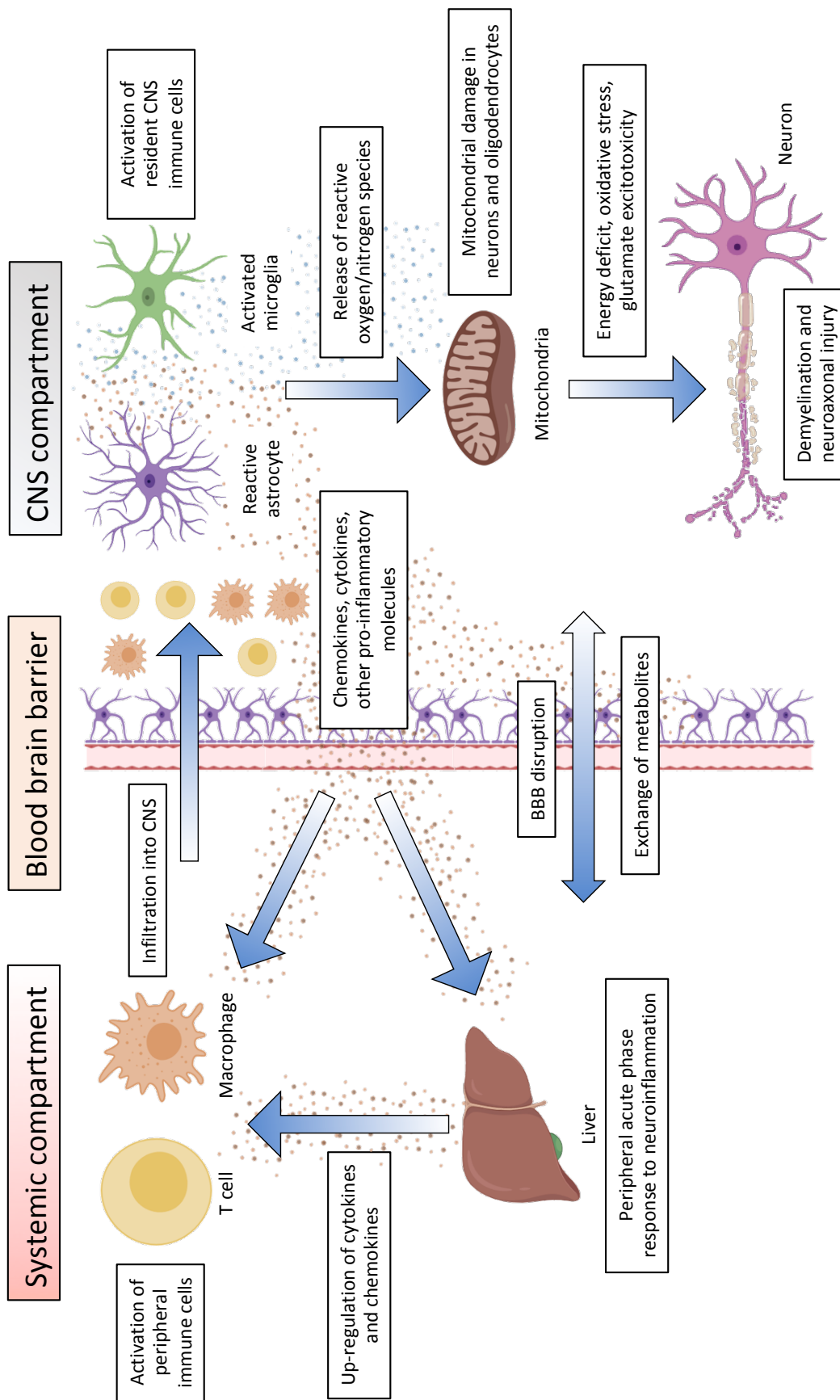


Figure 1.4: Metabolic perturbations arising from MS pathogenesis. The activation of peripheral T cells and macrophages commit them to specific intracellular pathways to produce adenosine triphosphate (ATP) – glycolysis with oxidative phosphorylation in T cells compared to glycolysis alone in macrophages. These activated immune cells infiltrate the CNS and initiate a neuroinflammatory cascade via the release of pro-inflammatory cytokines (IL-1 β , IL-17, IL-22, IFN- γ , TNF- α and CCL-2). This activates microglial and triggers reactive astrogliosis, which produces more inflammatory mediators and releases ROS and RNS via respiratory burst. The downstream effects of these reactive species are explained in detail within **Figure 1.6 B**. Cytokines like IL-1 β (via neutrophil-dependent processes) disrupt the BBB and tight junctions, which facilitates the exchange of small solutes between the CNS and the systemic circulation. The leaky BBB also augments the efflux of pro-inflammatory cytokines, chemokines, extracellular vesicles (which may carry microRNA and lipid signalling molecules), triggering the peripheral acute phase response that is mediated by the liver. The liver then increases the transcription of pro-inflammatory cytokine/chemokine genes with a corresponding increase in protein expression, perpetuating the inflammatory milieu. As the liver plays a major role in lipid, carbohydrate and amino acid metabolism, such a response would conceivably lead to alterations in the biosynthesis and degradation of these metabolite classes.

1.4.1.1 Immune cells reconfigure energy metabolic pathways upon activation

Regardless of the initial triggering mechanisms, the pathogenesis of MS involves the activation of peripheral T cells and monocytes/macrophages after antigenic presentation. Their activation commits them to specific intracellular metabolic pathways to generate energy. Activated M1 macrophages commits to glycolysis to generate adenosine triphosphate (ATP), and the majority of pyruvate is converted to lactate instead of being channelled into the tricarboxylic acid (TCA) cycle to drive oxidative phosphorylation, which produces more ATP compared to glycolysis (Pearce et al., 2013). This is known as the Warburg effect whereby glycolysis predominates even in the presence of aerobic conditions and functional mitochondria, a phenomenon well-described in cancer cells that is believed to promote cell proliferation (Liberti et al., 2016). Indeed, it has been shown the inhibition of glycolysis causes a shift towards an M2 macrophage phenotype (anti-inflammatory) with a corresponding increase in IL-10 production (Palsson-McDermott et al., 2015). In contrast, the metabolic configuration of activated T cells involves both aerobic glycolysis and oxidative phosphorylation, whereby pyruvate is both converted to both lactate as well as acetyl-CoA, the latter being a major substrate for the TCA cycle (Pearce et al., 2013). Activated T cells also utilise glutaminolysis, a process in which glutamine is converted to glutamate, then to α -ketoglutarate, and this is used as a substrate in the TCA cycle. A simplified overview of ATP generating energy pathways

is provided in **Figure 1.5**.

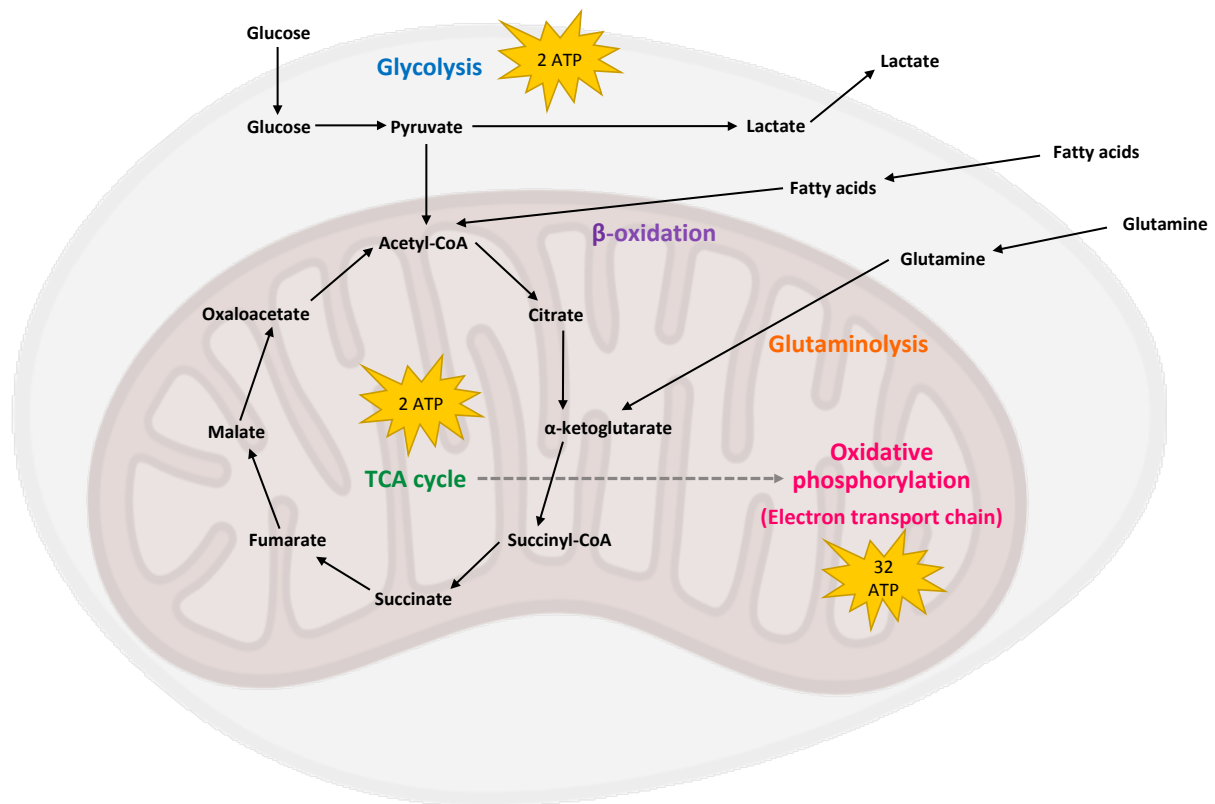


Figure 1.5: A simplified overview of ATP generating metabolic pathways. The grey dotted line indicates substrates produced by the TCA cycle that are required for oxidative phosphorylation.

These activated immune cells then cross the BBB into the CNS where they encounter the cognate antigen. The release of cytokines and chemokines recruits further peripheral inflammatory cells (monocytes/macrophages) and activates resident microglia and astrocytes. The activation of these CNS immune cells is likely to result in similar metabolic changes, as those seen in the peripheral immune cells. Indeed, it has been shown that activated BV-2 microglia cells had increased glucose uptake and glycolysis (Gimeno-Bayon et al., 2014), while starvation resulted in a reliance on glutaminolysis to generate ATP (Nagy et al., 2018). It has to be added that this activation cascade involves recruitment of phospholipids and amino acids to act as signalling molecules, regulators of membrane synthesis, lipid rafts and cytokine precursors (O'Neill et al., 2016). The activation of these

immune cells produces reactive oxygen and nitrogen radicals causing mitochondrial damage within neurons and oligodendrocytes, resulting in metabolic stress, energy deficiency, altered cell homeostasis, glutamate excitotoxicity and further oxidative radical production (Dendrou et al., 2015; Lassmann, 2014). A detailed description of these processes is given below, within a focus on neuronal-astroglial metabolic coupling.

1.4.1.2 Neuronal-astroglial metabolic coupling

Under normal physiological conditions, neurons and astrocytes exhibit metabolic coupling to meet neuronal energy and anti-oxidative requirements. Two major pathways are key to this metabolic coupling, namely the astrocyte-neuron lactate shuttle (ANLS) and the glutamate-glutamine cycle. These pathways and their disturbances have already been elegantly and extensively reviewed (Magistretti et al., 2015; Mahmoud et al., 2019; Robinson et al., 2019), thus the sections below describe their salient features relevant to this thesis (**Figure 1.6**).

As the name suggest, ANLS involves the shuttling of lactate from astrocytes to neurons via the monocarboxylate transporters (MCT). Aerobic glycolysis takes place within astrocytes whereby glucose is taken up by the glucose transporter (GLUT) 1, converted to pyruvate and then to lactate. Lactate is preferentially used a fuel source by neurons instead of glucose. It is first converted to pyruvate and incorporated into the TCA cycle for ATP production. This process allows glucose to be channelled to the pentose phosphate pathway (PPP). The PPP produces nicotinamide adenine dinucleotide phosphate (NADPH), which acts a substrate for the reduction of oxidised glutathione (GSSH) to glutathione (GSH) by glutathione reductase (GR). The GSH (a potent anti-oxidant) generated, together with glutathione peroxidase (GPx), reduces peroxides. In summary, the selective use of lactate by neurons help to minimise oxidative stress while meeting energy requirements.

The glutamate-glutamine cycle involves the exchange of these two metabolites between neurons and astrocytes. During excitatory neurotransmission, glutamate (the main excitatory

neurotransmitter) is released from the pre-synaptic membrane across the synaptic cleft, and activates ionotropic and metabotropic glutamate receptors. As an excess of glutamate is released, this must be removed to prevent neuronal excitotoxicity, a function performed by astrocytes through uptake via excitatory amino acid transporters (EAAT) 1 and 2. Within astrocytes, glutamate is converted to glutamine by the action of glutamine synthetase (GS), released into the brain interstitial fluid via sodium-coupled neutral amino acid transporter (SNAT) 3, and taken up by neurons through SNAT 1. Glutamine is then converted back to glutamate by glutaminase (GLS) within neurons, thus completing the cycle. Of note, the uptake of glutamate into astrocytes is associated with the influx of Na^+ , along its electrochemical gradient. Na^+ is in turn removed by the Na^+/K^+ ATPase, which is ATP-dependent, thus providing the stimulus for glucose influx into astrocytes via GLUT 1 and is then used as the main fuel source for astrocytes.

1.4.1.3 Metabolic 'de-coupling' during neuroinflammation and other toxic metabolites

During neuroinflammation, these key processes regulating metabolic coupling are disrupted. Activated microglia and reactive astrocytes release reactive oxygen (ROS) and nitrogen species (RNS) that damage the neuronal mitochondrial electron transport chain, resulting in a decrease in ATP production. An energy deficient state ensues and ATP-dependent processes e.g. Na^+/K^+ ATPase are compromised. This is aggravated by a reduction of lactate shuttled from astrocytes due to the downregulation of MCT on reactive astrocytes as they undergo functional modifications in response to inflammation. This neuronal lactate deficiency has sequential deleterious consequences: (1) less lactate-derived substrates to fuel the TCA cycle thus worsening the ATP deficit, (2) glucose is utilised preferentially for this purpose leading to lesser amounts available for the PPP for NADPH production, (3) decreased NADPH leading to diminished GSSH reduction to GSH, and (4) increased peroxide levels resulting in further mitochondrial/DNA damage.

Reactive astrocytes also exhibit impaired uptake of glutamate by the downregulation of EAAT expression. Excess glutamate in the synaptic cleft overstimulates the ionotropic receptors on the post-synaptic membrane and this induces excessive influx of Ca^{2+} via ionic channels. High intracellular Ca^{2+} lead to the opening of the mitochondrial permeability transition pore leading to mitochondrial depolarisation, reduced ATP generation and release of ROS and RNS. ATP-dependent Na^+/K^+ ATPase function is reduced and this in turn lead to the reversal of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger which increases intracellular Ca^{2+} even further, perpetuating an injurious cycle of energy deficit, oxidative stress and ionic imbalance. This also leads to the failure to maintain the Na^+ gradient required for the general of the action potential, causing conduction failure, axonal transport disruption and further axonal damage.

In addition to the above metabolic perturbations, it is shown that pathologically activated astrocytes with larger cell volumes have increased myoinositol, therefore it could serve as a marker for reactive astrogliosis/astrocytosis (Harris et al., 2015). Indeed, increased myoinositol levels have been noted in RRMS and CIS patients compared to controls using ^1H MRS of NAWM (Chard et al., 2002; Fernando et al., 2004).

Of interest is the role of the kynurenine pathway in MS pathogenesis and this has been reviewed extensively by Lim et al (Lim et al., 2010). Kynurenine is produced from tryptophan, and gives rise to several downstream metabolites that include kynurenic acid (KA) and quinolinic acid (QA). Using in vitro models, it has been shown that KA is neuroprotective as it (1) blocks the ionotropic glutamate receptors, thus preventing glutamate induced excitotoxicity, and (2) attenuates the excitotoxicity of QA. In contrast, QA is neurotoxic as it can (1) activate N-methyl-D-aspartate (NMDA) receptors in pathophysiological concentrations leading to excitotoxicity, (2) increase glutamate levels in the brain interstitial fluid by increasing neuronal glutamate release, decreasing astrocytic glutamate uptake and inhibiting astrocytic GS, (3) cause astrocyte cytotoxicity, and (4) cause lipid peroxidation. Most of the QA produced comes from activated macrophages and some from resident microglial, while

astrocytes are unable to synthesise QA. It is postulated that imbalance between the concentrations of KA and QA is present in MS and QA excess contributes to progressive axonal loss.

Figure 1.6: The ANLS and the glutamine-glutamate cycle under physiological and inflammatory conditions. **(A)** The ANLS involves the shuttling of lactate from astrocytes to neurons to be utilised preferentially as the fuel source for the TCA cycle, via its conversion to pyruvate, which is then oxidised to acetyl-CoA. This permits glucose to be directed towards the PPP to generate reducing substrates to neutralise oxidative stress. The glutamate-glutamine cycle involves the uptake of excess glutamate from the synaptic cleft by astrocytes during excitatory neurotransmission. Glutamate is converted to glutamine within astrocytes and released into the brain interstitial fluid where it is taken up by neurons and converted back to glutamate. **(B)** During neuroinflammation, astrocytes undergo functional modifications, which disrupt homeostatic metabolic coupling. Reduced MCT expression results in decreased lactate shuttling from astrocytes to neurons via the ANLS, resulting in glucose now being utilised as the main fuel source. As less glucose is channelled into the PPP, oxidative stress is not adequately neutralised. Reduced EAAT expression also impairs the uptake of glutamate by astrocytes. The excess glutamate leads to overstimulation of ionotropic glutamate receptors, causing an influx of Ca^{2+} , which damages mitochondria. ATP production decreases, compromising ATP-dependent processes like the Na^+/K^+ ATPase. Adapted and redrawn from (Magistretti et al., 2015) and (Robinson et al., 2019) with kind permission from the journals. LDH = lactate dehydrogenase

1.4.1.4 Metabolic products of tissue damage

The three main classes of lipids that make up the CNS myelin are cholesterol, glycosphingolipids and phospholipids (Podbielska et al., 2013). ROS released by activated microglia and astrocytes can cause lipid peroxidation of phospholipids containing poly-unsaturated fatty acids. Lipid peroxidation (via protein lipoxidation) further disrupts myelin, cell membranes, and enhancing pro-inflammatory activity (Ferretti et al., 2011; Gonzalo et al., 2012; Quintana et al., 2012). Hence, it is conceivable that demyelination by lipid peroxidation and oligodendrocyte injury (via oxidative damage and energy deficit) could lead to the release of these membrane/myelin lipid components, which are then detectable on a metabolomics platform.

N-acetylaspartate has been proposed as a CNS-specific metabolite, produced by neurons and transported to the cytoplasm of oligodendrocytes where aspartoacylase cleaves the acetate moiety to be used as substrates for myelin synthesis (Moffett et al., 2007). Reduced N-acetylaspartate is believed to represent neuroaxonal loss or neuronal stress, and it has been consistently showed to be decreased in both the white and grey matter in MS (Criste et al., 2006), hence it is possible that this could also be reflected in biofluids.

1.4.1.5 Metabolic derangements from BBB disruption

Under physiological conditions, the integrity of the BBB is maintained by the neurovascular unit, which consists of the cellular barrier (astrocytes, pericytes and endothelial cells), basement membrane, extracellular matrix and junctional components (Engelhardt et al., 2009; Zlokovic, 2011). The most important impedance of free solute transfer between the blood and CNS compartments comes from the tight junctions (with some contribution from the adherens junctions) that exist between endothelial cells (Alvarez et al., 2011; Mikitsh et al., 2014). These junctions are made up of transmembrane proteins (claudin, occludin and junctional adhesion molecule) that are connected to and anchored to their cytoplasmic counterparts (e.g. zonula occludens and cingulin). During neuroinflammation, the release of pro-inflammatory molecules and proteases (i.e. matrix metalloproteinase-9, IL-1 β , IL-17, IL-22, IFN- γ , TNF- α and CCL-2) is associated with BBB dysfunction in which the BBB becomes 'leaky' with a disruption of tight junctions (Alvarez et al., 2011). Alterations in the expression of tight/adherens junction proteins have been shown in the brains of MS patients (Kirk et al., 2003; Padden et al., 2007). Experimentally, it has been shown that intracranial injection of IL-1 β into rat striatum, via neutrophil-dependent processes, leads to the loss of zonula occludens-1 and occludin protein expression (Bolton et al., 1998), and tight junction morphology has been shown to be perturbed using electron microscopy in a murine experimental autoimmune encephalomyelitis (EAE) model of MS (Wolburg et al., 2005). This, in the case IL-1 β upregulation, is likely mediated by the increased expression of matrix metalloproteinase-9 (Lin et al., 2009), which in turn digests the tight junction proteins (Bauer et al., 2010). Therefore, it is highly likely that such disruptions to the BBB has the capacity for increased bi-directional exchange of small hydrophilic molecules.

1.4.1.6 Metabolic derangements from peripheral response to neuroinflammation

Experimentally, it is clear that in response to CNS injury, there is an accompanying acute phase response that is primarily mediated by the liver (Anthony & Couch, 2014). The mechanisms by which the presence of CNS injury is 'sensed' by the liver are postulated to be via circulating cytokines originating from immune cells in the brain, and recent evidence has also suggested the role of extracellular vesicles in mediating this process (Anthony & Couch, 2014; Couch et al., 2017). Within the liver, this leads to an upregulation of nuclear factor- κ B regulated genes and a consequential increase in pro-inflammatory cytokines and chemokines (e.g. CCL-2 and CXCL-1) (Campbell et al., 2005; Campbell et al., 2008), which in turn amplifies the activation of peripheral immune cells. It is hence likely that the molecular reconfiguration in the liver required to mount this acute phase response can produce metabolic perturbations (particularly in the blood), especially since the liver is the major organ involved in lipid, carbohydrate and amino acid metabolism. Indeed, it is well-known that in response of inflammation, elevated VLDL levels (from increased production and reduced hepatic clearance) are observed with increased oxidation of VLDL and LDL, while HDL adopts a pro-inflammatory configuration (Khovidhunkit et al., 2004). These changes result in an enhanced uptake of lipoproteins by macrophages, promoting atherogenesis.

1.4.1.7 Lipid mediators and lipid derangements during neuroinflammation

From the above sections, it is clear that alterations in lipids can occur as a result of degradation of cellular membrane and myelin, as well as in response to inflammation. However, it is increasingly recognised that lipids are not just mere bystanders in the inflammatory cascade, but rather exerts bioactive properties that mediate various inflammatory processes.

The reconfiguration of lipoproteins during inflammation is well-recognised, however lipoproteins are also involved in a wide array of physiological processes including cell signalling, lipid homeostasis, and mediating the acute phase response (Khovidhunkit et al., 2004). HDL is intrinsically anti-

inflammatory, and, via multiple mechanisms can potentially modulate monocyte and T cell responses (Jorissen et al., 2017). Previous studies have suggested that HDL particles are capable of crossing the blood brain barrier (Ferretti et al., 2011), and it has been shown that LDL is present in the parenchyma of early MS lesions (Newcombe et al., 1994), illustrating the potential of lipoprotein alteration within the brain in response to neuroinflammation. Indeed, the brain has its own unique lipoprotein environment, with astrocytes producing apolipoproteins and neurons displaying distinct lipoprotein receptor profiles (Wang et al., 2014). Mitochondrial damage, which occurs in MS, can lead to a reduction of β -oxidation of very long chain fatty acids (VLCFA). This in turn leads to increase β -oxidation/metabolism in peroxisomes which is intrinsically anabolic, leading to chain elongation of fatty acid (Senanayake et al., 2015). It is shown in rodent studies that high levels of VLCFA results in gliosis, inflammatory demyelination and axonopathy caused by the accumulation of VLCFA (Brites et al., 2009). There is currently considerable interest in the roles that prostaglandins, ceramides, hydroxyeicosatetraenoic acid and phosphatidic acids play in MS pathogenesis, likely via signalling mechanisms (Lotsch et al., 2018). Ceramide, a sphingolipid, is involved in various cellular signalling pathways (e.g. apoptosis, cell-cycle arrest). A study has showed increased CSF ceramide C16:0 and C24:0 in MS patients, and these were able to induce neuronal mitochondrial dysfunction and axonal damage (Vidaurre et al., 2014). Perhaps the most obvious example in MS of a bioactive lipid acting as an extracellular lipid mediator is that of sphingosine-1-phosphate (S1P). The effects of S1P are mediated by its cognate G-protein coupled receptors (five are known, termed S1P₁₋₅). These receptors are ubiquitous, being expressed by a variety of cells ranging from lymphocytes, neural cells, astrocytes and cardiomyocytes. The efficacious treatment (at least the inflammatory component) of MS by fingolimod is primarily through its modulatory effect (via functional antagonism) on S1P receptors present on lymphocytes (predominantly S1P₁) preventing their egress from peripheral lymphoid organs to the peripheral circulation, and thus preventing their (including the auto-reactive clone/s) infiltration into the CNS, although direct effects on neural cells has been

suggested due to the fact the fingolimod can cross the BBB and that neural cells also express S1P receptors (Chun et al., 2010).

1.4.2 Metabolomics profiling in animal models of MS

The potential of metabolomics to identify an MS-specific, and indeed a disease-state specific, metabolic biomarker signature comes from experimental evidence in rodent models of MS, of which the EAE model is most commonly used. These studies demonstrate that the summative metabolic perturbation of the various pathogenic processes described above allows for the separation of experimental animals from appropriate controls by the use of comprehensive biochemical profiling and multivariate mathematical modelling. Furthermore, by interrogating the important discriminatory metabolites and the pathways involved, these studies also allow for the discovery of novel metabolic pathways/connections associated with the disease process.

1.4.2.1 Intracranial cytokine injection

One of the earliest studies investigating the presence of metabolome from specific neuropathological changes was performed by Griffin et al (Griffin et al., 2004). Intra-striatal injections of recombinant adenovirus expressing TNF- α or IL-1 β complementary DNA were performed in Wistar rats; TNF- α injection resulted in neuroinflammation characterised by macrophage and T cell infiltration while IL-1 β injection led to neutrophil recruitment. Using ^1H NMR spectroscopy performed on urine collected at day 7, a predictive model could be built separating TNF- α , IL-1 β and controls (null adenovirus injection and naïve animals) with a predictive accuracy of 79%, and the identification of the discriminatory urine metabolites were attributable to a stress response. It is important to note that the animals injected with the cytokines did not demonstrate any clinical paralysis, therefore their distinctive urine metabolic signatures are independent of the physical consequences of neuroinflammation.

1.4.2.2 Myelin basic protein induced EAE

Using an EAE model induced by MBP in Lewis rats, mass spectrometry proteomics and ^1H NMR metabolomics were performed on CSF taken at the onset of disease (day 10) (Blanchet et al., 2011). Integration of proteomics and metabolomics data with a mid-level fusion architecture analysis approach allowed the distinction of MBP-EAE, control (injected with complete Freud's adjuvant [CFA] without MBP) and naïve animals from each other. MBP-EAE animals (compared to controls and naïve) showed higher levels of lysine, glyceric acid and citrate, with lower concentrations of glutamine, valine, sucrose, β -hydroxybutyrate, acetone, succinate and pantothenate. Using the same model on a targeted mass spectrometry metabolomics platform, Noga et al showed the CSF metabolome obtained from day 14 (disease peak) MBP-EAE rats was clearly different from control and naïve animals, and indeed from MBP-EAE animals sacrificed at day 10 (onset of disease) (Noga et al., 2012). A variety of metabolic changes occurred in MBP-EAE animals; disease onset was characterised by decreased glutamine, arginine, citrulline, ornithine and branched chain amino acids (BCAA), while CSF at the peak of disease showed increased γ -amino butyric acid (GABA), putrescine, taurine, asparagine and O-phosphoethanolamine. These metabolic changes reflect neuronal excitotoxicity, and disruptions in nitric oxide, polyamine synthesis, anti-oxidant generation, energy regulation and phospholipid metabolism.

An interesting study by Battini et al on the same model looked at the metabolic changes within intact CNS tissues (Battini et al., 2018). Using a ^1H high resolution magic angle spinning NMR spectroscopy approach, various CNS regions (brain, optic nerve, cervical cord, thoracic cord and lumbar cord) taken from MBP-EAE animals at peak of illness (day 12) showed distinct metabolic signatures as compared to naïve animals, allowing highly predictive models to be built. Of note, MBP-EAE rats showed raised glucose, lactate, phosphocholine, amino acids (serine, taurine, alanine, lysine, glutamine) and reduced N-acetylaspartate – as mentioned earlier, a metabolite that is

believed to be exclusively synthesised in neurons and its decrease suggests neuronal injury (Moffett et al., 2007).

1.4.2.3 MOG induced EAE

Using an EAE model with MOG as an immunogen in C57BL6 mice, a ^1H NMR metabolomics study on urine taken at disease peak (day 17) showed that MOG-EAE animals could be separated from a variety of controls (fingolimod treated MOG-EAE, CFA injected, CFA with fingolimod treatment, saline injected, saline with fingolimod treatment and naïve animals) (Gebregiworgis et al., 2013). MOG-EAE animals, as compared to all controls as well as to only fingolimod treated MOG-EAE animals, showed higher hippurate and fructose levels, with lower concentrations of citrate, oxoglutaric acid, taurine and urea. An plasma LC-MS and GC-MS metabolomics study using the same model observed that MOG-EAE animals could be distinguished from CFA injected animals (i.e. without MOG peptide) at the chronic phase of the model (day 45), with 100 metabolites differing between the 2 groups (mostly downregulated in diseased animals) (Poisson et al., 2015). Pathway analysis showed changes in unsaturated fatty acids (linolenic acid and linoleic acid), glutamine and glutamate, pentose pathway metabolites, tryptophan and arachidonic acid metabolism. In a separate study using a similar study design on urine, the same group demonstrated that 105 urine metabolites were significantly altered by EAE induction, again, most metabolites were decreased in diseased animals (Singh et al., 2019). Pathway analysis showed that the most affected pathways involved phenylalanine, tyrosine and tryptophan biosynthesis, ubiquinone and other terpenoidquinone biosynthesis, phenylalanine metabolism, arginine and proline metabolism, tyrosine metabolism, taurine and hypotaurine metabolism. The authors then explored the metabolite pathways that overlapped between both their plasma and urine metabolomics studies. Metabolites connected to the overlapping pathways consist of phenyl-acetyl-glycine, phenyl-pyruvate, tyrosine, 4-hydroxyphenylpyruvate, tyramine, 4-hydroxy-phenylacetate and gentisate – all these seven metabolites relate to phenylalanine catabolism. As phenylalanine catabolism generates

precursors (tyrosine and tyramine) for neurotransmitters (dopamine, noradrenaline adrenaline), thyroid hormones and melatonin, the authors postulated the alteration of phenylalanine catabolism with resultant changes in these metabolites have functional implications on the CNS.

It has to be noted that most studies performed have only looked at one time point, either during the disease peak or during the chronic phase, without exploring the metabolic fluxes as a function of the MOG-EAE disease course. A LC-MS metabolomics study by Lee et al investigated the plasma metabolome of MOG-EAE animals at days 10 (early EAE), 20 (disease peak) and 30 (chronic phase), as compared to naïve animals (Lee et al., 2019). Blood was obtained from the same animal (via retro-orbital vein puncture) throughout the experiment. A total of 49 metabolites were significantly altered at each time point or across time points between EAE and naïve animals. Glycerophospholipids and fatty acyls showed the greatest changes, followed by several bile acids, amino acids, triglyceride, carnitines and sphingolipids. Network analysis suggests the involvement of perturbed lipid metabolism, immune and inflammatory responses. The authors suggested that in the acute phase, the pro-inflammatory phenomenon was accompanied by an increase in a few lysophosphatidylcholine (lysoPC) and sphingomyelin, inferring that myelin damage has begun. As the disease progresses, anti-inflammatory-related mechanisms are triggered, causing a significant downregulation in lipids, including lysoPC, phosphatidylcholine (PC) and poly-unsaturated fatty acids. At the chronic stage, these effects are exacerbated and there is a gradual increase in tauroconjugates because of progressive nerve injury.

1.4.2.4 Other EAE models

Using guinea pig spinal cord homogenate as an immunogen in Lewis rats, Lutz et al employed ^1H NMR spectroscopy to profile the metabolic changes in brain and spinal cord (cervical, thoracic and lumbar) homogenates in EAE animals, compared to controls (CFA injection only) and naïve animals (Lutz et al., 2013). Animals were sacrificed at days 12 – 15 (peak of disease). The authors observed

that the EAE and control animals have largely similar CNS metabolome, which suggests that CFA may be the primary mediator of these changes, rather than neuroinflammatory changes. These changes seen in both EAE and control animals suggest the disruption of neuronal and astrocytic osmoregulation (decreased taurine, decreased N-acetylaspartate, increased myoinositol), membrane phospholipid accumulation (raised total phospholipid, decreased choline), production of inflammatory signalling molecules (increased ethanolamine-containing phospholipids, elevated ratio of choline to ethanolamine containing phospholipids), increased glycolysis and reduced oxidative phosphorylation (increased lactate, decreased succinate), and depletion of fuel stores (decreased β -hydroxybutyrate).

Relapsing-remitting EAE models have been used to explore the metabolic signatures associated with relapses and remission in EAE. One such model utilises proteolipid protein (PLP) as an immunogen in SJL mice, generating two distinct episodes of relapses and remissions, followed by a chronic phase. Using this model, Mangalam et al performed mass spectrometry metabolomics on plasma obtained at day 45 (chronic phase) (Mangalam et al., 2013). Forty-four metabolites differed between EAE and control (CFA injection alone) animals, a majority of which were upregulated. EAE animals had higher free fatty acid levels (predominantly medium-chain and long-chain fatty acids, likely due to higher lipolysis or breakdown of membrane lipids or lipolysis from adipose tissue and liver), myoinositol (a astroglial marker), acylcarnitines (a marker of incomplete fatty acid β -oxidation), alpha-tocopherol (anti-inflammatory), salicylate (anti-inflammatory) and indolepropionate (anti-inflammatory), while lower levels of citrulline and KA were observed. EAE induction was also associated with changes in glycolysis (decreased 3-phosphoglycerate), TCA cycle (decreased α -ketoglutarate) and glycaemic control (increased 1,2-anhydroglucitol) and histidine metabolism. Pathway analysis revealed changes in primary bile acid biosynthesis, taurine and hypotaurine metabolism, tryptophan and histidine metabolism, linoleic acid, D-arginine and D-ornithine metabolism pathways.

Using Biozzi mice and murine spinal cord homogenate for EAE induction, the Oxford group studied the urine and plasma metabolic profiles on ^1H NMR spectroscopy in a chronic-relapsing EAE model, characterised by biphasic relapses (Dickens et al., 2015). Biofluid samples were taken at days 10 (before disease onset), 14 (first relapse), 28 (remission) and 38 (second relapse). Using urine metabolomics data, both EAE and controls (CFA injection only) could be separated from naïve animals at day 10. However, no predictive model could be constructed by comparing EAE and control animals at this time point, and only at day 14 was a separation observed. At day 38, EAE animals could be distinguished from control and naïve animals, as well as within EAE animals across time points. The discriminatory urine metabolites separating EAE from CFA and naïve animals were citrate, creatine, taurine, trimethylamine-N-oxide, trimethylamine and choline. Interestingly, using plasma metabolomics data, EAE animals could be not separated from controls during clinically silent periods (days 10 and 28), but a distinction could be established during relapses (days 14 and 38). The discriminatory plasma metabolites separating EAE from the other two groups were fatty acids, lactate, glucose and phosphocholine. In general, plasma provided higher discriminatory capability compared to urine.

1.4.2.5 Summary and limitations of current studies

In summary, using ^1H NMR spectroscopy and mass spectrometry metabolomics approaches combined with mathematical modelling, diseased animals could be differentiated from controls, as well as from naïve animals. The pathways and metabolites identified point to disruptions in nitric oxide metabolism, membrane lipid breakdown, glial proliferation, neuronal excitotoxicity and injury, increased pro-inflammatory signalling, activation of anti-inflammatory cascades, energy dysregulation, oxidative stress, mitochondrial dysfunction and perturbed phenylalanine-tryptophan-tyrosine and taurine metabolism.

It is clear that several limitations are present in the above-mentioned studies, of which the most obvious is the confounding effect of paralysis, weight loss, food and water deprivation in EAE animals, which can affect the metabolome significantly. All EAE models are associated with clinical paralysis, and although it may be argued that this reflects human MS, it must also be noted that 90 – 95% of MS lesions are asymptomatic (Goodin et al., 2016), and that the EAE clinical manifestations are disproportionately severe. For example, the commonly used MOG-EAE model consistently results in complete hind-limb paralysis at the peak of disease. Therefore, the metabolomics studies in rodents to date are unable to disentangle the direct contributions arising from neuroinflammatory lesions from the functional sequelae of such lesions. Furthermore, although there are overlaps in the pathways implicated, there are significant variations in EAE induction protocols across studies, with different immunogens used and varying time points being investigated. Only two studies mentioned above had three or more time points, precluding the assessment of global metabolic fluxes, and indeed of individual metabolites, over the disease course. Despite the limitations mentioned, these lines of experimental evidence suggest that metabolic signatures associated with specific pathophysiological changes (be it disease specific or disease-state specific) could be established in vivo, providing support for those observations seen in human studies.

1.5 Review of metabolomics studies in human CNS IDD

The previous section has provided biological plausibility and in vivo evidence that an MS-specific metabolic biomarker signature, consisting of a combination of metabolites and lipid species, can be obtained in patients. Additionally, it is conceivable that by using strict within-disease subgroup definitions, metabolic signatures that inform on disease progression and inflammatory activity can also be defined, as well as signatures that characterise other CNS IDD such as AQP4-Ab and MOG-Ab associated CNS inflammatory diseases. The current section aims to review the clinical metabolomics studies that have been performed in human CNS IDD to date. As lipids are classed as metabolites,

studies focusing only on lipidomics (which is the large scale study of lipid species) are also included in this review for completeness.

1.5.1 Human CNS IDD

The majority of metabolomics studies in CNS IDD have been performed in MS. Most of these studies demonstrate that metabolomics could be used to reliably distinguish MS from both non-MS diseased controls as well as healthy controls (HC). There is also emerging evidence that metabolomics could be useful to differentiate between RRMS and progressive MS, to identify inflammatory activity and to separate MS from NMOSD. Studies investigating a single metabolite or standard lipid panel are not included, as the purpose of this review is to provide the background for the utility of large scale biochemical profiling to produce biomarker signatures in CNS IDD and to contextualise the results arising out of this thesis.

1.5.1.1 MS vs. healthy controls

There is robust evidence that MS can be differentiated from HC using a metabolomics/lipidomics approach coupled with multivariate statistical analyses, with predictive accuracies ranging from 60 – 100% and area under the curve (AUC) values ranging from 93 – 100%. Most studies compared RRMS to HC, and in studies whereby the MS group comprised of both RRMS and progressive MS, the majority of patients were RRMS. Although such a comparison is not clinically valuable, it can provide insights into the metabolic changes associated with MS pathophysiology.

As seen in rodent studies, the metabolites implicated relate mostly to the dysregulation of energy metabolism, mitochondrial dysfunction and increased oxidative stress. One of the first mass spectrometry metabolomics study in MS reported an increase of lactate, sorbitol and fructose in the CSF of MS patients (Regenold et al., 2008). These findings are indicative of mitochondrial dysfunction with an upregulation of extra-mitochondrial glucose metabolism, namely via anaerobic glycolysis

(hence the increased lactate) and the polyol pathway (hence the elevated sorbitol and fructose). Alterations of CSF metabolites linked to energy metabolism are confirmed in a ^1H NMR metabolomics study, showing lower citrate, acetate and glucose (metabolites linked to the TCA cycle and glycolysis) in RRMS patients (Kim et al., 2017). These observations are also reproduced using serum metabolomics, with an integrative metabolomics-transcriptomics study showing enrichment of pathways associated with mitochondrial dysfunction and apoptosis (Andersen et al., 2019). A well-powered serum metabolomics study showed an upregulation of purine metabolism with increased levels of oxypurines (reflecting mitochondrial dysfunction), elevated nitrites/nitrates (indicating nitrosative stress), increased malondialdehyde (a product of lipid peroxidation, implying oxidative stress) and a decrease in ascorbic acid (suggestive of decreased anti-oxidative capability) in MS compared to HC (Tavazzi et al., 2011). Elevations in oxypurines were again observed in another well-powered study by the same group (Lazzarino et al., 2017). Bhargava et al in two separate studies reported that RRMS patients displayed altered levels of plasma redox metabolites indicative of oxidative stress, and interestingly, in one of the studies, altered xenobiotic metabolism, which suggests the imbalance of gut microbiota (Bhargava et al., 2017; Bhargava et al., 2019).

Dysregulation of other pathways, some known to be associated with MS whilst others are novel, have been reported. A ^1H NMR serum metabolomics study showed increased serum scylloinositol in RRMS patients, which is likely to be related to glial proliferation/reactive astrogliosis (Moussallieh et al., 2014). An adequately powered ^1H NMR study reported low plasma tryptophan levels (as well as energy related metabolites) in MS patients, reflecting altered kynurenine metabolism as tryptophan is the metabolic precursor of kynurenine (Cocco et al., 2016). The perturbation of the kynurenine pathway in MS is further illustrated in a well-powered mass spectrometry study demonstrating that the serum kynurenine to tryptophan ratio increases over time within RRMS patients (Lim et al., 2017). These lines of evidence showing perturbed tryptophan metabolism are consistent with those observed in rodents (Mangalam et al., 2013; Poisson et al., 2015; Singh et al., 2019). Using a targeted

metabolomics approach, Haghikia et al reported an increase in serum asymmetric dimethylarginine (ADMA) in MS (ADMA being a strong inhibitor of neuronal nitric oxide synthase), reinforcing that disrupted nitric oxide metabolism is implicated in MS pathogenesis (Haghikia et al., 2015). Dysregulated BCAA metabolism has also been shown by metabolomics, characterised by low levels of plasma isoleucine, leucine and valine in MS patients (Kasakin et al., 2019). This is likely to be the result of BCAA catabolism to fuel the TCA cycle and/or BCAA consumption from immune activation. The same study also showed increased plasma glutamate, which likely reflects excess glutamate, a well-recognised phenomenon (i.e. glutamate excitotoxicity) in MS and other chronic neurodegenerative conditions. The low concentrations of BCAA have also been substantiated in CSF as well as in serum of MS patients by various groups (Mehrpour et al., 2013; Podlecka-Pietowska et al., 2019), consistent with rodent CSF studies (Noga et al., 2012). Using a GC-MS platform on plasma, Poddighe et al observed an enrichment of the asparagine biosynthesis pathway and also altered citrulline biosynthesis in RRMS patients who have been DMT free for 90 days, although the relevance of these metabolic pathways to MS pathogenesis is yet unclear (Poddighe et al., 2017).

With regards to lipidomics, both mass spectrometry and NMR spectroscopy approaches have demonstrated clear alteration of lipid profiles in MS patients as compared to HC. A ¹H NMR plasma lipidomics study showed that the LDL particle distribution of RRMS patients is shifted towards smaller LDL particles, as there is a decrease in large LDL together with a reduction in LDL size (Jorissen et al., 2017). In addition, low-BMI RRMS patients displayed a change towards increased levels of small HDL. Using functional assays, the investigators demonstrated that ATP-binding cassette transporter G1 (ABCG1)-mediated cholesterol efflux was reduced at both serum and cellular levels, and that small HDL from RRMS patients was not effective in suppressing an inflammation-induced transcriptional profile of pro-inflammatory cytokines. In another ¹H NMR lipidomics study on plasma, Gafson et al reported that 23 lipids within the VLDL and HDL sub-fractions were higher in RRMS (Gafson et al., 2018). Strong correlations were observed between VLDL-2 free cholesterol with

CCL-17 and with IL-7, suggesting that cytokines increase the production and reduce the clearance of VLDL. A well-powered serum LC-MS study by Villoslada et al observed that MS patients (as compared to HC) displayed disturbed sphingomyelin and lysophosphatidylethanolamine (PE) profiles, likely reflecting myelin destruction as well as activation of the immune system as phospholipids act as signalling molecules (e.g. for astrocyte proliferation), lipid rafts and cytokine precursors (Villoslada et al., 2017). Another well-powered LC-MS study coupled with various advanced machine-learning multivariate statistical algorithms showed that 8 lipid species were perturbed in MS, with the precursors of some of these lipid species being shown to be upregulated in leucocytes isolated from MS patients (Lotsch et al., 2018).

Other biofluids apart from CSF, serum/plasma have also been used for metabolic profiling and conclusions from these studies are consistent with those described earlier. A small study using urine on a ^1H NMR platform, demonstrated alterations in metabolites linked to the TCA cycle, ketone bodies and glycolysis, again pointing towards a dysregulation in energy metabolism (Gebregiorgis et al., 2016). An interesting study using lacrimal fluids metabolomics/lipidomics was able to distinguish MS from HC. PC, lysoPC and sphingomyelins classes were different in MS, most of which were lower in MS, suggesting membrane breakdown and the involvement of these lipid species in various inflammatory stimuli seen in MS (Cicalini et al., 2019).

1.5.1.2 MS vs. non-MS neurological controls

Several studies, instead of using HC, looked at comparing MS against non-MS neurological controls to find MS-specific metabolic signatures to aid in diagnosis. However, it has to be noted there is marked heterogeneity in these controls, ranging from thunderclap headache and Guillain Barre syndrome to idiopathic intracranial hypertension and dementia, many of which would not be clinically relevant comparisons. Also, the heterogeneity within these controls means that the discriminatory metabolites are likely to differ across studies and most of these studies have small

sample sizes (i.e. <20 subjects in each comparative group). Despite these study limitations, a clear distinction in the metabolic/lipidomic profiles of MS and non-MS controls could be established using multivariate statistical modelling, and the implicated metabolites remarkably agrees with the comparisons made against HC, relating to the disruption in energy metabolism, nitric oxide metabolism, kynurenine metabolism, cellular membrane damage, lipid peroxidation and glutamate excess, further supporting a MS-specific metabolic profile.

The first ^1H NMR metabolomics study in MS showed an increase in CSF lactate and reduced formate within RRMS patients in relapse as compared to non-MS neurological controls, although the control group was not well-defined (Simone et al., 1996). The increased in lactate was thought to be from infiltrated macrophages, which current knowledge suggests, results from the Warburg effect observed in macrophages upon activation. No differences in lactate were observed between RRMS in remission and neurological controls, although a reduction in formate was still noted. The reduction in formate was postulated to be due to disrupted formaldehyde metabolism related to choline-glycine cycle impairment. In another ^1H NMR CSF metabolomics study, Reinke et al was able to separate MS (comprising of RRMS, SPMS and CIS patients) from non-MS controls (mixed diagnoses) with a sensitivity of 93% and specificity of 71% by employing hierarchical cluster analysis, with the discriminatory metabolites again linked to dysregulation of energy metabolism. Subsequent studies corroborated these findings, and at the same time, allowing highly discriminatory diagnostic models to be built (de Oliveira et al., 2019; Podlecka-Pietowska et al., 2019).

Using a targeted metabolomics approach, Haghikia et al demonstrated an increase in CSF asymmetric dimethylarginine (ADMA) (a strong inhibitor of neuronal nitric oxide synthase) in MS compared to non-MS neurological controls, reinforcing that disrupted nitric oxide metabolism is implicated in MS pathogenesis (Haghikia et al., 2015). In paediatric MS, a mass spectrometry study showed that higher serum tryptophan and indole lactate was associated with a lower risk of developing paediatric MS, i.e. lower tryptophan levels in paediatric MS compared to non-MS

controls (Nourbakhsh et al., 2018), again implicating perturbed tryptophan metabolism in MS pathogenesis. Higher kynurenine level was correlated with relapse rates, and higher indole propionate was inversely associated with EDSS. Furthermore, it was shown that indole propionate and indole acetate (both anti-inflammatory metabolites) were positively correlated with the symbol digit modalities test scores. The finding that the indole metabolites and tryptophan are important metabolites is interesting as indole metabolites are produced by the action of gut commensals on tryptophan and thus suggests gut microbiota dysbiosis in paediatric MS.

As for lipidomics, a LC-MS study showed that compared to non-MS controls (variety of conditions including Guillain Barre syndrome and stroke) and HC, there is a decrease in lysoPC to PC ratio in the serum of MS patients, indicating altered phospholipid metabolism in MS (Del Boccio et al., 2011). The same group followed up with a CSF study showing that a valid predictive model could be constructed to distinguish RRMS patients from non-MS controls, with MS patients again demonstrating lower levels of certain lysoPC and PC species, lysophosphatidylinositol, phosphatidylinositol, phosphatidic acid, and an increase in glutamate (Pieragostino et al., 2015), with the AUC of individual metabolites ranging from 0.71 – 0.81. Another follow up CSF lipidomics study by the same group showed that MS and non-MS controls can be distinguish using multivariate models with an AUC of 0.942, once again showing the downregulation of sphingomyelin, PC and lysoPC species (Pieragostino et al., 2018). Functional assays showed that acid sphingomyelinase (the enzyme that catalyses the breakdown of sphingomyelins to ceramide and phosphocholine) expression and activity was increased in MS and this enzyme was enriched in CSF derived exosomes. An interesting, albeit small, study showed an increased in CSF 8-iso-prostaglandin 2 α (a lipid oxidation marker) in MS patients compared to non-inflammatory neurological controls (Gonzalo et al., 2012). Western blotting showed an increase in protein expression of neuroketals, malondialdehyde-lysine and carboxymethyl-lysine (proteins damaged by lipid peroxidation) in the CSF and a corresponding increase in antibodies against these damaged proteins. A follow up study

by the same group observed an upregulation of diglycerides and a downregulation of triglycerides in the CSF of MS patients compared to non-MS neurological controls, pointing towards a defect in the acyl-CoA:diacylglycerol acyltransferase enzyme, which is related to a worse insulin sensitivity (Nogueras et al., 2019). Other smaller studies have similarly reported a variety of lipid species changes in the CSF of MS patients related to altered fatty acid synthesis/metabolism in the CNS as well as membrane disruption (Podlecka-Pietowska et al., 2019).

1.5.1.3 Within RRMS – stratifying by disease activity, disability status and drug effect

Simone et al using a ^1H NMR platform found increased CSF lactate in RRMS patients with GAD-enhancing lesions compared to those without GAD-enhancing lesions, and the CSF lactate levels correlated with lactate levels derived from ^1H magnetic resonance spectroscopy (MRS) of those GAD-enhancing lesions (Simone et al., 1996). As mentioned, this is likely due to macrophage activation and mitochondrial dysfunction. Lutz et al also made the same observations using a similar approach in the same biofluid. They investigated CIS patients (although looking at their inclusion criteria, most would have been defined as RRMS by the latest criteria) found increased lactate and β -hydroxybutyrate in patients with GAD-enhancing lesions compared to those without (Lutz et al., 2007). The higher β -hydroxybutyrate is interesting, especially when it is now recognised that astrocytes can perform gluconeogenesis when glucose levels are low. Therefore, this indicates an energy deficient state in which fatty acids are converted to ketone bodies via β -oxidation by astrocytes, and the ketone bodies are transported to neurons to be used as energy substrates. A CSF mass spectrometry metabolomics study by Park et al showed that MS relapses are characterised by alterations in fatty acid metabolism, along with arginine-proline and glutathione perturbations (Park et al., 2016). On the ^1H NMR platform, a reduction in CSF isoleucine and valine characterised MS relapses, and again, this is likely to be related to energy deficiency and immune activation (Kim et al., 2017). Villoslada et al reported that various serum PC and lysoPC species as well as arachidonic acid were the main discriminatory metabolites between RRMS patients who had relapses and those

without relapses during a 2-year period (Villoslada et al., 2017). The same study was also able to build a valid predictive model separating patients with Extended Disability Status Scale (EDSS) <3.0 from those with EDSS >4.5, with tryptophan and glutamate amongst other discriminatory metabolites. An interesting study using mass spectrometry assessed the metabolic effect of starting dimethyl fumarate (DMF) in plasma. It showed that a multitude of metabolic changes occurred, with decreased fatty acids and increased lysolipids, phospholipids and plasmalogens, after commencing DMF (Bhargava et al., 2019). The reduction in fatty acids correlated with the decrease in CD8 positive effector memory cells as well as the increase in CD8 positive naïve T cells, and these effects could be explained by the activation of hydroxycarboxylic acid by DMF.

1.5.1.4 Progressive MS vs. RRMS and controls

Given the clinical difficulty in establishing progressive MS, especially early secondary progressive MS, studies have been undertaken to explore the metabolic signatures of disease progression. The first metabolomics study to distinguish RRMS and SPMS patients was a ^1H NMR study from Oxford showing that using serum metabolomics was able to distinguish these two MS stages with a predictive accuracy of 87% and an AUC of 0.94 (Dickens et al., 2014). Top discriminatory metabolites revealed increases in lipoprotein and β -hydroxybutyrate in RRMS while SPMS is characterised by raised phosphocholine, N-acetyl species and glucose. A well-powered study in RRMS and progressive MS showed that serum phosphatidylethanolamine (PE) containing VLCFA and plasmalogen ethanolamine containing docosahexaenoic acid were most elevated in SPMS, followed by PPMS and RRMS (disease duration ≥ 13 years), RRMS (disease duration <13 years), and was the lowest in HC (Senanayake et al., 2015). These lipid derangements are likely to be explained by the reduction of fatty acid β -oxidation due to mitochondrial dysfunction. The same study also showed higher levels of serum gastrointestinal tract acids (anti-inflammatory long chain fatty acids) in RRMS (disease duration <13 years), followed by RRMS (disease duration ≥ 13 years) with the lowest levels in SPMS patients, reflecting an inability to sustain protective responses in the progressive stages of MS. In

another well-powered targeted metabolomics focusing on purine metabolism, nine serum metabolites were found to be different between MS and controls (Lazzarino et al., 2017). Using these discriminatory metabolites, a composite biomarker score was computed, with the highest scores observed in SPMS and PPMS while lower scores were seen in RRMS. This biomarker score also correlated with EDSS and MRI parameters of neurodegeneration, suggesting that this metabolite composite score reflects increasing energy deficiency and mitochondrial dysfunction in progressive MS. A small study looking at SPMS and HC observed that four CSF metabolites were increased in SPMS on a multivariable regression model, and amongst them, increased ADMA and glycine, implicating nitric oxide metabolism and oxidative stress respectively (Carlsson et al., 2020).

With regards to the kynurenine pathway, a targeted metabolomics study discovered that serum QA was the highest in PPMS followed by SPMS and then RRMS patients (Lim et al., 2017). Indeed, the QA to kynurenic acid (KA) ratio was higher in progressive MS patients compared to RRMS. The authors suggest that activated macrophages are a major source of QA, mediating excitotoxicity at the NMDA receptor. A small CSF study also implicated the kynurenine pathway in progressive MS. Using a global LC-MS metabolomics approach to compare RRMS and SPMS, valid predictive models could be constructed and interrogation of the discriminatory metabolites revealed increased indolepyruvate which plays a role in kynurenine/tryptophan metabolism (Herman et al., 2018). In the same study, using a combination of 11 variables that included clinical, metabolites and proteins, an AUC of 0.97 was achieved. In a separate study by the same group using the same cohort of patients, multivariate models with AUC of 0.92 could be constructed, and again, tryptophan metabolism (amongst others like phenylalanine and pyrimidine metabolism) was implicated in SPMS (Herman et al., 2019). A global metabolomics/lipidomics study with multivariate analysis showed that PPMS and RRMS could be distinguish with an AUC of 0.70 – 0.79, with increase in tiglyceronine in PPMS (Stoessel et al., 2018). Compared to HC, PPMS had lower levels of tryptophan, γ -Linolenic acid and lysoPC (20:0). Interestingly, lysoPC (20:0) levels decreased steadily over time in PPMS

suggesting perturbations in the glycerophospholipids and linoleic acid pathways, which could be due to membrane disruptions. Interesting, the decrease in lysoPC species was also noted in the chronic phase of the MOG-EAE model (Lee et al., 2019). These lines of evidence suggests that there is indeed a metabolic difference between RRMS and progressive patients, which could be useful in understanding the pathophysiology of progression leading to potential druggable targets. The studies also support the use of metabolomics/metabolites to distinguish these MS subgroups for objective inclusion in progressive MS trials and also to help to identify early progression in the clinic.

1.5.1.5 NMOSD vs. MS and controls

A global metabolomics study on the ^1H NMR platform in CSF showed that valid predictive models could separate NMOSD from HC, and that the changes relate to energy dysregulation, however, the proportions of antibody-positive patients were unknown (Kim et al., 2017). A small study by Haghikia et al demonstrated an increase in serum ADMA (a strong inhibitor of neuronal nitric oxide synthase) and arginine (the metabolic precursor of nitric oxide) with lower nitrate and nitrite in NMO patients compared to HC, indicating impaired nitric oxide availability presumably caused by the inhibitory action of ADMA on nitric oxide synthase (Haghikia et al., 2015). Six out of nine NMO patients in that study were AQP4-Ab positive. Moussallieh et al, using ^1H NMR metabolomics showed that a valid predictive model could be built to distinguish RRMS from NMO patients with increased serum scylloinositol in RRMS patients (AUC of scylloinositol = 0.982), which is likely to be related to glial proliferation/reactive astrogliosis (Moussallieh et al., 2014). However 50% of the NMO group were AQP4-Ab negative. The same study also reported increased serum acetate in NMO compared to MS and HC, which the authors postulated is due to impaired uptake/metabolism by astrocytes. A GC-MS study by Park et al showed that NMO relapses are characterised by alterations in CSF fatty acid metabolism, along with phenylalanine and cyanoamino acid perturbations (Park et al., 2016). Again, the AQP4-Ab status of these patients within the study was not known. A small study using urine metabolomics showed a reduction in creatinine, β -hydroxybutyrate, 3-hydroxyisovalerate,

methyilmalonate, and an increase in oxaloacetate in AQP4-Ab compared to RRMS patients, although it is difficult to conclude which pathways are up/downregulated in the respective diseases (Gebregiworgis et al., 2016).

The first study to properly characterise the differences between RRMS, AQP4-Ab and MOG-Ab patients was performed in Oxford, using a ^1H NMR platform on plasma (Jurynczyk, Probert, et al., 2017). RRMS could be distinguish from AQP4 with a predictive accuracy of 89% while the accuracies for the RRMS vs. MOG and AQP4-Ab vs. MOG-Ab models were both 73%. It is important to note the distinction of AQP4-Ab NMOSD and RRMS was independent of AQP4-Ab antibody titre. Indeed, when patients who were seronegative for AQP4-Ab at study sampling (but previously positive at initial diagnosis) were used as a predictive set, the model classified all these patients correctly, i.e. accuracy of 100%. It was also observed that each disease displayed a distinct set of discriminatory plasma metabolites relative to the other two diseases.

1.5.1.6 Summary and limitations of current studies

In summary, metabolomics with multivariate modelling could be used to robustly distinguish CNS IDD from controls and appears to be promising in discriminating RRMS from progressive MS. The identified discriminatory metabolites suggest energy deficiency, oxidative stress, mitochondrial dysfunction, altered lipoprotein function, cellular membrane disruption, perturbed kynurenine/tryptophan metabolism, glutamate excitotoxicity and disrupted nitric oxide metabolism in MS patients, with some processes showing worsening perturbation with disease progression. It must be stated that all these processes are occurring simultaneously in patients, in a correlated and inter-dependent manner, thus metabolomics as a platform together with mathematical modelling can provide a summative and collective snapshot of all these biochemical processes.

However, there are several important limitations within the clinical metabolomics studies performed thus far. Apart from a few well-powered studies, the majority of studies have very small sample sizes

with less than 20 patients in the main exploratory group, precluding the generalisability of the mathematical models built. Earlier metabolomics studies also employed univariate analyses, which are clearly not suitable for high dimensional, correlated metabolomics data. Even when multivariate analyses were used, only few studies specified the use of cross-validation methods and iterative independent test sets, which, are crucial in predictive modelling to provide a precise measure of the predictive capabilities. Of particular concern is the lack in consistency with regards to sample-handling, especially when it is known that time to blood centrifugation and freeze-thaw cycles can alter the metabolic profile substantially (this is further elaborated in the Introduction of Chapter 7). Most studies do not detail this critical step, which is an obvious potential confounder, especially when samples from different centres were used or when samples from sourced from the community or biobanks.

As metabolomics is a relatively new approach in CNS IDD, such ‘teething’ problems are not unexpected. Future studies would need to address these limitations, validate previous findings and to explore new scenarios in which metabolomics could be useful, specifically with regards to identifying early disease progression, inflammatory activity and refining CNS IDD classifications. Additionally, as almost all metabolomics studies in CNS IDD are cross-sectional, future studies would need to explore the changes in metabolite (and their signatures) concentrations temporally, with respect to the clinical question of interest.

1.6 Basic principles of nuclear magnetic resonance spectroscopy

NMR spectroscopy is the analytical technology for metabolomics employed in this thesis. The following sections aim to provide a basic and intuitive introduction to the principles of liquid state NMR spectroscopy, rather than an in-depth explanation of quantum mechanics of spins in magnetic fields, which is difficult to visualise by conventional means. The textbooks by Hore (Hore, 2015) and Keeler (Keeler, 2010) served as references for these explanations.

The ability to utilise NMR spectroscopy is dependent on a few fundamental components: (1) the presence of a non-zero spin magnetic nucleus, (2) application of an external magnetic field leading to (3) the generation of differing nuclear magnetic moments resulting in (4) an energy difference between the different quantised energy states which can be (5) measured using radiofrequency pulses to induce transitions between the different energy states.

1.6.1 Spin angular momentum

Magnetic nuclei possess an intrinsic angular momentum known as spin angular momentum (also called spin). Only nuclei having a non-zero spin quantum number (e.g. ^1H , ^{13}C , ^{15}N) exhibit spin angular momentum, while nuclei having a zero spin quantum number (^{12}C , ^{16}O) possess no spin and are thus invisible on NMR spectroscopy. The spin quantum number is determined by the number of unpaired nucleons and only nuclei that have an uneven number of protons or neutrons have a non-zero spin quantum number and thus have spin angular momentum.

1.6.2 Magnetic dipole moment

The presence of spin angular momentum produces a magnetic dipole moment, therefore nuclei that possess spin can be thought of as small electromagnets. Expectedly, the magnitude of the magnetic dipole moment is proportional to the magnitude of the spin angular momentum, and their vectors are parallel to each other.

The ^1H nucleus is by far the most popular nucleus for NMR spectroscopy because of its high natural abundance (99.985%) and its high magnetogyric ratio. The high magnetogyric ratio results in a relatively higher NMR resonance frequency and a greater energy difference between the two quantised energy states, therefore increasing its NMR signal (see section 1.6.3).

In the absence of an external magnetic field, the nuclear spins are orientated isotropically (i.e. uniformly distributed in all directions) (**Figure 1.7 A**). However, the application of an external

magnetic field (B_0) causes the nuclear spins to align in the direction of B_0 , akin to a compass aligning in a north-south axis (**Figure 1.7 B**). For the ^1H nucleus, there are two possible alignments in an external magnetic field; one parallel to B_0 and one anti-parallel to B_0 . The ratio of parallel ('spin-up') and anti-parallel ('spin-down') spins are nearly identical but not exactly, with a slight excess of spins parallel to the external magnetic field, resulting in a net macroscopic magnetisation (M_0) parallel to the external magnetic field. Of note, 'spin-up' nuclei have a lower energy level while 'spin-down' nuclei have a higher/upper energy level.

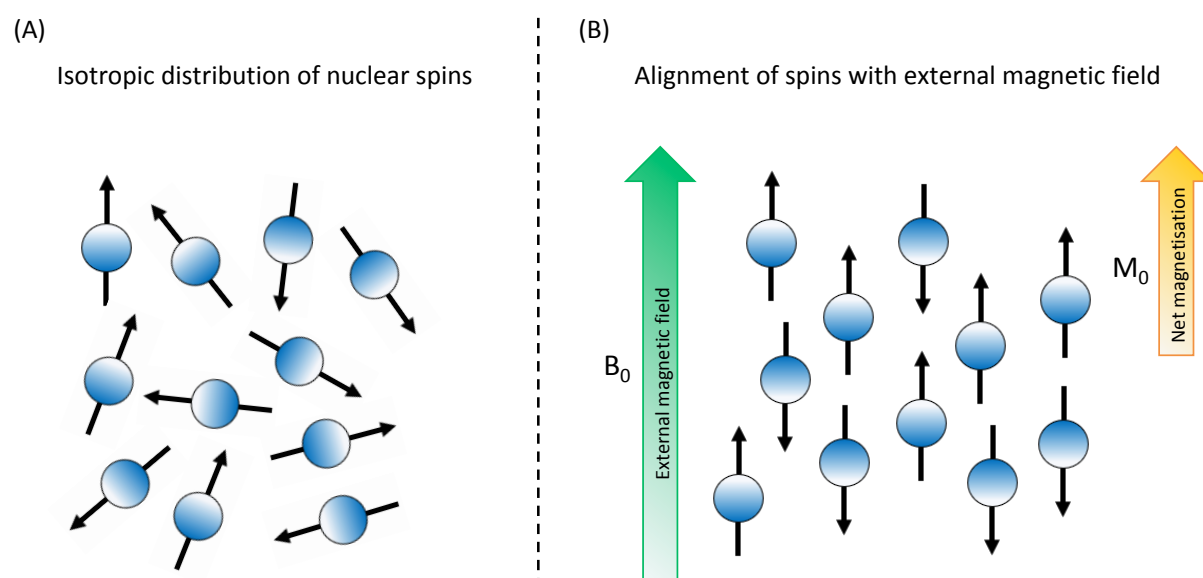


Figure 1.7: Distribution of spins with and without an external magnetic field. **(A)** In the absence of an external magnetic field, the nuclear spins are orientated isotropically, and thus there is no net magnetisation. **(B)** Application of an external magnetic field (B_0) results in the nuclear spins adopting either a parallel or anti-parallel orientation (in the case of a spin $\frac{1}{2}$ nuclei). A small excess of parallel spins results in a net magnetisation (M_0) parallel to the external magnetic field.

1.6.3 Energy levels and resonance frequency

1.6.3.1 Energy levels

From the previous section, it is evident that the application of an external magnetic field results in a slight excess of parallel/'spin-up' nuclei that have a lower energy state, with a slight deficit of anti-

parallel/'spin-down' nuclei with a higher/upper energy state. This results in an energy difference , ΔE , between the two energy states.

NMR spectroscopy in essence involves the application of a radiofrequency pulse (as a source of electromagnetic energy) which promotes the 'spin-up'/lower energy nuclei to a 'spin-down' upper energy state (**Figure 1.8**). The radiofrequency pulse is then turned off and the system is allowed to equilibrate, returning to the baseline energy level.

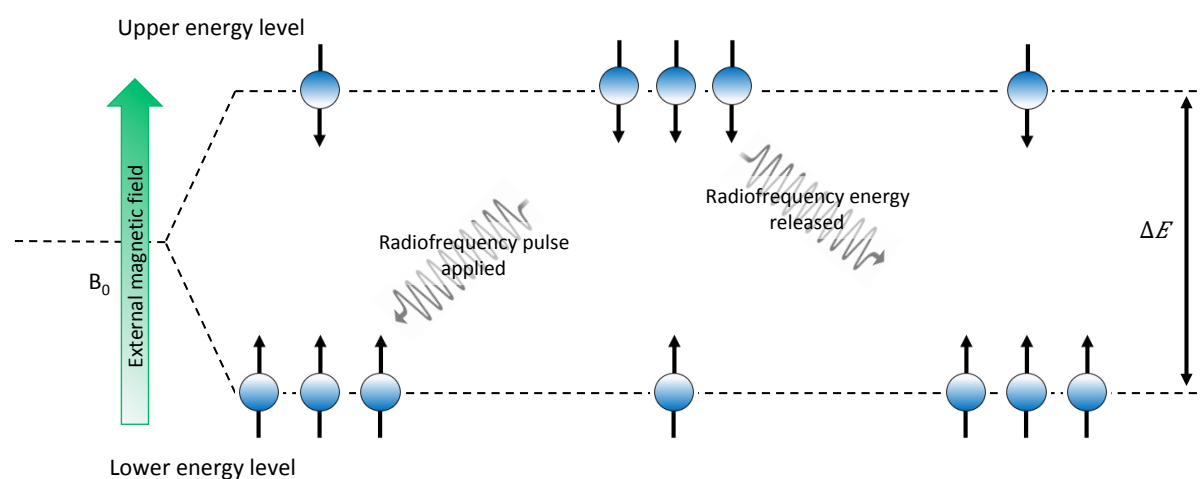


Figure 1.8: Schematic representation of quantised energy levels in NMR spectroscopy. The application of an external magnetic field (B_0) results in a slight excess of 'spin-up' nuclei that exist in a lower energy level with a corresponding deficit in 'spin-down' nuclei with an upper energy level. ΔE refers to the energy difference between the two energy states. The application of a radiofrequency pulse promotes 'spin-up' nuclei from a lower energy state to the higher energy state. The radiofrequency pulse is then switched off. During energy re-equilibration, the nuclei return to their lower energy level state and the excess energy is then emitted and detected.

1.6.3.2 Resonance frequency

The resonance frequency (ν), which is specific for each nuclear species, is given by the equation:

$$\nu = \frac{\gamma B_0}{2\pi}$$

where γ is the magnetogyric ratio of the nuclear species and B_0 is the magnitude of the external magnetic field.

The energy difference (ΔE) and the resonance frequency (ν) is thus related to each other by this equation:

$$\Delta E = h\nu$$

where h is the Planck's constant.

The magnetic field (B) experienced by a nucleus within an atom or a molecule differs marginally from the applied external magnetic field (B_0), therefore its exact resonance frequency is determined by the chemical environment of the nucleus. In other words, different nuclei (of the same nuclear species) within the same molecule can have varying resonance frequencies due to their differing chemical environment causing variation in the magnetic field experienced (see section **1.6.5** for the basis of differing chemical environment). The exact resonance frequency (also known as the NMR frequency) of a particular nucleus in a molecule is thus given by this equation:

$$\nu = \frac{\gamma B}{2\pi}$$

where γ is the magnetogyric ratio of the nuclear species and B is the magnitude of the magnetic field experienced by the particular nucleus.

1.6.4 Radiofrequency pulse and free induction decay

1.6.4.1 Application of the radiofrequency pulse

When an external magnetic field (B_0) is applied along the z-axis, the nuclear magnetic moments/spins, will precess around this axis (**Figure 1.9**). As the nuclear magnetic moments precess about the z-axis, it maintains a constant angle with respect to B_0 and therefore a constant projection

onto B_0 . The net magnetisation (M_0) is therefore parallel to the z-axis and B_0 . This motion is similar to that of the axis of a gyroscope and is known as Larmor precession. The rate of this precession is thus known as the Larmor frequency (ν_0) and is given by this equation:

$$\nu_0 = -\frac{\gamma B_0}{2\pi}(1 - \sigma)$$

where γ is the magnetogyric ratio of the nuclear species, B_0 is the magnitude of the external magnetic field and σ is the shielding constant (see section 1.6.5 for a description on shielding).

Similarly, the Larmor frequency can be expressed as its angular counterpart, the Larmor angular frequency, given by this equation:

$$\omega_0 = 2\pi \nu_0 = -\gamma B_0 (1 - \sigma)$$

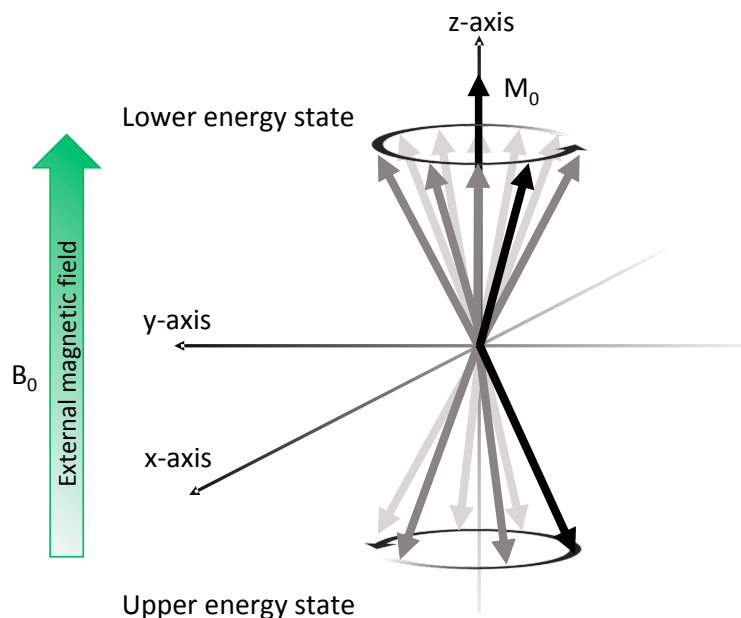


Figure 1.9: Application of an external magnetic field (B_0) causes the nuclear spins to precess about the z-axis with the net magnetisation (M_0) parallel the z-axis and B_0 .

It is the Larmor frequency that is detected by the NMR spectrometer. However, in order to observe the Larmor frequency, the net magnetisation must be perturbed away from the z-axis. This is

achieved by the application of a radiofrequency pulse (at the Larmor frequency) at an angle perpendicular to the external magnetic field, which will rotate the equilibrium magnetisation towards the x-y plane (**Figure 1.10**). If the radiofrequency pulse is applied for a sufficient length of time, the magnetisation is completely rotated from the z-axis to the x-y plane. This radiofrequency pulse is thus called a 90° excitation pulse because the net magnetisation is perturbed by 90° and is now precessing in the x-y plane.

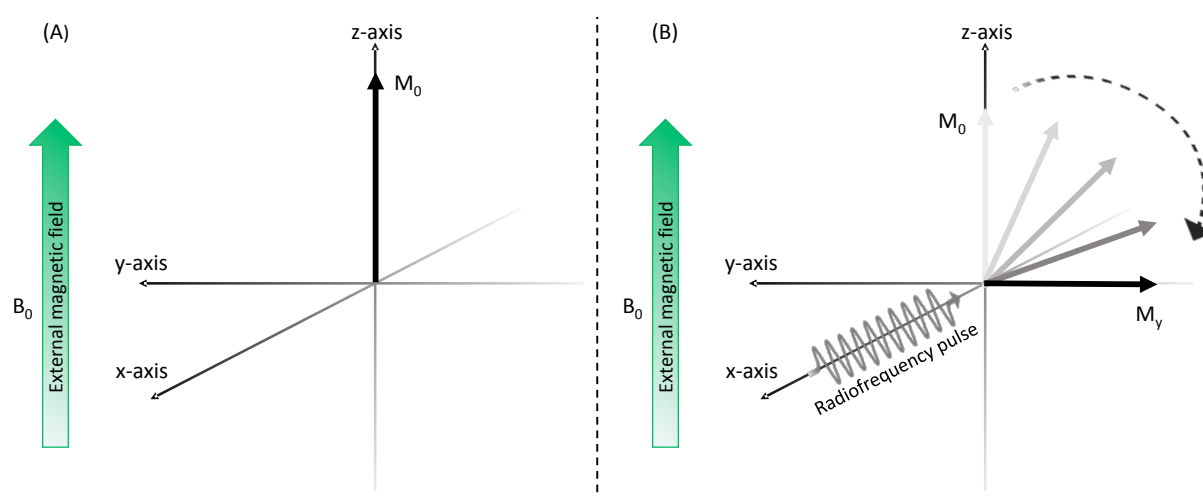


Figure 1.10: 90° excitation pulse. (A) At thermal equilibrium, the net magnetisation (M_0) is along the z-axis. (B) The application of a radiofrequency pulse along the x-axis at the Larmor frequency causes the net magnetisation to rotate, and if applied for a sufficient length of time, this will allow the net magnetisation (M_y) to be transferred onto the y-axis. The magnetic moments/spins are now precessing in the x-y plane.

1.6.4.2 Free induction decay

As the precession of the nuclear magnetic moments/spins in the x-y plane represents a state of disequilibrium, the net magnetisation returns to the direction of the z-axis in the absence of the radiofrequency pulse (**Figure 1.11**). The precessing magnetisation (back to equilibrium) will induce a corresponding oscillating voltage in the detection coil. This time-domain signal, i.e. the free induction decay, is digitised and then Fourier transformed in order to obtain a frequency spectrum of the NMR signal, i.e. the NMR spectrum, expressed as parts per million (ppm).

NMR experiments thus differ in their pulse sequences, which in turn vary in their number, duration, strength, phases, frequencies and timings. These can be precisely controlled by a modern NMR spectrometer. To use an intuitive analogy, the whole sequence of excitation and relaxation is akin to striking (radiofrequency pulse) bells (nuclei) of varying sizes (different chemical shifts) at the same time and then observing the sound produced (NMR resonance) diminishing over time (free induction decay).

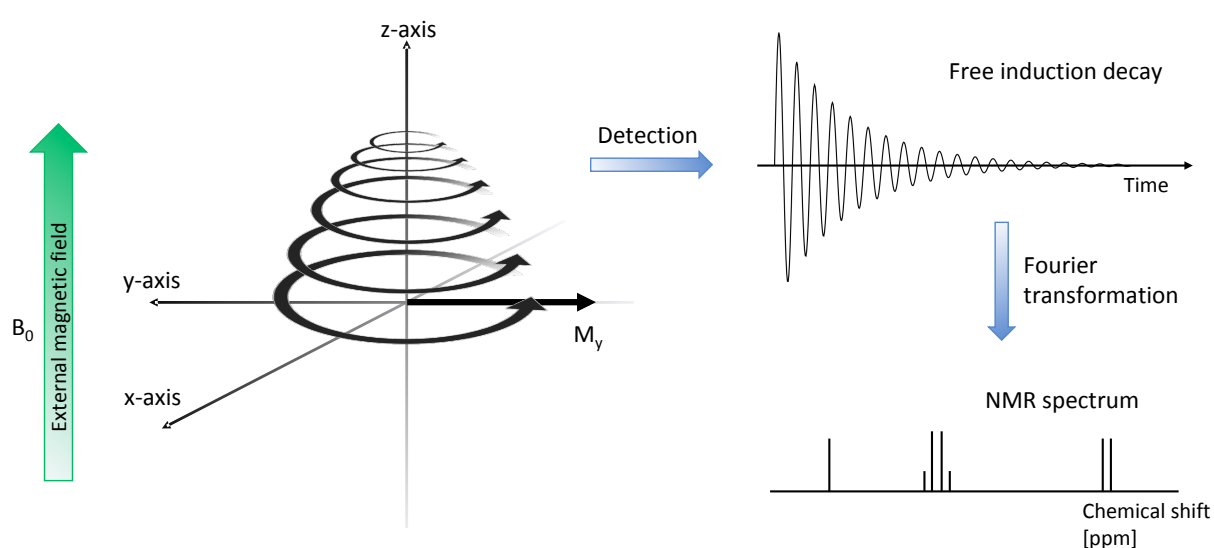


Figure 1.11: The magnetisation vector returns to the equilibrium state, i.e. along the z-axis. The precession of the nucleus creates an electric current which is detected by a receiver coil as a free induction decay. Fourier transformation of the free induction decay transforms the time domain within the free induction decay into a frequency domain, producing an NMR spectrum.

1.6.5 Origin of chemical shift

As mentioned in 1.6.3.2, chemical shifts arise because the magnetic field (B) experienced by a nucleus is slightly different from the applied external magnetic field (B_0) due to its chemical environment. This is because each nucleus in the magnetic field behaves like a small electromagnet producing a local magnetic field, which opposes the external magnetic field. In addition, there is magnetic field produced by the electron shell surrounding a nucleus, which 'shields' the nucleus from the external magnetic field. A 'shielded' nucleus has a lower resonance frequency and appears

shifted 'upfield' (i.e. rightwards) on the NMR spectrum. In contrast, 'de-shielded' nuclei with lower electron density have higher resonance frequencies and appear shifted downfield (i.e. leftwards) on the NMR spectrum. Therefore, it is evident that 'shielding' is determined by the electronic structure of the molecule in the vicinity of the nucleus. This is the origin of the chemical shift and is an essential component of NMR spectroscopy as it gives separately detectable signals for each nucleus within a molecule. When the sample contains nuclei with different chemical shifts, each chemical environment gives rise to a unique magnetisation vector that precesses at an offset frequency appropriate for its chemical shift.

1.6.6 Introduction to the NMR spectrum

In the following sections, a brief introduction to the NMR spectrum will be given using lactate, a common biofluid metabolite, as an example to provide a practical explanation and interpretation of the NMR concepts of chemical shift, integration and splitting.

1.6.6.1 Effect of chemical shift on NMR spectrum

The chemical structure of lactate is shown in **Figure 1.12**. There are two NMR visible functional groups of ^1H nuclei, circled in red and in blue. The three ^1H nuclei (labelled 1, 2, and 3) in the methyl group (circled in red) have the same chemical environment, i.e. each are shielded equally by their electron shell, therefore they have lower resonance frequencies and they appear 'upfield' on the NMR spectrum (at 1.33 ppm). In contrast, the ^1H nucleus labelled as 4 has a different chemical environment, one that is less shielded as it is located close to oxygen atoms which are electro-negative, hence drawing the electron shell away from the ^1H nucleus. This results in a higher resonance frequency and its signal appears 'downfield' (at 4.11 ppm). The ^1H nuclei in the hydroxyl (-OH) groups do not typically appear on liquid state NMR because it transfers to one of the lone pairs on the oxygen of the water molecule.

1.6.6.2 Splitting

The NMR signal is split because of the effect of neighbouring ^1H nuclei. Nuclei separated by up to three bonds interact with each other via the bonding electrons and behave like a row of magnets exerting force on each other. In the example given in **Figure 1.12**, the methyl group (containing the ^1H nuclei labelled 1, 2 and 3) interacts with the ^1H nucleus (labelled 4). This interaction causes splitting in both their NMR signals and the number of peaks is equals to $n + 1$, where n is the number of neighbouring ^1H nucleus. As the methyl group has only one ^1H neighbour, its signal will be split into two, i.e. a doublet. In contrast, the ^1H nucleus (labelled 4) has three ^1H neighbours, thus its signal is split into four, i.e. a quartet.

1.6.6.3 Integration

From the above, it is clear that three ^1H nuclei contribute to the doublet signal at 1.33 ppm, while the quartet at 4.11 ppm is composed of the signal from a single ^1H nucleus. The integration (area under the spectra), also known as the integral value, of the doublet and the quartet will thus be in the ratio of 3 : 1, as they are contributed by three ^1H and one ^1H nuclei respectively. The integral value of each peak within the signal of each functional group is calculated using Pascal's triangle (**Figure 1.13**). A doublet thus have two equal peaks, while a quartet will have intensities in the ratio of 1 : 3 : 3 : 1. Therefore by understanding the nature of chemical shift, splitting and integration of the different functional groups, it is possible to determine the chemical structure of an entire molecule.

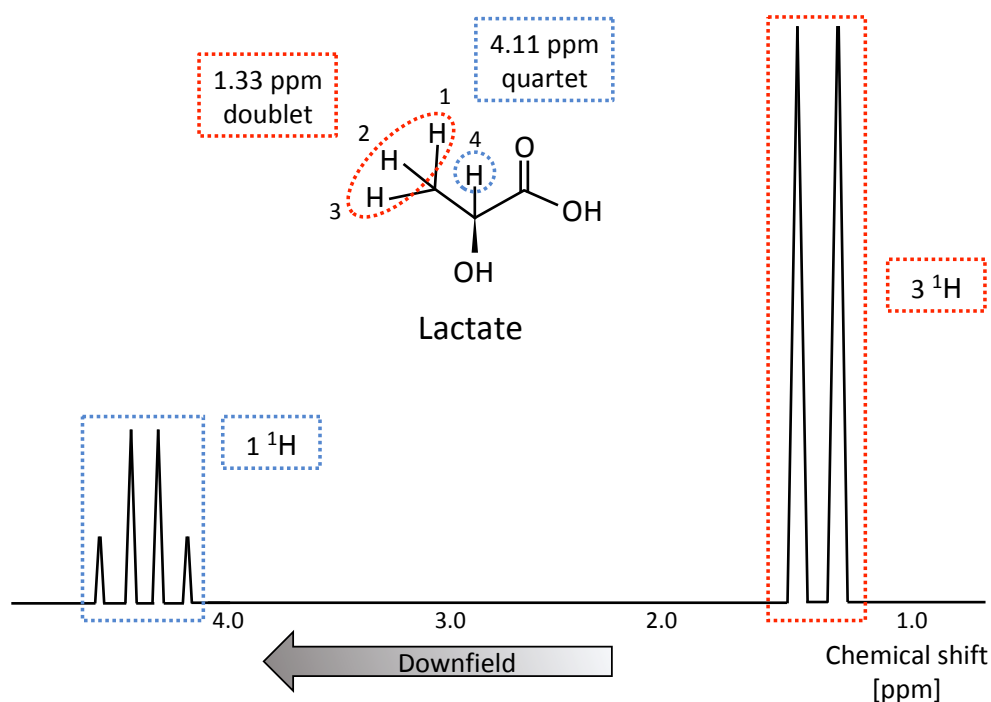


Figure 1.12: Chemical structure and NMR spectrum of lactate. The three ¹H nuclei (methyl group circled in red) appear as a doublet centred at 1.33 ppm on the NMR spectrum while the single ¹H (circled in blue) appears as a quartet downfield, centred at 4.11 ppm. The signal widths are not drawn to scale for illustrative purposes.

$n = 0$	1	Singlet
$n = 1$	1 1	Doublet
$n = 2$	1 2 1	Triplet
$n = 3$	1 3 3 1	Quartet
$n = 4$	1 4 6 4 1	Quintet
$n = 5$	1 5 10 10 5 1	Sextet

Figure 1.13: Pascal's triangle. n refers to the number of neighbouring ¹H nuclei. The number of peaks a signal will have is equal to $n + 1$. For example, an n of 1 will produce a doublet signal, i.e. two peaks with the same intensities in the ratio of 1 : 1. An n of 3 will produce a quartet, i.e. four peaks with intensities in the ratio of 1 : 3 : 3 : 1.

1.6.6.4 NMR spectra of human blood

The NMR spectrum shown in **Figure 1.12** demonstrates the NMR signals arising from a single molecule, lactate. Expectedly, the NMR spectrum of human blood is more complex as it contains hundreds of NMR visible metabolites, each giving one or more signal resonances. It is therefore very

information rich and a single spectrum can be thought of as a unique blood metabolic signature of an individual. **Figure 1.14** shows an example of an NMR spectrum obtained from human serum.

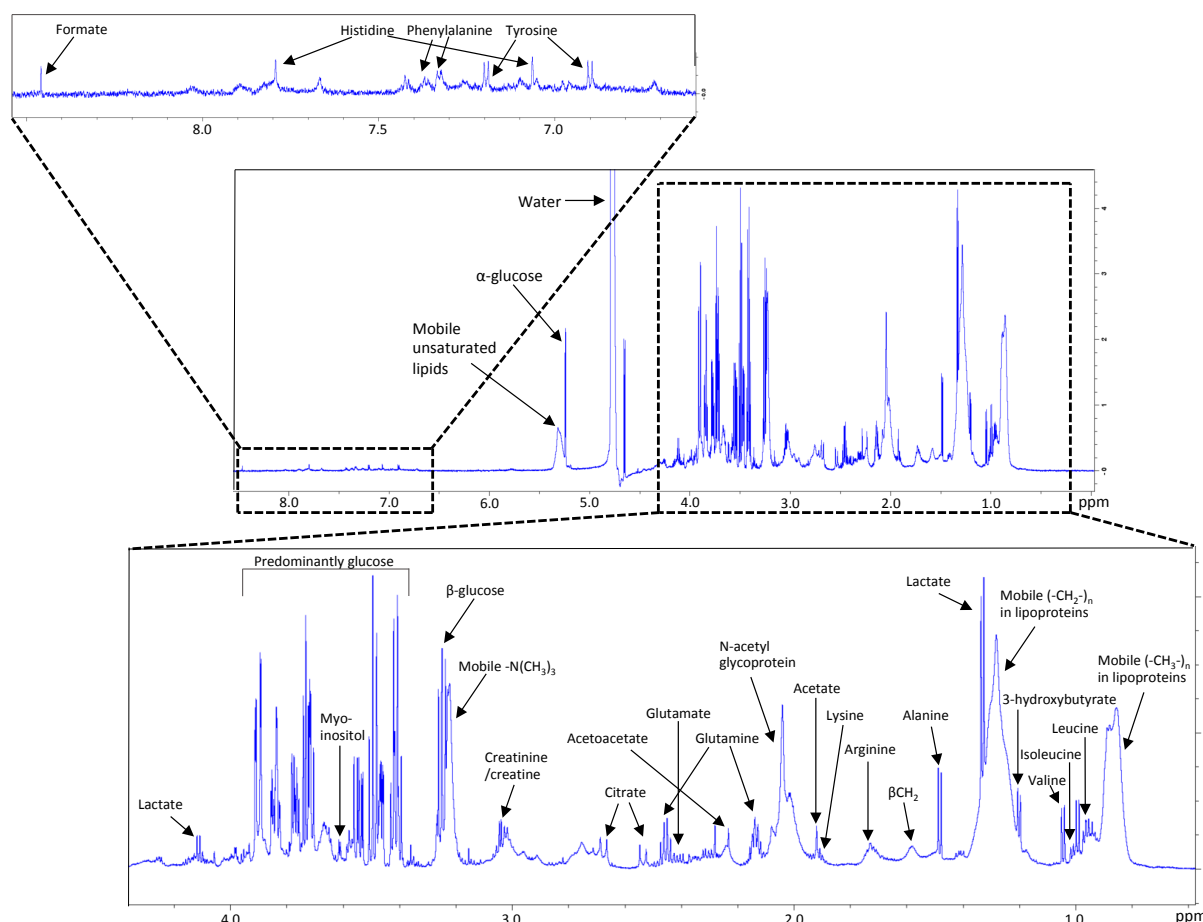


Figure 1.14: ^1H NMR spectrum of human serum using the CPMG pulse sequence with D_2O as NMR solvent. The middle panel shows the entire spectrum, the upper panel shows the metabolites ‘downfield’ of the water signal and the lower panel shows the metabolites ‘upfield’ of the water signal.

1.6.6.5 Metabolite assignment

Despite the apparent complexity of NMR spectra derived from human biofluids, most metabolite assignments can be readily made from knowledge of their chemical shifts and splitting patterns, and by comparing these to known literature values and metabolite databases, e.g. the human metabolome database (HMDB) and the Kyoto encyclopaedia of genes and genomes (KEGG) (Kanehisa et al., 2012; Wishart et al., 2018). For peaks/signals with unknown metabolite assignments

or if there are overlapping regions between peaks, a few approaches are available to interrogate this. Firstly, spiking experiments can be performed whereby a known candidate compound is being spiked into the sample. If the chemical shift of the spiked compound agrees with the chemical shift of the unknown, this confirms the presence of the compound in the sample. Secondly, several pulse sequences exist such as one-dimensional (1D) correlation spectroscopy (COSY) and 1D total correlation spectroscopy (TOCSY) that selectively excites one signal (i.e. the unknown one), and the resulting magnetisation can be transferred via bonds from the excited spins (arising from ^1H nuclei) to the other spins/ ^1H nuclei. In this way, only signals that then appear are those that are in the same spin system (i.e. the same molecule/compound) as the excited nuclei. Lastly, two-dimensional (2D) NMR experiments can be performed in which a second dimension is added which allows correlated signals (either through a direct bond/series of bonds e.g. COSY and TOCSY, or through space e.g. nuclear overhauser effect spectroscopy [NOESY]) to be identified. A simplified representation and explanation of this is given in **Figure 1.15**.

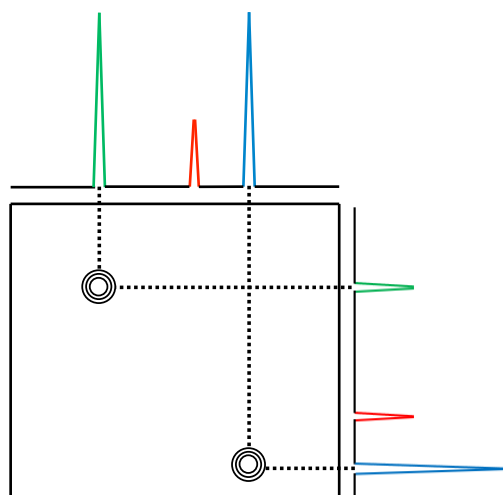


Figure 1.15: Simplified representation of a 2D NMR experiment. The combination of two 1D NMR spectra allows correlated peaks to be identified. Peaks in the 2D spectrum are only present if there is a correlation between peaks in the 1D spectra, either through a direct bond or a series of bonds or through space, depending on the NMR experiment used. The example above shows two peaks on the 2D NMR spectrum, corresponding to two correlations present, i.e. the green peaks are from one molecule and the blue peaks are from another molecule. If a peak (red peak) in the 1D spectrum does not correlate with any of the peaks in the second 1D spectrum, there is then no resultant peak on the 2D spectrum. For simplicity, peak splitting is ignored in this representation.

Chapter 2: Materials and Methods

2.1 NMR experimental workflow

The following sections describe the entire NMR experimental workflow, from sample processing to NMR spectra acquisition and processing.

2.1.1 Sample-handling protocols

2.1.1.1 Blood processing

For blood metabolomics, it is crucial that the centrifugation of blood samples for erythrocyte separation is performed in a timely and standardised manner as delay in this process alters the blood metabolome due to ongoing in vitro enzymatic activity in the blood tube (Jobard et al., 2016). The most marked change relates to the increase in lactate to glucose ratio as glucose levels fall and lactate rises as a result of low oxygenation, which activates anaerobic glycolysis (Malm et al., 2016). The metabolic derangements resulting from delayed centrifugation is further reviewed in the Introduction of Chapter 6, the aim of which was to investigate the effect of blood sample-handling variations on the diagnostic accuracy of the RRMS vs. SPMS metabolomics test.

During venepuncture, blood was collected into red-top (BD™ Vacutainer™ 367837) and green-top lithium-heparin tubes (BD™ Vacutainer™ 367375) for serum and plasma collection respectively. Of note, there are no appreciable differences between the serum and plasma metabolome on the ^1H NMR spectroscopy platform (Deprez et al., 2002; Teahan et al., 2006). The *optimised* blood sample-handling protocol used in this thesis is consistent with those frequently employed in the metabolomics literature and involved the following steps (Bervoets et al., 2015; Yin et al., 2015). Once collected, blood was left to stand for 30 minutes (to give sufficient time for clot formation in the case of serum) at room temperature after which it was centrifuged at 1,300 *g* for 10 minutes at

room temperature using a benchtop centrifuge (Medifuge™, Thermo Fisher Scientific Inc.) for erythrocyte separation to obtain serum/plasma. The serum/plasma was then immediately aliquoted into polypropylene cryotubes (Crystal clear™, STARLAB UK Ltd) and stored at –80 °C until NMR sample preparation.

For rodent (rat) samples (see Chapter 4), the same *optimised* protocol was followed to retrieve serum after blood withdrawal via cardiac puncture.

2.1.1.2 CSF processing

Rat CSF was used for metabolomics analysis in this thesis. Once collected, CSF was immediately centrifuged at 15,000 *g* for 10 minutes at room temperature (Heraeus™, Thermo Fisher Scientific Inc.). The CSF was then promptly aliquoted into cryotubes (Crystal clear™, STARLABS UK Ltd) and stored at –80 °C until NMR sample preparation.

2.1.2 NMR sample preparation

For NMR sample preparation, human serum/plasma was thawed at room temperature followed by ultra-centrifugation at 100,000 *g* for 30 minutes at 4 °C (Beckman Coulter Optima™, with TLA-110 fixed-angle rotor). Two hundred microlitres of the supernatant was then diluted with 400 µL of 75 mM sodium phosphate buffer, to make up a total volume of 600 µL. The sodium phosphate buffer was prepared by dissolving 62.5 mM of anhydrous sodium phosphate dibasic powder (CAS number 7558794) and 12.5 mM of anhydrous sodium phosphate monobasic powder (CAS number 7558807) in deuterium oxide (D₂O) (CAS number 7789200), giving a final pH of 7.4 (all three reagents were obtained from Sigma-Aldrich, Dorset, UK). D₂O was used as the NMR solvent for all NMR experiments.

Rat serum NMR samples were prepared in the same way as the above, without the ultra-centrifugation step. Two hundred and forty-five microlitres of serum was buffered with 305 µL of 75

mM sodium phosphate buffer, giving a final volume of 550 μL . For rat CSF NMR sample preparation, 80 μL of CSF was thawed and then buffered with 470 μL of sodium phosphate buffer to a final volume of 550 μL .

The buffered NMR samples were then stored at $-80\text{ }^{\circ}\text{C}$ until NMR experiments. Immediately before NMR experiments, the NMR samples were thawed at room temperature, briefly vortexed, and then transferred to a 5 mm borosilicate glass tube (Norell™ 502-7) via a glass pipette.

2.1.3 NMR spectroscopy experiments

All NMR experiments were performed using a 700-MHz Bruker (Bruker BioSpin GmbH, Rheinstetten, Germany) AVIII spectrometer operating at 16.4 T equipped with a ^1H [$^{13}\text{C}/^{15}\text{N}$] TCI cryoprobe at the Department of Chemistry, University of Oxford. Sample temperature was regulated at 310 K.

Various NMR experiments can be used to analyse complex biofluids such as serum, plasma and CSF. The two most commonly used experiments in NMR metabolomics are the 1D NOESY and CPMG pulse sequences. A serum/plasma spectrum obtained using the NOESY pulse sequence consists of resonances from both proteins and metabolites. This precludes the accurate measurement of metabolite signals as the broad resonances from proteins (especially high molecular weight proteins such as albumin) affect the spectral baseline. To suppress the contribution of these protein derived broad resonances for improved metabolite quantification, an NMR-edited pulse sequence, such as the CPMG sequence is required. The CPMG pulse sequence allows for the signals from the larger protein molecules to relax before detecting the longer-lived signals from metabolites. Therefore, the CPMG experiment is widely used in serum/plasma NMR metabolomics while the 1D NOESY experiment is used for samples such as CSF and urine, whereby the NMR signals are intrinsically narrow with a flat baseline (due to low concentrations of proteins and lipids) allowing for accurate metabolite measurement.

For all serum/plasma NMR samples used in this thesis, ^1H NMR spectra were first acquired using a 1D NOESY presaturation scheme for suppression of the water resonance with a 2 second (s) presaturation, 8 data collections, an acquisition time of 1.5 s, and a fixed receiver gain (**Figure 2.1 A**). Following this, a spin-echo Carr-Purcell-Meiboom-Gill (CPMG) sequence was applied, with a τ interval of 400 μs , 80 loops, 32 data collections, an acquisition time of 1.5 s, a relaxation delay of 2 s and a fixed receiver gain, was used to suppress broad signals arising from large molecular weight serum/plasma components (e.g. albumin). In this way, the CPMG pulse sequence retains resonances from small molecular weight metabolites and mobile side chains of lipoproteins, providing an accurate measurement of these parameters in the serum/plasma sample (**Figure 2.1 B**). All serum/plasma spectral data acquisitions in this thesis were therefore performed using the CPMG pulse sequence, as per standard NMR metabolomics literature (Soininen et al., 2009).

For rat CSF samples used in this thesis, ^1H NMR spectra were acquired using a 1D NOESY presaturation scheme for attenuation of the water resonance with a 2 s presaturation, 64 data collections, an acquisition time of 1.5 s, and a fixed receiver gain. This pulse sequence was used as the small amounts of large macromolecules in CSF means that there is no observable distortion of the spectral baseline (**Figure 2.1 C**), consistent with the methods used in other NMR metabolomics studies on CSF (Kim et al., 2017; Mandal et al., 2012; van der Sar et al., 2015), and has better signal to noise ratio compared to the CPMG pulse sequence. All CSF spectral data acquisitions in this thesis were therefore performed using the 1D NOESY pulse sequence.

Free induction decays of the pulse sequences were zero-filled by a factor of 2 and multiplied by an exponential function corresponding to 0.30 Hertz (Hz) line broadening prior to Fourier transformation. For quality control (QC), pooled serum/plasma samples were spread throughout the run to monitor technical variation. This was done by pooling samples from healthy individuals, thoroughly mixing the pooled samples and then aliquoting the mixture into many individual aliquots. Each QC NMR sample was then prepared from each aliquot, therefore in this way, all the QC samples

are the same. During the NMR run, the QC samples were spread throughout to observe if there was any technical variation arising from the NMR spectrometer whilst using essentially the same sample. ^1H COSY spectra were acquired on at least one sample in each disease classification to aid in metabolite identification. When required, further confirmation was achieved by 1D TOCSY or 2D TOCSY NMR experiments, or with spiking experiments with known candidate compounds. Metabolite assignments were performed by referencing to literature values and the HMDB (Wishart et al., 2018).

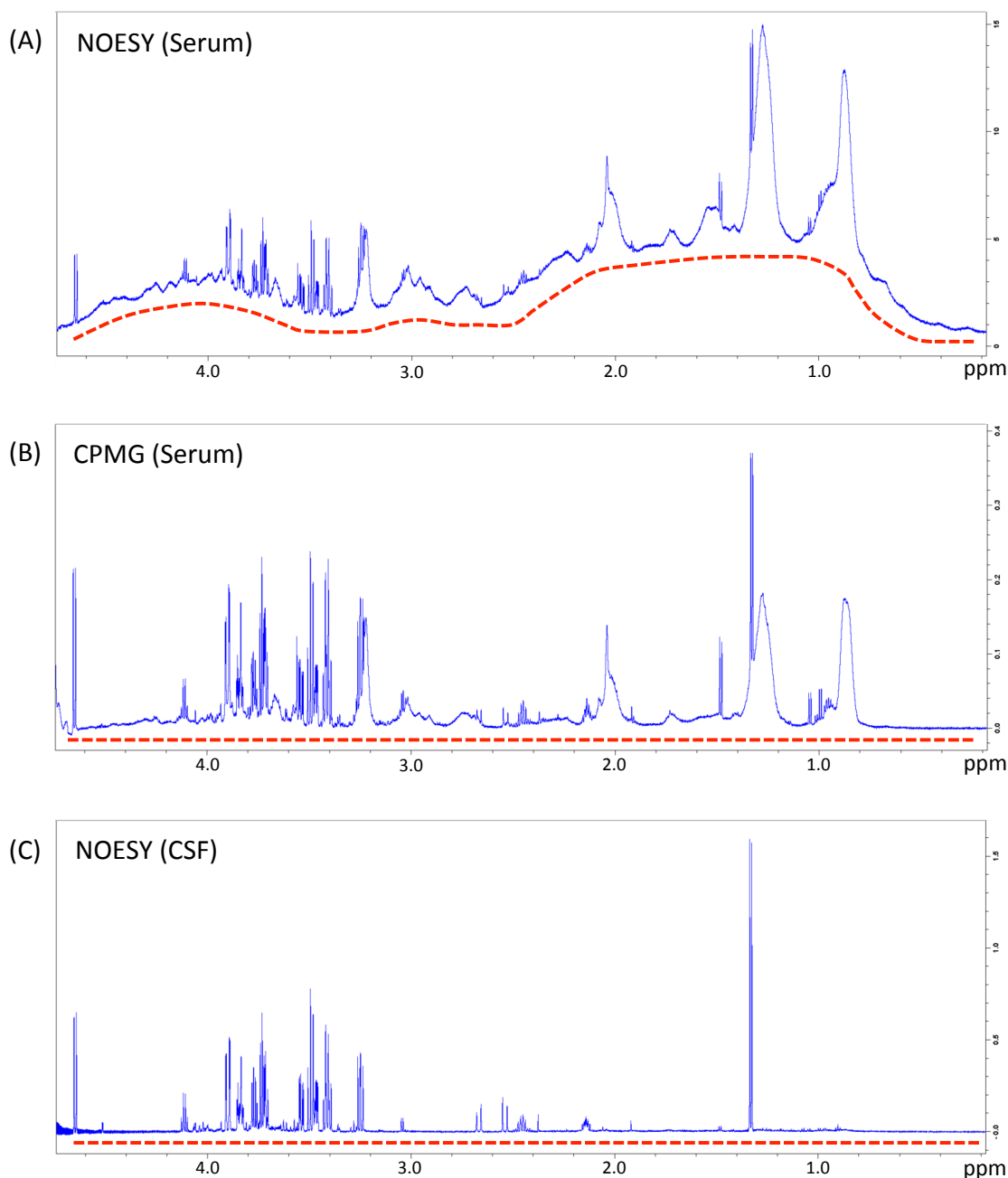


Figure 2.1: $1D\ ^1H$ NMR experiments using different pulse sequences on different biofluids. (A) NMR spectrum obtained from NOESY presaturation pulse sequence on serum show broad signals with elevated baseline (red dashed line) arising from macromolecules (e.g. albumin) in the blood. This precludes accurate quantification of small low molecular weight metabolites in the blood. (B) Application of the CPMG pulse sequence suppresses these broad signals, resulting in a flat baseline (straight red dashed line) from which accurate measurements of low molecular weight metabolites could be obtained. (C) As there are miniscule amounts of macromolecules in a clean CSF sample (i.e. without blood contamination), there is no perturbation of the spectral baseline (straight red dashed line). Thus, the spectrum obtained from the NOESY sequence is optimal for measuring metabolites present in the CSF, and in addition, signal to noise ratio is better in the NOESY sequence compared to the CPMG sequence.

2.1.4 NMR spectral data processing

All spectra were processed in Topspin 3.6.1 (Bruker BioSpin GmbH, Rheinstetten, Germany) in which they were phased, baseline corrected using a third degree polynomial, and chemical shifts referenced to the lactate doublet resonance centred at chemical shift 1.33 ppm, a commonly used method of spectra referencing (Lu et al., 2018; Pontes et al., 2019; Verwaest et al., 2011). No internal chemical standards were used for spectra referencing as the chemical shifts of these internal standards can change with varying pH and there is also line broadening of their signals arising from protein binding (an issue with protein-rich biofluids such as serum and plasma) (Alum et al., 2008). The NMR spectra were then visually inspected for errors in baseline correction, referencing, spectral distortion, or contamination with exogenous products (e.g. ethanol, propylene glycol). Processed spectra were then transferred to ACD/Labs Spectrus Processor Academic Edition 12.01 (Advanced Chemistry Development, Inc., Toronto, Canada) whereby the water signal from 4.20 – 5.20 ppm was excluded due to baseline distortion, as were signal-free noise regions. The remaining regions of the spectra between 0.80 – 4.20 ppm and 5.20 – 8.50 ppm were split into 0.02 ppm fixed-width bins (**Figure 2.2 A**). This binning procedure not only allows quantitative data to be extracted from the spectrum, it also masks subtle chemical shift differences and filters noise in spectra (Worley et al., 2013). Integral values of each individual spectral bin were obtained and normalised to the integral value of the entire spectrum (i.e. constant-sum-normalisation) such that the integral value of the whole spectrum is 1 (Worley et al., 2013). As there are inevitably small variations in pipetting, this sum-normalisation technique is able to correct for variable dilutions arising from slight differences in the biofluid and buffer volumes. The integral values were then used as quantitative variables, expressed in arbitrary units (AU), representing the columns in the data matrix (X), while the rows represent the different samples/subjects (**Figure 2.2 B**). This data matrix was used for multivariate statistical analysis (see section **2.2.1**).

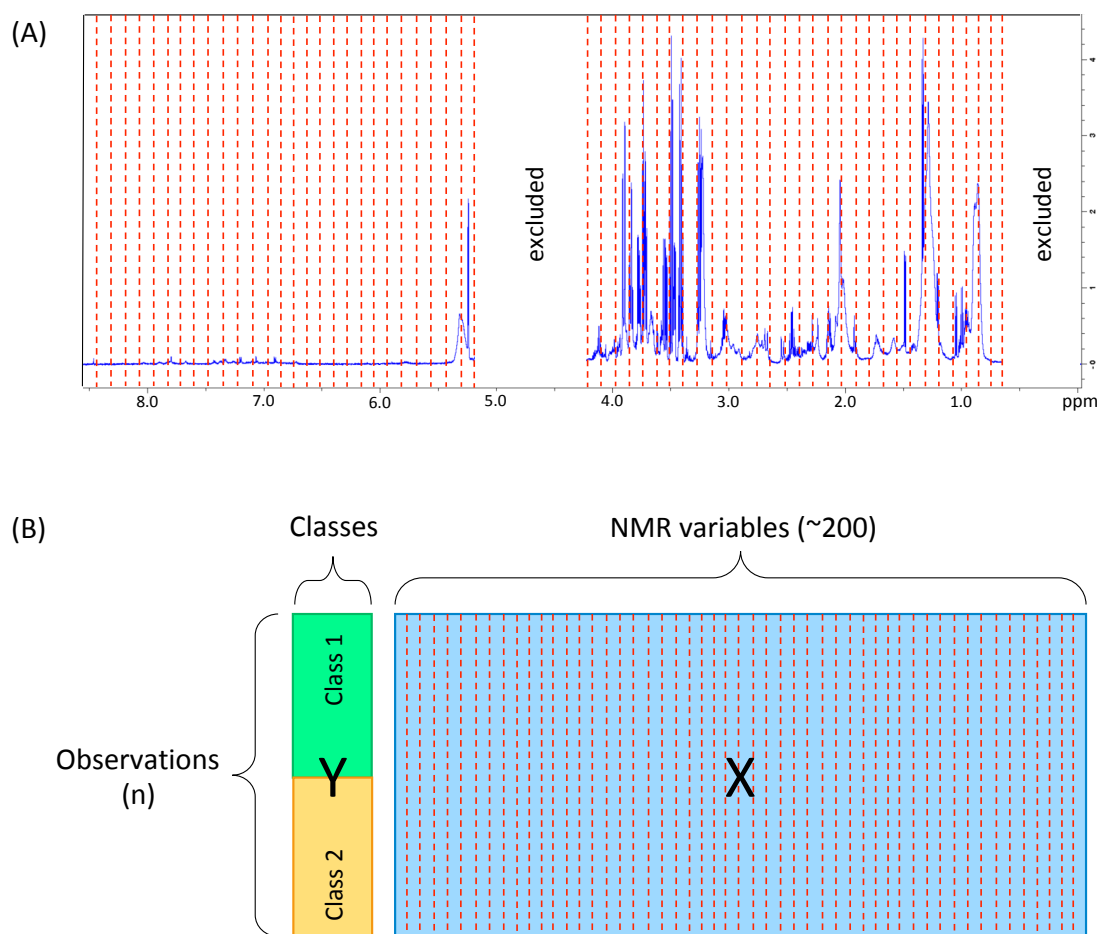


Figure 2.2: Spectral binning and generation of the data matrix. **(A)** Spectral regions between 0.80 – 4.20 ppm and 5.20 – 8.50 ppm were split into 0.02 ppm wide bins. Note that for illustration purposes, the width of the bins is not drawn to scale. The water signal (4.20 – 5.20 ppm) was removed due to baseline distortion as well as regions that contained no signal (for illustrative representation, only the signal-free region from 0.00 – 0.80 ppm is shown). The integral value of each bin was normalised to the integral value of the entire spectrum (constant-sum-normalisation). **(B)** The integral values of each spectrum was then imported into the data matrix (X) as columns (i.e. one column = integral value of an individual bin). Subjects/samples make up the rows of the data matrix (i.e. one row = one subject). This data matrix (X) can be condensed down by using unsupervised dimension reduction approaches like principal component analysis (PCA). Alternatively, it can be paired with a binary matrix (Y) corresponding to the class membership of the observations to be used in supervised, class-based analysis like orthogonal partial-least square discriminant analysis (OPLS-DA).

2.2 Statistical analysis

2.2.1 Multivariate statistical analysis

Two multivariate statistical approaches were used in this thesis: principal component analysis (PCA) and orthogonal partial-least square discriminant analysis (OPLS-DA). These are essentially dimension reduction techniques to enable the user to better visualise the dataset by combining discriminatory variables into components that explain the largest proportion of the variance within the data matrix (Worley et al., 2013). This allows the user to observe the presence of clusters as well as the separation between clusters/classes. Furthermore these techniques permit the identification of the most important variables accounting for the clustering as well as those separating the classes. Of note, these techniques are suitable to be used in correlated variables, which is often the case in metabolomics as well as in clinical research datasets.

2.2.1.1 Principal component analysis

PCA is an unsupervised (i.e. without defining class membership) multivariate analysis approach to identify a set of variables (i.e. principal components, hence the name) accounting for the greatest variation present in the dataset (Worley et al., 2016). As the analysis is unsupervised, clustering of subjects/samples (if any) on the PCA scores plot is in no way influenced by the user but rather is wholly dependent on the variables present within the data matrix (X) alone. Following the generation of the PCA scores plot, the loadings plot can be created to visualise the variables responsible for clustering. A variable's position on the loadings plot corresponds to its high contribution to the observations/subjects whose scores are found in a similar position in the PCA scores plot (Worley et al., 2013). Additionally, variables that cluster together in a loadings plot are likely to be correlated. In this thesis, SIMCA software (MKS Data Analytics Solutions, Umetrics, Sweden) was used for PCA.

2.2.1.2 Orthogonal partial-least square discriminant analysis

While PCA is a useful technique to identify potential clusters by unsupervised dimension reduction, it is a less ideal approach to recognise specific class-based differences as class separations on the PCA scores can only be revealed if within-class variation is sufficiently less than between-class variation (Worley et al., 2013). To explore variables (more specifically, a vector combination of them) accounting for class separation between user-defined class memberships, supervised multivariate analysis such as OPLS-DA is required. OPLS-DA is essentially a supervised extension of PCA, paired with a data matrix (Y) which specifies class membership of each observation/subject, such that the predictive OPLS-DA scores and loadings used to estimate Y consist of variation directly correlated with Y and free of interfering structured variation (Worley et al., 2013). Additionally, an orthogonal signal correction technique is applied in OPLS-DA in which unwanted spectral variations of data that do not contribute to prediction is removed, therefore optimising classification and visualisation (Cho et al., 2008).

To establish the predictive ability of the OPLS-DA model, it is important to perform cross-validation of the model using an independent test set (Worley et al., 2013). Indeed, a sufficiently large ensemble of training and independent test sets is required to provide the best possible estimation of the predictive capability of the OPLS-DA models generated from the data matrix. An ‘*n*-fold’ cross-validation scheme was employed in this thesis (Broadhurst et al., 2006). This validation scheme involves multiple iterations of splitting the data into training and testing sets, which ensures that systematic bias is avoided. This iterative process is explained in detail below and illustrated in **Figure 2.3**. For this thesis, R software (R foundation for statistical computing, Vienna, Austria) was used for OPLS-DA (R Core Team (R Foundation for Statistical Computing V, 2013), using in-house R scripts and the *ropls* package (Thevenot et al., 2015).

The first step is to correct for unequal class sizes (Brereton et al., 2014; Thevenot et al., 2015). This involves the random sampling of observations from the larger class to match the number of observations in the smaller class. The metabolomics data is then shuffled randomly and split into ten equal, mutually exclusive partitions (i.e. 'folds'); nine partitions go into a training data set (90% of data) and one partition is used as a test data set (10% of data) (Broadhurst et al., 2006). The '10' in 10-fold external cross-validation thus refers to the partitioning of the data set into 10 data strata/folds.

The training data set is first used to build the OPLS-DA model using 7-fold internal cross-validation. This 7-fold cross-validation scheme was used as this was the default number of folds in the *ropls* script (Thevenot et al., 2015). To do this, the training data is split into two sets, a training set (6 data folds) and a validation set (1 data fold) (Broadhurst et al., 2006; Thevenot et al., 2015). The training set is used to build a model by which the goodness of fit of the model is determined by R^2 (i.e. the goodness of fit statistic). The R^2 is a mathematical index to determine how well the model 'fits' the observed data, i.e. the percentage of the response variable variation that is explained by the model (maximum value of 1.0). The validation set is then projected into the model that was built using the training set and the goodness of prediction is assessed by Q^2 (i.e. the goodness of prediction statistic). The Q^2 is an index frequently used in mathematical predictive modelling to determine how well the model predicts future data, i.e. the consistency between predicted and original data. A Q^2 of ≥ 0.4 (maximum value is 1.0) is conventionally taken to be a cut-off for biological relevance (Westerhuis, 2008). Optimisation is done using the Q^2 and R^2 statistics to determine the optimal number of components and learn the underlying discriminatory patterns between the classes under consideration (Eriksson, 2001). This is done by increasing the number of latent variables, which sees both the R^2 and Q^2 increase, until a point at which they diverge and it is at this point that the model is assumed to have reached its optimal predictive capability (Broadhurst et al., 2006). The OPLS-DA model is trained 7 times (i.e. 7-fold internal cross-validation), each time holding out one of the folds

as an internal validation set. The Q^2 values for the 7 validation steps are then averaged to provide an overall Q^2 value for that particular model (Broadhurst et al., 2006).

After the OPLS-DA model is built, the test data set is then used as an independent predictive set (thus the word 'external' in the term 10-fold external cross-validation) to determine the predictive ability of the OPLS-DA model that has been constructed using the training data set. This predictive ability is quantified by calculating the accuracy, sensitivity and specificity of each model from the predicted classifications of the external independent test data set. It is important to note that the training and test data sets are completely independent of each other, i.e. the test data set is not used to build/train the OPLS-DA model and conversely, the training data set has not 'seen' the test data set, therefore there is no bias in this approach.

Following this, another separate stratum of data (containing 10% of the data) is taken out and used as the test data set, while the test data set that has already been used above is now inserted back to make up 90% of the data to be used as training data set to build another OPLS-DA model. This process is repeated 10 times (hence the term 10-fold external cross validation), so that all observations in the data matrix have been used as the test set once.

The above process of random class size balancing and random partitioning of the data into ten strata followed by 10-fold external cross-validation is then repeated 100 times. This ensures an honest and representative approach to estimate the predictive ability of the OPLS-DA models in assigning class membership using the data matrix present. A critique of OPLS-DA models is that the predictive accuracies could be artificially elevated because class information is intentionally used to remove variability of the input data orthogonal to the response (Park et al., 2011). In this thesis, in order to validate the OPLS-DA models generated, a random permutation method using random class assignment was applied. This is done by generating a null distribution obtained by randomly permuting the class assignment and then performing the OPLS-DA modelling using the same

metabolomics data matrix (X) (Westerhuis, 2008). As the class assignments to the observations are entirely random, the mean accuracy obtained is essentially the same as chance (i.e. ~50% predictive accuracy). The distribution of the ensemble of predictive accuracies of the defined class assignment OPLS-DA models is then compared to the distribution (i.e. null distribution) of the random class assignment OPLS-DA models using the Kolmogorov-Smirnov test, a non-parametric statistical test that compares the cumulative distributions of two data sets (Frey, 2018), with statistical significance set at two-sided $p < 0.05$. The discrimination between the two defined classes is only validated if the distribution of the predictive accuracies of the defined class OPLS-DA models is significantly different from the distribution of the predictive accuracies generated from the randomly permuted models. Of note, the number of defined class assignment OPLS-DA models and the random class assignment OPLS-DA models are the same. In summary, this validation scheme can help to avoid overfitting and help assess the generalisability of the model to previously unseen datasets.

To identify which are the most discriminatory variables driving the separation between classes, variable importance in projection (VIP) scores derived from the OPLS-DA models are computed. A VIP score is a summary measure of the overall contribution of each variable (from the X data matrix) to the OPLS-DA models, summed over all components and weighted according to the Y variation accounted for by each component. The higher the VIP score, the greater the contribution a variable makes to the discriminatory model. Although a VIP score ≥ 1 has been proposed as a cut-off to identify the important discriminatory metabolites, data matrices are diverse and thus this threshold should not be universally applied to all OPLS-DA models (Akarachantachote N., 2014). In this thesis, in order to establish this cut-off point, a VIP score ranking plot was first constructed. The threshold is then determined by identifying either: (1) the inflexion point (the point at which the VIP ranking plot makes an acute angle), or (2) an abrupt drop in VIP score.

In this thesis, OPLS-DA was employed to interrogate the metabolomics spectral data to identify metabolic differences between patient groups (e.g. RRMS vs. SPMS patients, RRMS vs. antibody-

positive NMOSD), while the VIP scores were used to identify the top metabolites accounting for the separation between groups. For rodent metabolomics analyses (e.g. DTH animals vs. control animals) where there were lesser number of observations, the data matrix is first partitioned according to the number of observations in the smaller group, such that the test set (which represents one stratum of the data) consisted of two observations, i.e. one from each class. The training set is used to build the OPLS-DA model using 'leave one out internal cross-validation', after which the test set is applied to it to obtain the predictive indices. A graphical representation of this is shown in Error! Reference source not found..

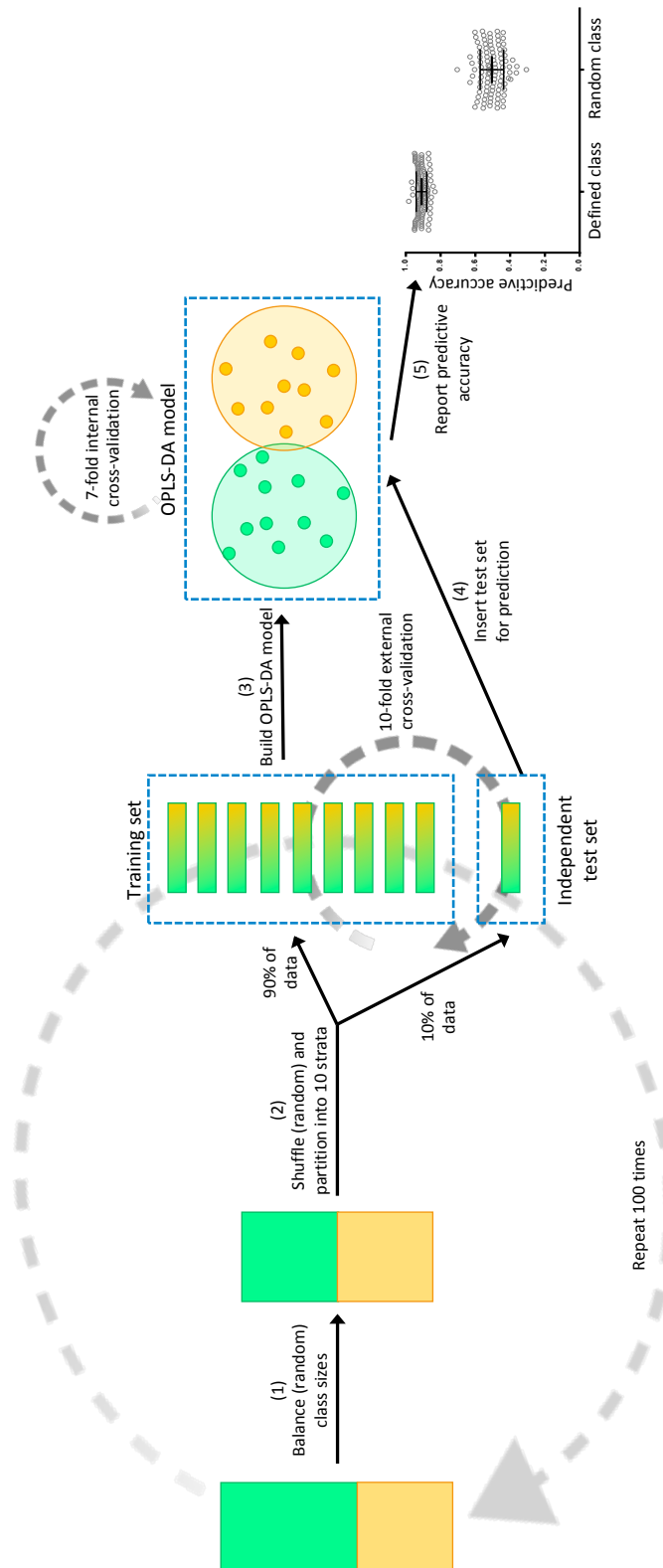


Figure 2.3: Schematic representation of OPLS-DA and cross-validation strategy. After (1) balancing class sizes, (2) the data matrix is shuffled randomly and split into ten partitions/folds. The training set (nine strata/90% of the data) is used to (3) construct the OPLS-DA. (4) The test set (one stratum/10% of the data) is then inserted into the OPLS-DA model and (5) the predictive accuracy is determined, along with other diagnostic indices i.e. sensitivity and specificity. This is repeated to a total of ten times, i.e. 10-fold external cross-validation, to ensure that all observations have been in the test set and that all data has been utilised. The process then repeats itself from the start (i.e. random selection for class size balancing) for another 100 times.

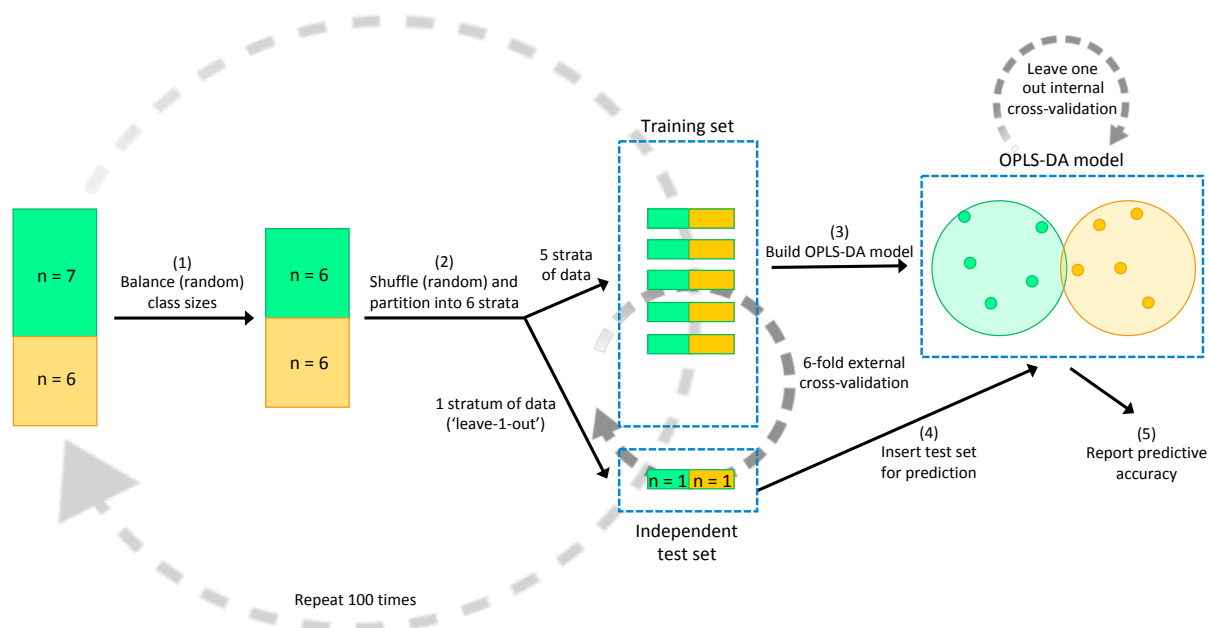


Figure 2.4: Cross-validation strategy for rodent metabolomics data using two groups, one with $n = 7$ and one with $n = 6$, as an example. At step (2), the data matrix is partitioned into the number that equals to the number in the smaller group, i.e. in this case, six. Five of the data strata is used to build the OPLS-DA model using a leave one out internal cross-validation strategy, while one stratum, which contains one observation from each class, is used as the independent test set. The fold numbers for the external cross-validation is adjusted accordingly.

2.2.2 Univariate and logistic regression analysis

All other statistical analyses (comparative, logistic regression, receiver operating curve [ROC], correlation analyses) were performed with STATA software (Release 14, College Station, TX: Statacorp LP, USA) and GraphPad Prism (version 6, California, USA). Comparative analyses between two groups were performed using Mann-Whitney U test or two-sample t-test as appropriate for continuous variables. For comparisons between three groups, one-way ANOVA or Kruskal Wallis test were used, with pair-wise post-hoc corrections using Bonferroni/Tukey and Dunn tests respectively. Chi-square or Fisher exact tests were used for categorical variables depending on the size of the expected frequency, with Bonferroni correction when comparing ≥ 3 groups. Two-way ANOVA was used to compare across groups and time points/treatment, with Sidak's test for multiple comparison corrections. Pearson's or Spearman's correlation was used to explore correlations depending on data normality. Two-tailed p values of <0.05 were considered statistically significant.

2.3 Clinical cohorts and clinical data collection

Clinical data and metabolomics blood samples used in this thesis were collected from two patient cohorts.

The recruitment for the MT cohort (used in Chapter 3) was completed and the metabolomics data acquired just before the start of this DPhil project. This cohort consisted of 298 samples, of which 263 were unique individuals. Patients were recruited from the Neuroscience Clinics, John Radcliffe Hospital (West Wing), Oxford, as well as from the Oxford Centre for Enablement, Nuffield Orthopaedic Hospital, Oxford, under ethical approval from the Oxford Research Ethics Committee C (REC references: 10/H0606/56 and 16/SC/0224A).

The MET cohort (used for Chapters 5 and 6) was established by the DPhil candidate upon DPhil commencement, under the governance and ethical approval from the Oxford Radcliffe Biobank (NRES Committee South Central – Oxford C, REC reference: 09/H0606/5+5). In addition to the sites listed above, patients were also recruited from the Horton General Hospital, Banbury. At the time of the writing of this thesis, metabolomics and clinical data were available from 632 samples, of which 512 were unique.

Clinical and demographic data were obtained from medical notes and patient interviews. Reviews of clinical MRI were supplemented by neuroradiologic reports. Current EDSS was assessed on the day of recruitment, prior (~60 to 90 minutes) to metabolomics blood sampling. Scores related to optic, brainstem, pyramidal, cerebellar and sensory functions were obtained directly during physical examination while scores pertaining to bladder/bowel and cerebral function were derived from patient interviews, as was ambulatory distance.

Patients with comorbidities were included in analyses as it is important that the findings are generalisable. As mentioned, the OPLS-DA statistical approach constructs discriminatory models

based on the factor of interest while minimising the variation introduced by other factors, such as comorbidities. There was an extensive range of diseases that were classed as comorbidities. Only diseases that were persistent (i.e. not transient) and were biologically expected to produce metabolic changes were classed as comorbidities. The more common comorbidities included asthma, diabetes, hypertension, hyperlipidaemia, psoriasis, depression, anxiety disorder, hyper/hypothyroidism, ulcerative colitis, gout, polycystic ovarian syndrome, ischaemic heart disease, breast cancer, epilepsy, and migraine. Transient past events were not treated as comorbidities. These mainly included surgical treatment for fractures, appendicectomy, salpingectomy, ovarian cystectomy, as well as previous treated/recovered events such as deep vein thrombosis, glandular fever, zoster, renal calculi, and peptic ulcers. All patients suspected to have acute infection (e.g. urinary tract infection, acute zoster flare, upper respiratory tract infection) were not recruited.

Chapter 3: Diagnostic utility of metabolomics in clinical syndromes overlapping MS and NMOSD

3.1 Introduction

The discovery of AQP4-Ab in patients presenting with NMO and Asian optico-spinal 'MS' by Lennon et al has led to a paradigm shift in the diagnosis and treatment of CNS IDD (Lennon et al., 2005). CNS AQP4 water channels are concentrated at the astrocytic end feet which are critical in maintaining the integrity of the BBB and brain-CSF barrier (Jarius et al., 2008). It is now known that ~70% of NMOSD patients have serum AQP4-Ab (Waters et al., 2012), and its presence specifies a condition that is clinically and pathophysiologically distinct from MS. Human histopathological studies have shown that AQP4-Ab is a primary astrocytopathy, characterised by a marked loss of AQP4 and glial fibrillary acidic protein (GFAP) immunoreactivity, prominent complement activation, immunoglobulin deposition, an inflammatory infiltrate consisting of mostly eosinophils, neutrophils and macrophages, with a disproportionately lesser amount of neuroaxonal loss and demyelination (Lucchinetti et al., 2014). Experimental studies have demonstrated the pathogenicity of AQP4-Ab on cell culture platforms as well as in rodent ex vivo and in vivo models, primarily mediated by AQP4-Ab activation of the complement system and subsequent complement-dependent cytotoxicity (Bradl et al., 2009; Hinson et al., 2007; Phuan et al., 2012; Saadoun et al., 2010; Zhang et al., 2011).

More recently, antibodies against conformational MOG have followed suit. MOG is a glycoprotein located on the outermost layer of the myelin sheath, expressed by oligodendrocytes, and is highly conserved between species (Reindl et al., 2019). It is widely used as an immunogen in EAE, indeed, the MOG-EAE model has been used to investigate the efficacy of MS DMT (Papadopoulos et al., 2010; Schilling et al., 2006). Earlier studies revealed the presence of MOG-Ab in MS patients, however, these studies only detected antibodies against linear/non-conformational epitopes of MOG which were later found to be not clinically relevant (Reindl et al., 2019). With the use of cell-

based assays expressing conformational MOG, it is now known that MOG-Ab is associated with a wider range of clinical phenotypes including NMOSD (Mader et al., 2011), recurrent/bilateral optic neuritis (Ramanathan et al., 2014), acute disseminated encephalomyelitis (Jurynczyk, Messina, et al., 2017b), and the recently recognised isolated cortical disease (Ogawa et al., 2017), but importantly, not with MS in both adults and children (Ketelslegers et al., 2015; Waters et al., 2015). Emerging evidence from human histopathological shows that patients with MOG-Ab have pattern II 'MS' pathology implying humoral-mediated mechanisms (Spadaro et al., 2015). This view is supported by animal and cell culture based mechanistic studies suggesting that MOG-Ab have pathogenic capability (Dale et al., 2014; Kinzel et al., 2016; Peschl et al., 2017; Saadoun et al., 2014). Indeed, the cytokine profile of MOG-Ab and AQP4-Ab are similar (but both different to MS) (Kaneko et al., 2018), suggesting that they share similar inflammatory/immune responses that are primarily humoral mediated, such that they are both now regarded as antibody-mediated diseases, and despite having overlapping clinical phenotypes, are immunopathologically distinct from MS.

Diagnosis is straightforward when AQP4-Ab or MOG-Ab are present in the context of an appropriate clinical presentation. The conundrum arises when patients are seronegative for both antibodies yet exhibit clinical features suggestive of NMOSD, especially in patients who do not fulfil the current diagnostic criteria. Patients having only optic neuritis with poor recovery, or only longitudinally extensive transverse myelitis (LETM), or a mix of MS and NMOSD features can be challenging to diagnose as no single feature outside of the antibody tests are diagnostic. It is clear that such patients have heterogeneous conditions: seronegative NMOSD fulfilling the 2015 diagnostic criteria, and those outside of the 2015 criteria involve several pathologies such as (1) MS with atypical clinical features (Whittam et al., 2017), (2) true antibody-mediated conditions whereby either AQP4-Ab or MOG-Ab are negative outside of the acute attack (a common feature with MOG-Ab disease) or if tested while on immunosuppressive treatment (Jarius et al., 2016; Jurynczyk, Messina, et al., 2017a; Jurynczyk, Probert, et al., 2017), (3) other undiscovered pathogenic antibodies or other cell-

mediated disorders such as CNS sarcoidosis (Flanagan et al., 2016), or monophasic post-infectious conditions. Indeed, the differential diagnosis of LETM is extensive (Kitley et al., 2012). This challenge in diagnosing antibody-negative NMOSD patients was demonstrated when disagreement on the diagnosis was shown amongst experts in this field, and in fact the diagnostic criteria was not used in every case by any of them (Jurynczyk et al., 2016).

In the absence of defined diagnoses within antibody-negative patients, targeted therapy is not possible and may present a risk as many MS therapies (i.e. natalizumab, fingolimod, beta-interferon, alemtuzumab, glatiramer acetate, dimethyl fumarate) have been reported to aggravate antibody-mediated NMOSD (Ayzenberg et al., 2016; Azzopardi et al., 2016; Kleiter et al., 2012; Min et al., 2012; Palace et al., 2010; Shimizu et al., 2010; Spadaro et al., 2016; Stellmann et al., 2017; Wildemann et al., 2017; Yamout et al., 2017). The differing treatment response is not unexpected considering the different pathogenesis in MS and antibody-mediated CNS IDD. Therefore, determining which antibody-negative patients have MS and which have antibody-mediated pathologies is hugely important.

Previously, the Oxford group found that by using conventional brain MRI data coupled with supervised multivariate analysis, MS could be accurately distinguished from: (1) AQP4-Ab NMOSD, (2) MOG-Ab disease, and (3) antibody-mediated NMOSD (combining AQP4-Ab NMOSD and MOG-Ab disease) (Jurynczyk, Geraldes, et al., 2017). While the application of these algorithms to the brain MRI data from antibody-negative patients might be expected to distinguish between MS vs. antibody-mediated CNS IDD for these individuals, such an approach requires an assumption that there are only two phenotypic subgroups within antibody-negative patients (grouped by either MS or antibody-mediated CNS IDD brain MRI features), with no inference to underlying pathophysiology. Furthermore, a significant proportion of antibody-mediated CNS IDD patients do not have brain lesions (Hyun et al., 2017; Matthews et al., 2013), restricting the applicability of these algorithms in a clinical setting. Similarly, using supervised multivariate analysis of plasma

metabolomics data, the Oxford group was able to construct accurate algorithms distinguishing RRMS from AQP4-Ab NMOSD and MOG-Ab disease (Jurynczyk, Probert, et al., 2017). Indeed, the distinction of AQP4-Ab NMOSD from RRMS was independent of antibody titre and each disease was characterised by a specific set of discriminatory metabolites with respect to the other two diseases. The application of these algorithms to plasma metabolomics data from antibody-negative patients will not be appropriate as this again assumes that only MS or antibody-mediated CNS IDD pathologies exist within these patients. As antibody-negative patients consist of a heterogeneous group of MS, antibody-mediated and other conditions, an extensive range of brain MRI, spinal MRI and clinical data focusing on features known to distinguish MS from NMOSD is first needed for comprehensive phenotypic classification within this group of patients. Molecular markers reflecting disease mechanisms are then required to independently validate these phenotypic classifications.

3.1.1 Aims of Chapter 3

The main aim of this chapter is to classify a group of difficult-to-diagnose, antibody-negative patients into those whose underlying pathology are antibody-mediated and those who are likely to have MS. The specific aims are:

1. To explore the metabolic separation of established RRMS vs. antibody-mediated NMOSD (Ab-NMOSD) (i.e. combining known AQP4-Ab NMOSD and MOG-Ab disease) and to identify the top discriminatory metabolites.
2. To elucidate if there are spontaneous clusters within antibody-negative patients based upon their clinical and MRI features using unsupervised PCA, and to explore if these clusters appear to segregate into plausible disease-specific groups (i.e. 'MS-like' or 'NMOSD-like').
3. To validate that these spontaneous clusters are likely to be representing underlying pathological processes by applying the metabolic discriminators of MS vs. Ab-NMOSD.

As mentioned in the Methods, Section 2.3, blood samples and metabolomics data used in this Chapter have already been acquired just prior to the commencement of the candidate's DPhil project. The candidate's individual contributions to this work include: (1) design and conceptualisation of the study, (2) acquisition of all clinical data, (3) analysis of all metabolomics and clinical data as well as their interpretation. The findings arising from this chapter has been published (Yeo et al., 2019).

3.2 Methods

3.2.1 Patients and clinico-radiological data

The study workflow is outlined in **Figure 3.1**

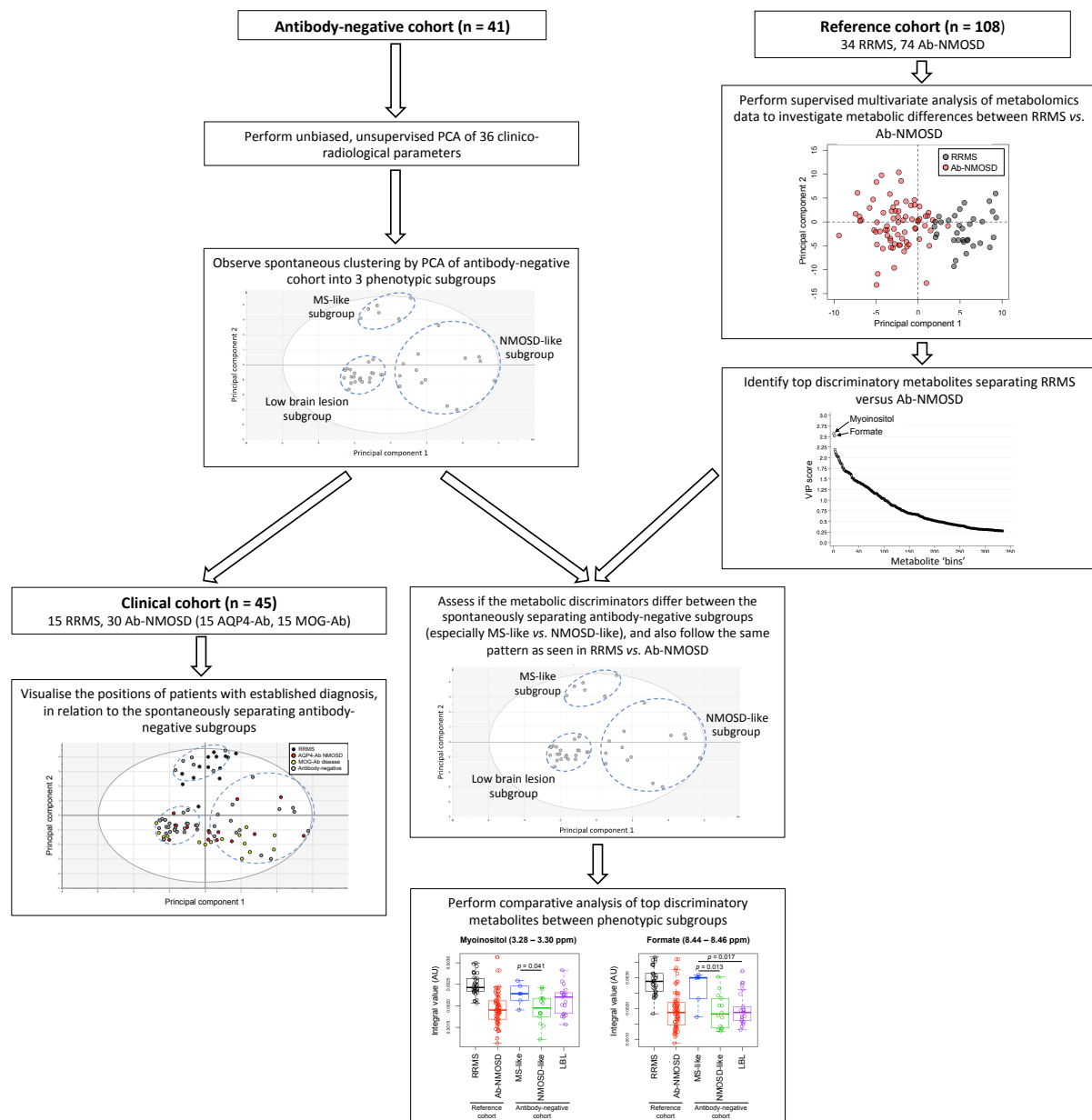


Figure 3.1: Outline of the study workflow.

3.2.1.1 Antibody-negative cohort for PCA model building using clinico-MRI features

Forty-one antibody-negative patients were recruited from the Oxford national NMO service at the John Radcliffe Hospital from November 2013 to September 2015 (i.e. MT cohort). All patients were out of relapses and were referred by their primary neurologists for possible NMOSD and none had typical MS. Serum in all patients were negative on multiple occasions for both AQP4-Ab and MOG-Ab, tested by cell-based assays as previously described (Waters et al., 2015; Waters et al., 2012).

Clinico-radiological data was obtained from medical notes and reviews of clinical MRI scans were supplemented by neuroradiological reports. Thirty-six pre-defined clinico-radiological parameters were collected, focusing on features that have been described to distinguish between MS and NMOSD (Jurynczyk et al., 2015; Jurynczyk, Germalde, et al., 2017; Kim et al., 2015; Matthews et al., 2013) (**Table 3.1**). These parameters were scored as present if a patient ever had that clinico-MRI feature. This clinico-radiological dataset was used for unsupervised multivariate PCA for unbiased pattern-recognition to identify phenotypic subgroups within the antibody-negative patients.

Table 3.1: Details of the 36 clinico-radiological parameters and their definitions. MAGNIMS = Magnetic resonance Imaging in Multiple Sclerosis; IPND = International Panel for NMO diagnosis.

1. Any transverse myelitis
2. LETM • Cord lesion ≥ 3 vertebral segments
3. T ₁ hypointensity with corresponding T ₂ hyperintensity in acute stage of cord lesion
4. Cord lesion spanning cervical medullary junction
5. Predominant central cord involvement
6. Conus involvement
7. EDSS ≥ 6 at nadir of any attack
8. Any optic neuritis
9. Severe optic neuritis • Visual acuity ≤ 0.1 at nadir
10. Simultaneous bilateral optic neuritis • ≤ 1 month between the two clinical episodes
11. Simultaneous optic neuritis and transverse myelitis • ≤ 1 month between the two clinical episodes
12. Long segment optic neuritis • Involvement of $\geq$ half the length of optic nerve visualized on MRI
13. Optic chiasm involvement
14. Area postrema syndrome (clinical)
15. No brain lesion
16. 1 – 3 brain lesions
17. ≥ 4 brain lesions
18. Dawson's fingers • Finger-like projections orientated perpendicularly to the walls of the lateral ventricles
19. Lesion touching body of lateral ventricle
20. Inferior temporal lesion

21. Corpus callosum lesion
22. Diffuse splenial lesion
23. Fluffy (poorly demarcated) infratentorial lesion
24. Lesion adjacent to 4 th ventricle
25. Lesion adjacent to 3 rd ventricle
26. Periaqueductal lesion
27. Area postrema lesion (radiological)
28. Hypothalamic/thalamic lesion
29. Tumefactive lesion • ≥ 2cm T₂ hyperintense lesion with GAD enhancement
30. Cortical/Juxtacortical lesion
31. Juxtacortical S or U shaped lesion
32. Fulfill 2016 MAGNIMS dissemination in space criteria
33. Fulfill 2015 IPND seronegative NMOSD criteria
34. Disability progression independent of relapses
35. Unmatched CSF OCB
36. Co-existing autoimmunity and/or autoantibodies

3.2.1.2 Clinical cohort of RRMS and Ab-NMOSD patients for visualisation of known diagnostic clusters within PCA model

The same 36 clinico-MRI parameters were collected from 45 patients with established diagnosis (RRMS [n = 15], AQP4-Ab, [n = 15], MOG-Ab [n = 15]), randomly selected from the Oxford MS/NMO research database. This data was used as a predictive set and inserted into the PCA model that was built using the clinico-MRI data from antibody-negative patients, allowing corroboration of phenotypic subgroups (if any) with known diagnostic clusters.

3.2.1.3 Reference cohort of RRMS and Ab-NMOSD patients for plasma metabolomics discriminatory analysis

Plasma metabolomics spectral data from an independent cohort of 108 patients (i.e. MT cohort) with established diagnosis (RRMS [n = 34], AQP4-Ab [n = 54], MOG-Ab [n = 20]) was used to build discriminatory OPLS-DA models to identify metabolites separating RRMS from Ab-NMOSD (i.e.

AQP4-Ab combined with MOG-Ab patients). Sample collection protocols were identical for both the reference cohort and antibody-negative cohort.

MOG-Ab status was determined prior to metabolomics blood sampling, i.e. the anti-MOG positivity was already established before the metabolomics study. This meant that some MOG-Ab patients were still seropositive while others became seronegative after their attack (a common phenomenon in MOG-Ab disease) at the time point of metabolomics blood sampling. The criterion to establish anti-MOG seropositivity was a fluorescence visual score of ≥ 1 (range 0 – 4) on the live cell based assay expressing full-length MOG incubated with serum diluted at 1:20.

3.2.1.4 Blood collection, serum processing and NMR sample preparation

The *optimised* protocol, as described in Material and Methods, was used for pre-analytical sample-handling. Two hundred microlitres of plasma was used for metabolomics.

3.2.1.5 CPMG spectra data acquisition

All ^1H NMR experiments for global metabolomics were performed in-house at the Department of Chemistry, University of Oxford using a 700-MHz Bruker AVIII spectrometer, with the CPMG relaxation editing pulse sequence for spectra acquisition. Technical specifications of the NMR experiments and details of data handling are as described in Materials and Methods. NMR experiments were performed at the same time for both the reference cohort and antibody-negative cohort. In all, 335 metabolite ‘bins’ were available for multivariate statistical analysis.

3.2.1.6 Multivariate statistical analysis

Unsupervised multivariate PCA was employed to identify potential subgroups within the antibody-negative cohort using clinico-imaging data. As the analysis is unsupervised, clustering (if any) is in no way influenced by the user but rather is wholly dependent on the clinico-MRI data alone.

Furthermore, the PCA approach allows the inclusion of correlated variables which reflects the actual, real-life clinico-MRI (often correlated) data gathered by a neurologist when seeing a patient. This approach was used to analyse the 36 pre-defined clinico-radiological parameters (binary data) to evaluate the degree of clustering between the 41 antibody-negative patients based on clinico-MRI features, enabling clusters (if any) to be identified. Loadings plots were generated to visualise the clinico-radiological parameters responsible for clustering.

OPLS-DA was used to interrogate the CPMG spectral data set to identify metabolic differences between RRMS and Ab-NMOSD. Details of the OPLS-DA approach are as described in Materials and Methods.

3.2.1.7 Univariate statistical analysis

Analysis of other clinico-imaging and metabolomics data were performed with STATA software (Release 14, College Station, TX: Statacorp LP) and R software. Chi-square tests or Fisher's exact tests were used for categorical variables as appropriate, while two-sample t-test/one-way ANOVA with Tukey's post-hoc correction or Mann Whitney U/Kruskal Wallis tests were used for continuous variables as appropriate. Two-tailed p values of <0.05 were considered statistically significant.

3.3 Results

3.3.1 PCA of clinico-radiological data within the antibody-negative cohort

To identify potential phenotypic subgroups within antibody-negative patients, unsupervised PCA of the 36 specified clinico-radiological parameters were performed. The PCA scores plot is shown in **Figure 3.2**. Each point in the plot represents all 36 clinico-radiological parameters from one patient; points closer to one another are more clinically alike. Two clusters of data points were apparent on visual inspection of the PCA scores plot – a bottom left cluster and a top cluster. There remained some data points that were observed to group loosely compared to the other two clusters. As such,

the antibody-negative cohort was grouped into three patient clusters (dashed blue circles) depending on their relative positions to each other on the PCA plot (**Figure 3.2 A**). Alternative ways/clusters to group the patients were also considered (e.g. the seven data points right of the y axis along the first component could be grouped together with the bottom left cluster) or increasing the number of subgroups although doing this (in an already small sample size) would mean a loss of power for subsequent subgroup comparative analysis. The observations drawn from the PCA clustering suggested a distinct clinical profile for each cluster and the reason accounting for the clustering was explored.

The variable loadings plot of the PCA was constructed to identify the variables driving the clustering (**Figure 3.2 B**). The variables driving the top cluster are features characteristic of MS (Arrambide et al., 2018; Matthews et al., 2013), while the ones defining the bottom right cluster are more typical of NMOSD (Jurynczyk et al., 2015; Kim et al., 2015; Wingerchuk et al., 2015). The bottom left cluster is characterised by no or low brain lesion load. This allowed the classification of these three phenotypic clusters into an MS-like subgroup, an NMOSD-like subgroup, and a low brain lesion (LBL) subgroup (**Figure 3.2 A**), with the most important variables listed in the inset.

To corroborate these phenotypic assignments with patients with established diagnosis, the 36 clinico-radiological parameters were collected from patients in the clinical cohort of known RRMS and Ab-NMOSD. Insertion of this data set confirmed that most of the RRMS patients clustered with the MS-like subgroup, while the majority of the AQP4-Ab NMOSD and MOG-Ab disease patients clustered to the NMOSD-like subgroup (**Figure 3.2 C**). It is interesting to note the clustering of AQP4-Ab and MOG-Ab patients – this is consistent with previous studies showing that AQP4-NMOSD and MOG-Ab disease in adults have largely identical clinical presentations and cannot be distinguished on conventional MRI (Hyun et al., 2017; Jurynczyk, Gerales, et al., 2017). Of note, some RRMS, AQP4-Ab NMOSD and MOG-Ab disease patients clustered with the LBL subgroup, highlighting that these diseases have overlapping clinico-radiological features.

Taking these observations in totality, PCA of clinico-radiological data within the antibody-negative cohort identified three phenotypically distinct subgroups; an MS-like subgroup (n = 6), an NMOSD-like subgroup (n = 14) and an LBL subgroup (n = 21). **Table 3.2** shows the demographic and clinical data of the antibody-negative patients grouped by the three PCA-defined subgroups, as well as the proportions of patients having each of the 36 clinico-radiological parameters.

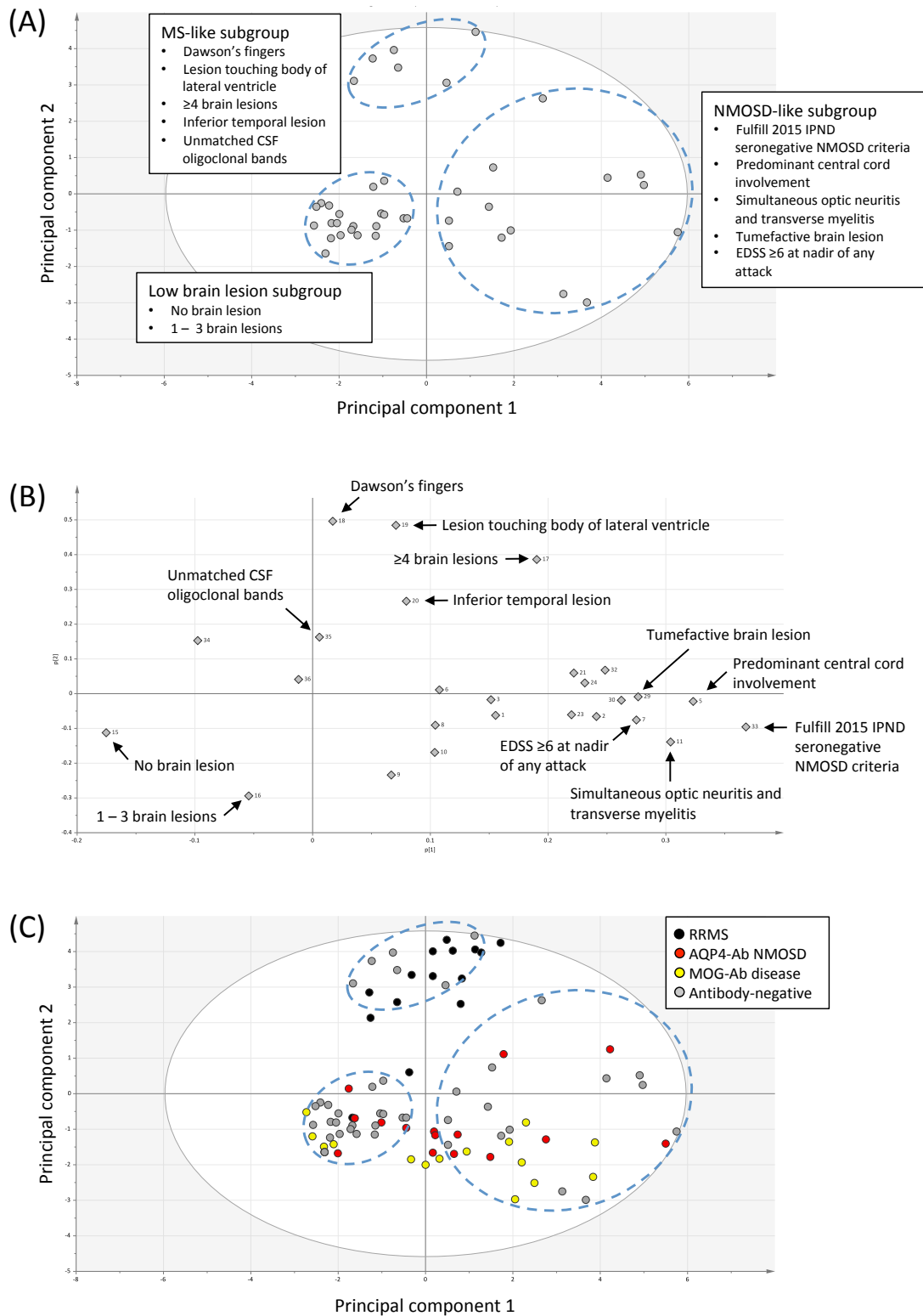


Figure 3.2: Identification of phenotypic subgroups within the antibody-negative cohort by PCA using clinico-radiologic data. (A) Spontaneous separation of antibody-negative patients into three distinct clusters using the 36 pre-defined clinico-radiologic parameters alone (dashed blue circles). (B) Variable loadings plot of the clinico-radiologic parameters allows visualisation of parameters responsible for patient clustering. Each parameter is represented by a grey diamond. The number beside each diamond corresponds to the number listed in **Table 3.1**. This enables the three phenotypic clusters to be classified as an MS-like subgroup, an NMOSD-like subgroup, and an LBL subgroup (panel A inset). (C) Insertion of clinico-radiologic data from the clinical cohort of patients with RRMS, AQP4-Ab NMOSD, and MOG-Ab disease into the PCA scores plot shows corroboration of the phenotypic subgroups with known diagnostic clusters.

Table 3.2: Demographic and clinico-radiological data within the antibody-negative cohort grouped according to the three PCA-defined subgroups. *Calculated with the onset attack included, and restricted to patients with at least one year interval between the onset attack and sampling. **Glatiramer acetate. ARR = annualised relapse rate.

	MS-like (n = 6)	NMOSD-like (n = 14)	LBL (n = 21)
Age at sampling, median (range), years	54.2 (37.5 – 71.5)	38.6 (24.4 – 70.4)	45.7 (23.0 – 59.7)
Female, No. (%)	3 (50.0)	8 (57.1)	13 (61.9)
Duration of disease (disease onset to sampling), median (range), years	5.4 (1.3 – 17.4)	3.4 (0.0 – 17.5)	5.2 (0.2 – 20.6)
ARR, median (range)*	0.2 (0.1 – 0.7)	0.7 (0.2 – 1.7)	0.3 (0.1 – 1.1)
Interval between last attack to sampling, median (range), years	5.4 (1.0 – 17.4)	1.8 (0.2 – 13.8)	3.2 (0.2 – 15.2)
Interval between disease onset to latest MRI brain, median (range), years	5.3 (0.5 – 17.4)	3.0 (0.3 – 17.7)	4.5 (0.003 – 14.0)
Interval between disease onset to latest MRI spine, median (range), years	2.5 (0.3 – 17.4)	3.0 (0.6 – 17.7)	4.5 (0.2 – 17.3)
On immunosuppressant, No. (%)	0 (0.0)	8 (57.1)	6 (28.6)
Azathioprine	-	5 (35.7)	3 (14.3)
Mycophenolate mofetil	-	2 (14.3)	2 (9.5)
Methotrexate	-	1 (7.1)	1 (4.8)
On prednisolone, No. (%)	1 (16.7)	7 (50.0)	5 (23.8)
On MS disease modifying therapy, No. (%)	0 (0.0)	0 (0.0)	1 (4.8)**
The 36 clinico-radiological variables used for PCA multivariate analysis	-	-	-
Any transverse myelitis, No. (%)	4 (66.7)	14 (100.0)	16 (76.2)
LETM, No. (%)	1 (16.7)	12 (85.7)	5 (23.8)
T ₁ hypointensity with corresponding T ₂ hyperintensity in acute stage of cord lesion, No. (%)	0 (0.0)	5 (35.7)	1 (4.8)
Cord lesion spanning cervical medullary junction, No. (%)	0 (0.0)	1 (7.1)	1 (4.8)
Predominant central cord involvement, No. (%)	2 (33.3)	13 (92.9)	4 (19.0)
Conus involvement, No. (%)	2 (33.3)	4 (28.6)	1 (4.8)
EDSS ≥6 at nadir of any attack, No. (%)	1 (16.7)	12 (85.7)	2 (9.5)
Any optic neuritis, No. (%)	2 (33.3)	11 (78.6)	9 (42.9)
Severe optic neuritis, No. (%)	0 (0.0)	6 (42.9)	6 (28.6)
Simultaneous bilateral optic neuritis, No. (%)	0 (0.0)	5 (35.7)	2 (9.5)
Simultaneous optic neuritis and	0 (0.0)	5 (35.7)	0 (0.0)

transverse myelitis, No. (%)			
Long segment optic neuritis, No. (%)	0 (0.0)	0 (0.0)	1 (4.8)
Optic chiasm involvement, No. (%)	0 (0.0)	0 (0.0)	0 (0.0)
Area postrema syndrome, No. (%)	0 (0.0)	2 (14.3)	0 (0.0)
No brain lesion, No. (%)	0 (0.0)	0 (0.0)	7 (33.3)
1 – 3 brain lesions, No. (%)	0 (0.0)	6 (42.9)	12 (57.1)
≥4 brain lesions, No. (%)	6 (100.0)	8 (57.1)	2 (9.5)
Dawson's fingers, No. (%)	6 (100.0)	2 (14.3)	0 (0.0)
Lesion touching body of lateral ventricle, No. (%)	6 (100.0)	3 (21.4)	0 (0.0)
Inferior temporal lesion, No. (%)	2 (33.3)	1 (7.1)	0 (0.0)
Corpus callosum lesion, No. (%)	1 (16.7)	6 (42.9)	3 (14.3)
Diffuse splenial lesion, No. (%)	0 (0.0)	2 (14.3)	0 (0.0)
Fluffy infratentorial lesion, No. (%)	0 (0.0)	3 (21.4)	0 (0.0)
Lesion adjacent to 4 th ventricle, No. (%)	1 (16.7)	5 (35.7)	0 (0.0)
Lesion adjacent to 3 rd ventricle, No. (%)	0 (0.0)	2 (14.3)	0 (0.0)
Periaqueductal lesion, No. (%)	0 (0.0)	2 (14.3)	0 (0.0)
Area postrema lesion, No. (%)	0 (0.0)	2 (14.3)	0 (0.0)
Hypothalamic/thalamic lesion, No. (%)	0 (0.0)	1 (7.1)	0 (0.0)
Tumefactive lesion, No. (%)	0 (0.0%)	3 (21.4%)	0 (0.0)
Cortical/Juxtacortical lesion, No. (%)	1 (16.7)	6 (42.9)	2 (9.5)
Juxtacortical S or U shaped lesion, No. (%)	0 (0.0)	2 (14.3)	0 (0.0)
Fulfill 2016 MAGNIMS Dissemination in Space criteria, No. (%)	4 (66.7)	10 (71.4)	5 (23.8)
Fulfill 2015 IPND seronegative NMOSD criteria, No. (%)	0 (0.0)	12 (85.7)	0 (0.0)
Disability progression independent of relapses, No. (%)	3 (50.0)	1 (7.1)	4 (19.0)
Unmatched CSF OCB, No. (%)	4 (66.7)	7/13 (53.8)	9/18 (50.0)
Co-existing autoimmunity and/or autoantibodies, No. (%)	2 (33.3)	3 (21.4)	5 (23.8)

3.3.2 Distinguishing RRMS vs. Ab-NMOSD (reference cohort) using OPLS-DA on plasma metabolomics data

While unsupervised PCA of extensive clinico-radiological data is able to identify distinct phenotypes within the antibody-negative cohort, pathophysiological relevance at a molecular level with respect to the reference diseases (i.e. MS pathology vs. antibody-mediated pathology) is lacking. Thus, in order to investigate if plasma metabolomics can identify metabolic biomarkers separating the antibody-negative phenotypic subgroups with inference to their underlying pathologies, discriminatory metabolic markers in the reference cohort of known RRMS and Ab-NMOSD patients were obtained. OPLS-DA was used to build discriminatory models using metabolomics spectral data to distinguish between RRMS and Ab-NMOSD within the reference cohort. A representative OPLS-DA scores plot was generated (**Figure 3.3 A**). Each point in the plot represents all metabolomics data from one patient; points closer to one another are more metabolically similar. A clear separation between RRMS and Ab-NMOSD was observed on the scores plot. This separation was validated as the mean accuracy (of the ensemble of accuracies) of the disease groups model was significantly greater than the mean accuracy of the random class assignment model (mean $\pm$ SD, $80.7 \pm 4.2\%$ vs. $52.3 \pm 7.6\%$; $p < 0.0001$) (**Figure 3.3 B**).

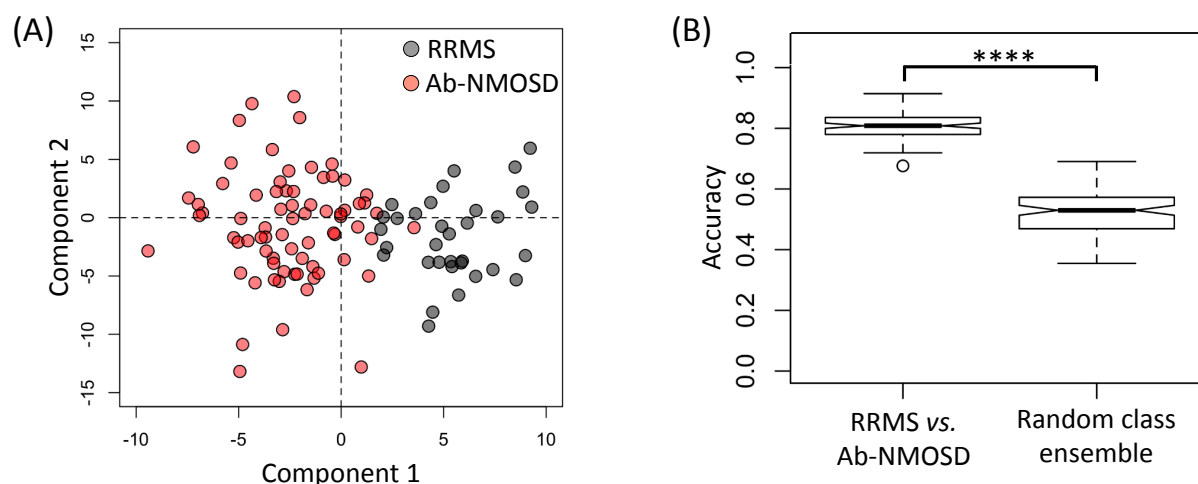


Figure 3.3: OPLS-DA models generated from CPMG metabolomics spectral data of RRMS vs. Ab-NMOSD patients from the reference cohort. **(A)** Representative OPLS-DA scores plot (RRMS = black circles, Ab-NMOSD = red circles). **(B)** Box plots of predictive accuracies from the OPLS-DA models of RRMS vs. Ab-NMOSD patients, against random class assignment. ****indicates $p < 0.0001$ by Kolmogorov-Smirnov test.

3.3.2.1 Identifying discriminatory metabolites from the RRMS vs. Ab-NMOSD OPLS-DA models

Next, to identify the most important metabolites driving the separation between RRMS and Ab-NMOSD, VIP scores were generated. Ranking of VIP scores revealed that myoinositol and formate (both metabolites being higher in RRMS) were the two most important metabolites driving this separation (**Figure 3.4 A**), with a VIP score of 2.57 and 2.51 respectively. ROC analysis revealed high diagnostic accuracies, as measured by AUC, of myoinositol (AUC = 0.914, 95% CI 0.862 – 0.967; $p < 0.0001$) and formate (AUC = 0.907, 95% CI 0.849 – 0.965; $p < 0.0001$) (**Figure 3.4 B**).

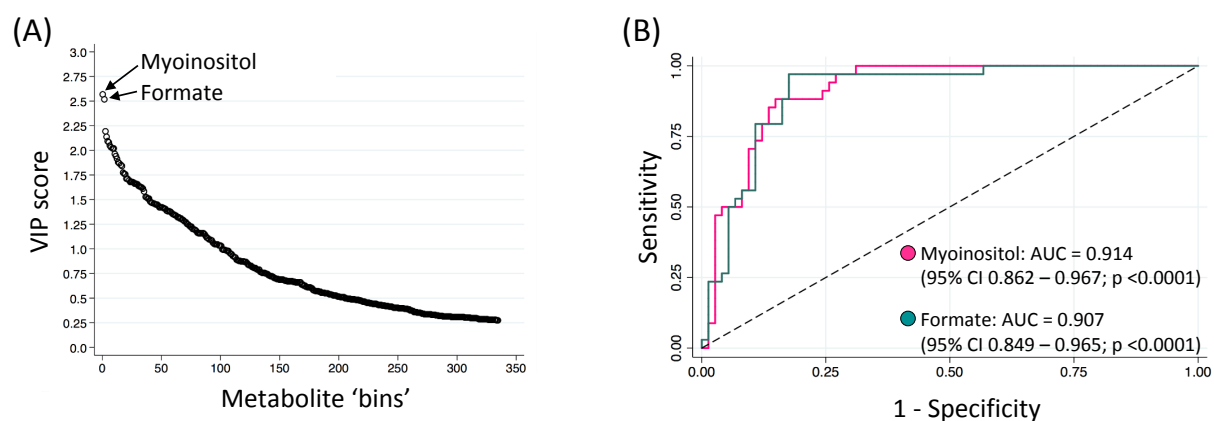


Figure 3.4: (A) VIP score ranking plot obtained from the OPLS-DA models of RRMS vs. Ab-NMOSD patients identifies myoinositol and formate as the top two discriminatory metabolites. (B) On ROC analysis, both metabolites, individually, show high AUC in distinguishing RRMS and Ab-NMOSD.

3.3.3 Comparing myoinositol and formate levels between the MS-like and the NMOSD-like subgroups within the antibody-negative cohort

As myoinositol and formate could accurately discriminate between RRMS and Ab-NMOSD, it was explored if these metabolites are also different between the MS-like and NMOSD-like clinico-radiological subgroups within the antibody-negative cohort. Myoinositol was significantly higher in the MS-like subgroup compared to the NMOSD-like subgroup (mean $\pm$ SD, 0.0023 ± 0.0002 AU vs. 0.0019 ± 0.0003 AU; $p = 0.041$) (**Figure 3.5 A**). Formate was also significantly elevated in the MS-like subgroup vs. the NMOSD-like subgroup (0.0027 ± 0.0006 AU vs. 0.0019 ± 0.0006 AU; $p = 0.010$). On one-way ANOVA, formate was significantly different across the three subgroups [$F(2, 38) = 5.02$; $p = 0.012$]; post-hoc comparisons using Tukey's test showed formate to be higher in the MS-like subgroup compared to the NMOSD-like subgroup ($p = 0.013$), as indeed when compared to the LBL subgroup (0.0027 ± 0.0006 AU vs. 0.0020 ± 0.0005 AU; $p = 0.017$) (**Figure 3.5 B**). Taking successive discriminatory metabolites with VIP scores ≥ 1.75 (before the second 'drop off' in VIP scores, see **Figure 3.4 A**) showed similar trends in separating the MS-like from NMOSD-like subgroups (**Figure 3.6**). Next, we explored if the MS-like and NMOSD-like patients were metabolically similar to RRMS and Ab-NMOSD patients respectively. Using all CPMG metabolomics spectral data, it was not

possible to distinguish MS-like from RRMS patients, as well as NMOSD-like from Ab-NMOSD patients (Figure 3.7).

In summary, the two most discriminatory metabolites obtained from the OPLS-DA model of RRMS vs. Ab-NMOSD were also significantly different between the MS-like and NMOSD-like subgroups (and in the same direction) within antibody-negative patients. This suggests that the MS-like and NMOSD-like subgroups have different underlying pathologies, akin to their respective reference diseases (i.e. RRMS and Ab-NMOSD).

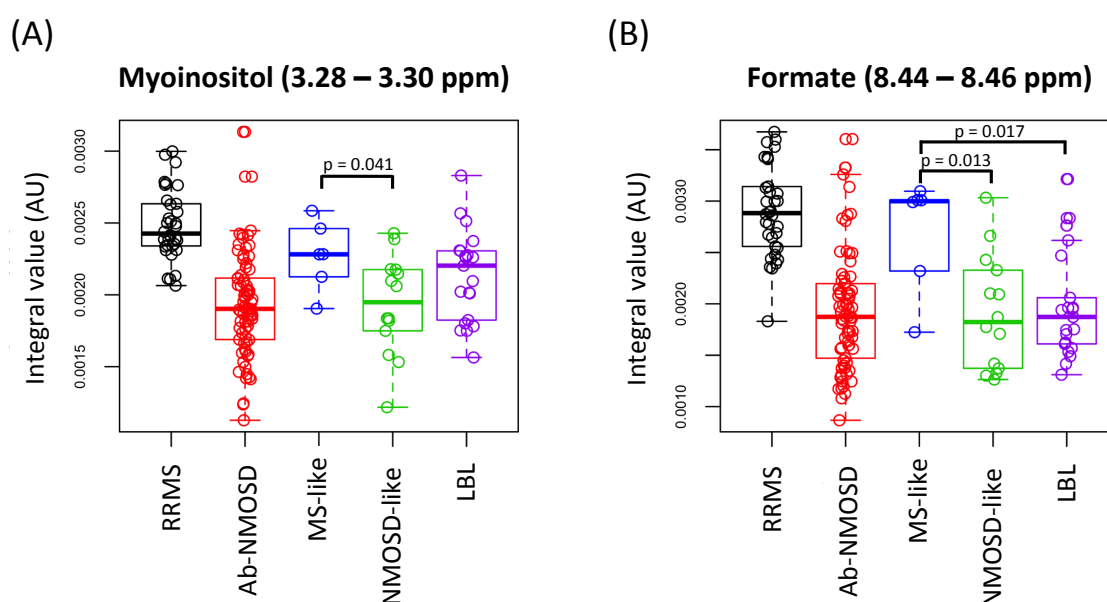


Figure 3.5: Box plots comparing myoinositol and formate levels between MS-like and NMOSD-like subgroups within the antibody-negative cohort. Both (A) myoinositol and (B) formate are significantly higher in the MS-like subgroup compared to the NMOSD-like subgroup. On one-way ANOVA, (B) formate was significantly different across the three subgroups and post-hoc comparisons using Tukey's test showed formate to be significantly higher in the MS-like subgroup compared to the NMOSD-like subgroup, as well as to the LBL subgroup. *p* values shown in (B) are from one-way ANOVA with post-hoc multiple comparison corrections. Box plots of myoinositol and formate in RRMS and Ab-NMOSD patients are constructed from the same data used to generate the AUC graphs in Figure 3.4 B.

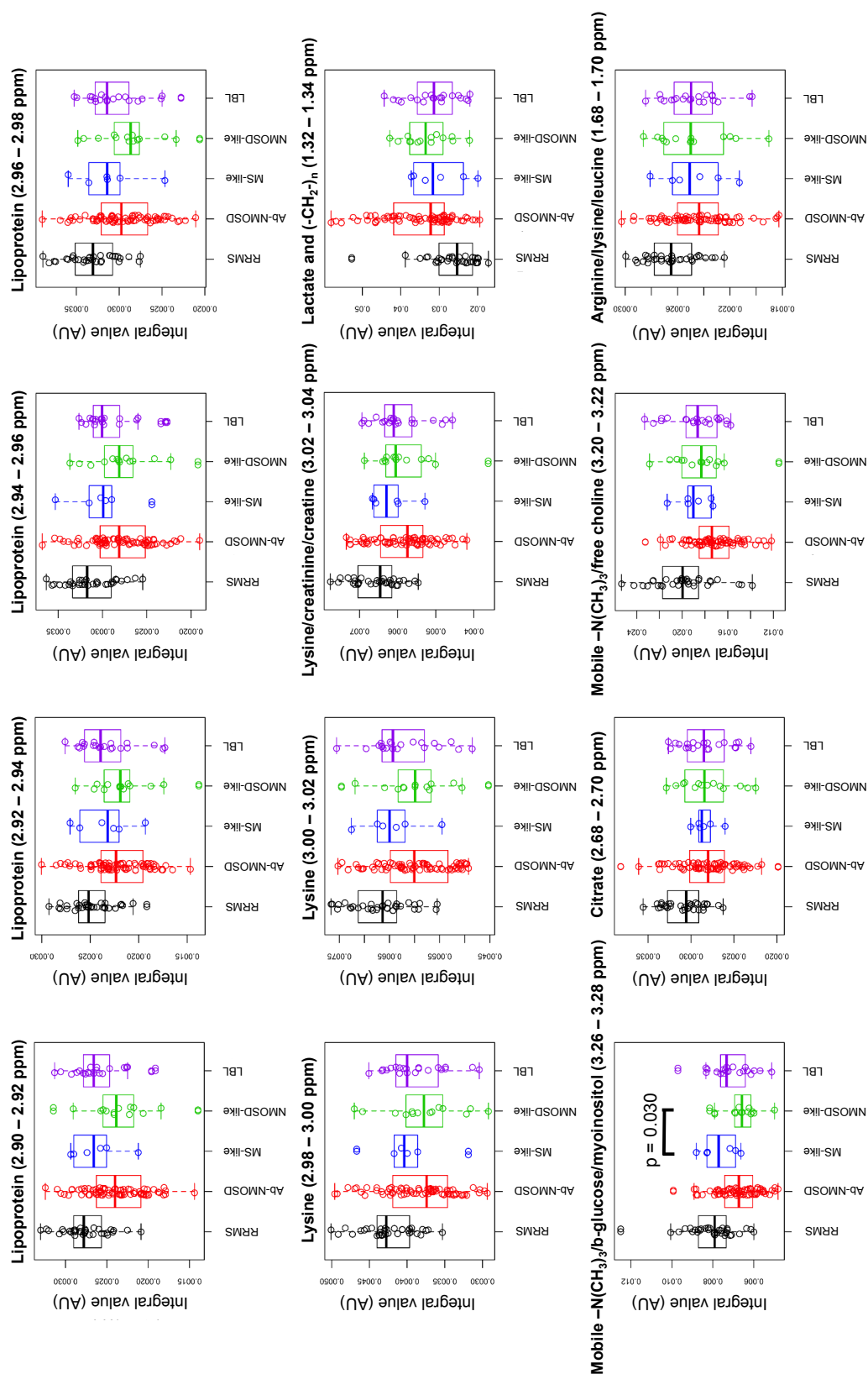


Figure 3.6: Box plots of other discriminatory metabolites (VIP score ≥ 1.75). Other discriminatory metabolites trend in the same direction when comparing the MS-like to NMOSD-like subgroups, as with RRMS to Ab-NMOSD. This trend becomes less clear with lower VIP scores as shown by the last three metabolite bins in the panel; citrate (2.68 – 2.70 ppm, VIP score 1.87), mobile $-N(CH_3)_3$ /free choline (3.20 – 3.22 ppm, VIP score 1.85), arginine/lysine/leucine (1.68 – 1.70 ppm, VIP score 1.75).

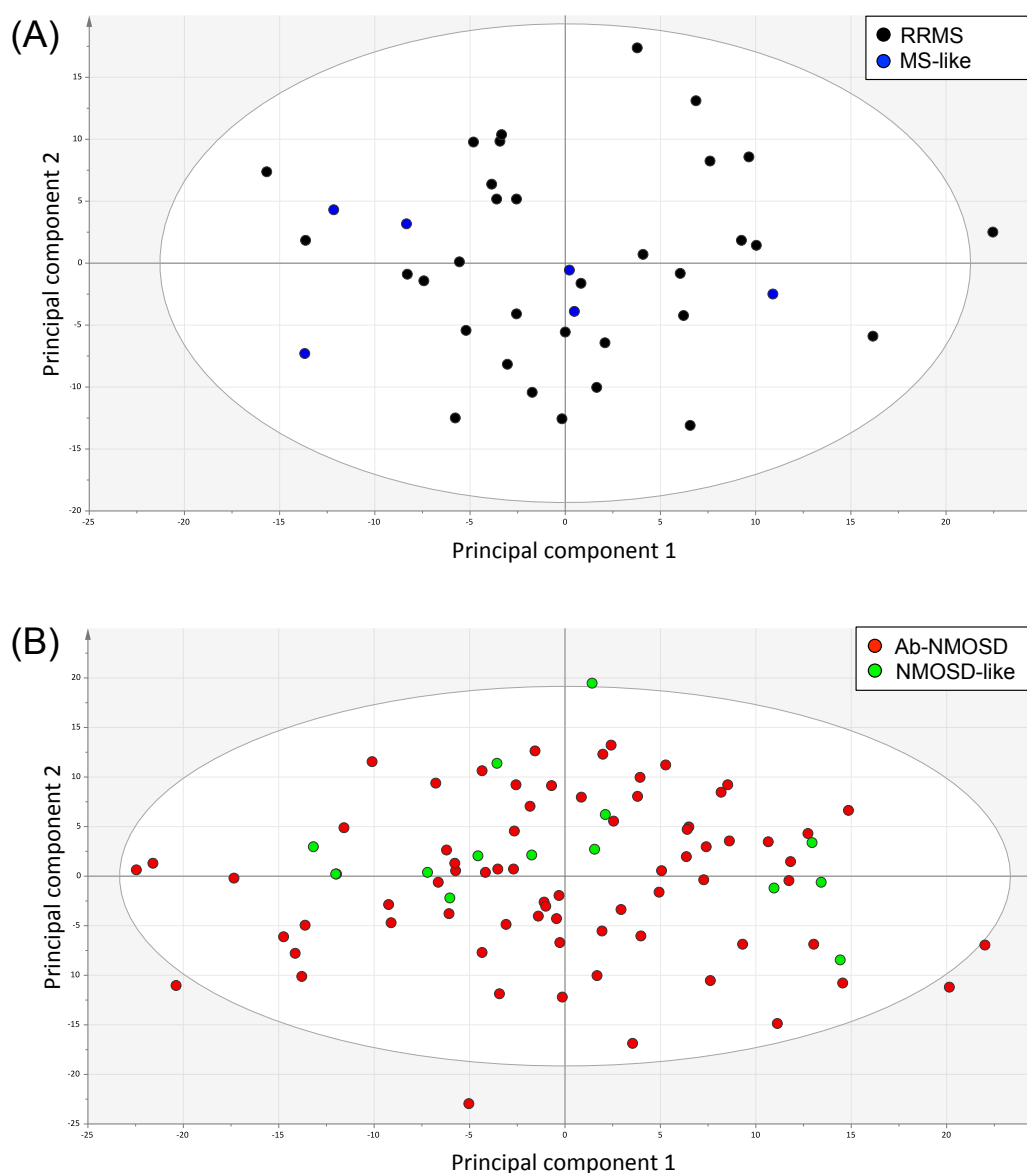


Figure 3.7: PCA scores plots of metabolomics spectral data comparing MS-like to RRMS patients, and NMOSD-like to Ab-NMOSD patients. There was no clustering of (A) MS-like patients separate from RRMS patients, as well as (B) NMOSD-like patients separate from Ab-NMOSD patients.

3.3.4 Addressing potential confounders for the identified metabolite biomarkers

As a higher proportion of patients in the NMOSD-like and LBL subgroups were on immunosuppressants and prednisolone at the time of plasma sampling compared to the MS-like subgroup (Table 3.2), it was explored if these accounted for the differences in myoinositol and formate levels. By combining the NMOSD-like and LBL subgroups, myoinositol and formate levels of patients on immunosuppressants were compared to patients not on immunosuppressants. Similar

analysis was performed for prednisolone use. There were no statistically significant differences in both metabolites stratified by immunosuppressant or prednisolone use; myoinositol by immunosuppressant use (on immunosuppressant, 0.0020 ± 0.0002 AU vs. off immunosuppressant, 0.0021 ± 0.0004 AU; $p = 0.384$); myoinositol by prednisolone use (on prednisolone, 0.0020 ± 0.0004 AU vs. off prednisolone, 0.0021 ± 0.0003 AU; $p = 0.224$); formate by immunosuppressant use (on immunosuppressant, 0.0019 ± 0.0005 AU vs. off immunosuppressant, 0.0020 ± 0.0005 AU; $p = 0.714$); formate by prednisolone use (on prednisolone, 0.0017 ± 0.0005 AU vs. off prednisolone, 0.0020 ± 0.0005 AU; $p = 0.111$). In fact within the NMOSD-like subgroup alone, patients on immunosuppressants had higher levels of myoinositol (on immunosuppressant, 0.0020 ± 0.0002 AU vs. off immunosuppressant, 0.0018 ± 0.0005 AU; $p = 0.370$) and formate (on immunosuppressant, 0.0021 ± 0.0006 AU vs. off immunosuppressant, 0.0016 ± 0.0002 AU; $p = 0.143$), and this would, if anything, reduced the discriminatory power of the metabolites. Similar analyses were performed for age, gender, disease duration, and interval since last attack with no significant differences/correlations in the levels of both metabolites based on these parameters.

3.4 Discussion

The above findings confirmed that distinct phenotypic subgroups exist within the antibody-negative cohort using PCA pattern-recognition techniques coupled with extensive clinico-radiological data, without any *a priori* assumptions of their clinical diagnosis. Application of the two most discriminatory metabolites (myoinositol and formate) between RRMS and Ab-NMOSD to the antibody-negative cohort confirmed that these same metabolites distinguished between the antibody-negative subgroups that were MS-like and NMOSD-like. This suggests that the phenotypic separation by PCA is pathophysiologically meaningful and the clinico-MRI features shown in **Figure 3.2** (inset) are pathologically relevant for classification. This has the potential to guide diagnostic and therapeutic decisions when seeing antibody-negative patients in the clinic.

Myoinositol is a component of the cell membrane and myelin, and is involved in intracellular signalling in many CNS cells (Rae, 2014). More importantly, it has been recognised as a marker of astrocyte activation and proliferation (Harris et al., 2015). Low myoinositol levels have been observed in AQP4-Ab NMOSD compared to MS after transverse myelitis using ^1H MRS of the spinal cord, reflecting astrocytic necrosis (Ciccarelli et al., 2013). Conversely, high myoinositol levels have been noted in RRMS and CIS patients compared to controls using ^1H MRS of NAWM, indicating reactive astrocytosis/astrogliosis (Chard et al., 2002; Fernando et al., 2004). Unlike AQP4-Ab NMOSD, MOG-Ab disease is not an astrocytopathy and GFAP is not elevated in the CSF (Kaneko et al., 2016). Although accurate quantification of astrocytes has not been performed in MOG-Ab disease in view of the small number of cases with histopathology, it is likely that extent of gliosis as seen in MS (resulting from ongoing chronic neuroinflammation) does not occur in MOG-Ab disease (Shu et al., 2019), and this may explain the reduced levels of myoinositol in MOG-Ab disease with respect to MS. This needs further pathological verification. The findings of higher myoinositol levels in RRMS and MS-like patients compared to Ab-NMOSD and NMOSD-like patients are in agreement with these observations. With regards to clinical metabolomics studies, Reinke et al found that CSF myoinositol levels were higher in MS patients compared to non-MS controls on ^1H NMR spectroscopy (Reinke et al., 2014). Using a similar platform, Moussallieh et al showed higher levels of scylloinositol (the stereoisomer of myoinositol) in RRMS compared to NMO patients. The authors postulated that this is also likely to be reflective of increased glial proliferation as other studies have demonstrated a correlation of myoinositol and scylloinositol concentrations (myoinositol being the more abundant isomer) (Moussallieh et al., 2014).

Formate causes mitochondrial damage by inhibiting cytochrome c oxidase resulting in disruption of the electron transport chain and production of ROS (Nicholls, 1976). Formate-induced cytotoxicity has been demonstrated in rat hippocampal cultures as well as in retinal (human and rat) cell cultures (Kapur et al., 2007; Treichel et al., 2003). Interestingly, methanol poisoning is mediated by formate,

producing optic nerve demyelination and subsequent progressive retinal axonal loss in humans (Nurieva et al., 2018; Sharpe et al., 1982). As mitochondrial dysfunction is a major process implicated in MS pathogenesis (Witte et al., 2014), it is of interest to note the higher formate levels in MS patients. It was observed in a ^1H NMR metabolomics study that CSF formate was higher in MS and NMOSD patients compared to HC, with no statistical difference between MS and NMOSD patients (Kim et al., 2017). How formate is involved in MS pathogenesis, if at all, as a primary mediator or as part of an injurious cascade will require further mechanistic studies.

The McDonald criteria was developed to identify individuals with a first MS-like clinical attack who are at increased risk of developing MS. However, as mentioned in the thesis Introduction, further modifications to the criteria in the last two decades has led to an increase in sensitivity (referenced to the development of CDMS), but with a trade-off of lower specificity. Moreover, it could be argued that a diagnostic criteria should also encompass components that permit the exclusion of competing diagnoses to prevent the misdiagnosis of MS (Solomon, 2019), as other conditions can mimic MS clinically and radiologically, as well as demonstrating DIS and DIT. In view of the lack of accuracy of the McDonald criteria to separate MS from NMOSD (Asgari et al., 2011; Chan et al., 2011; Pittock et al., 2006), the Oxford group has previously attempted to better delineate MS from Ab-NMOSD using conventional MRI parameters. Distinctive MRI brain features of MS include Dawson's fingers, inferior temporal lobe lesion and lesion adjacent to the body of the lateral ventricle (Jurynczyk, Geraldes, et al., 2017; Matthews et al., 2013), which interestingly, are also the variables driving the MS-like subgroup in this current study. The Oxford group has also previously shown that blood-based metabolomics can accurately separate MS from controls, and from AQP4-Ab NMOSD and MOG-Ab disease (Dickens et al., 2014; Jurynczyk, Probert, et al., 2017). The current study combines both approaches by using metabolomics to give pathological support to the spontaneously separating clinico-radiological phenotypes. Of note, the clinico-MRI phenotypic PCA classification identified the

2015 seronegative NMOSD criteria as the most important distinguishing NMOSD-like variable, independently supporting these criteria.

The main limitation of the current study is the small sample size due to the rarity of antibody-negative patients. Despite this, the findings still show a remarkable similar pattern of discriminatory metabolites in the MS-like against the NMOSD-like subgroups, as seen in RRMS against Ab-NMOSD patients. The study methodology is optimised to compare two subsets, and in the antibody-negative group there will be multiple disorders, hence the focus of the study was on the two phenotypic subgroups which appeared to represent MS-like and antibody-mediated-like pathology. The third phenotypic subgroup (from the PCA approach) contained patients with lower brain lesion load without any 'MS-like' or 'NMOSD-like' discriminators, and pathologies amongst this subgroup will include antibody-mediated pathologies, MS, other cell-mediated disorders such as CNS sarcoidosis, and monophasic post-infectious conditions. In view of the mixed conditions within the LBL subgroup, it was kept separate for analysis. Clinico-pathological classification within this LBL subgroup will be particularly challenging. However, in patients with 1 – 3 brain lesions who have 'MS-like' or 'NMOSD-like' discriminators, the identified clinico-radiological discriminators are still potentially useful, as illustrated by 43% of NMOSD-like patients having 1 - 3 brain lesions. Future validation of our findings is needed in an independent cohort of antibody-negative patients. Another limitation of this study was the lack of accurate information on social and dietary habits, prandial status, vitamin D levels, and BMI. However, it is likely that these potential confounders would have been evenly distributed across comparative groups and the OPLS-DA modelling approach would have maximised the variation according to class assignment, hence the variation introduced by potential confounders would have been comparatively smaller. There was also no systemic logistical bias (e.g. need for fasting bloods in a specific group) that could have accounted for disease separation, in particular, for RRMS vs. Ab-NMOSD patients.

3.4.1 Chapter summary

The study demonstrates the strength of computational modelling of clinico-MRI features, which cannot be done in a consistent and unbiased way by clinicians in the clinical setting given the huge amount of data available for each patient. In addition, the findings also highlight the use of metabolomics in supporting PCA phenotypic classification, with disease-specific pathogenic inference. By selecting a diagnostically challenging group of patients, the current study is able to identify useful clinical and radiological characteristics that support some antibody-negative individuals having likely MS and others with likely antibody-mediated pathology. As the MRI parameters are not time restricted, these observations are more useful to apply in clinical practice. Prospective work to study treatment responses and long-term outcome, along with CSF metabolomics analysis and samples taken during relapses may further improve this classification, especially in patients within the LBL subgroup.

Chapter 4: Serum and CSF metabolomics in a focal DTH model of MS

4.1 Introduction

Chapter 3 illustrated that an RRMS-specific blood metabolic signature, relative to Ab-NMOSD (i.e. combining AQP4-Ab and MOG-Ab NMOSD), is present. This constellation of metabolites is likely to reflect the total MS lesion burden, which comprises of both inflammatory and quiescent lesions, as well as the systemic response that accompanies MS-specific neuroinflammatory and neurodegenerative processes. This metabolic signature is therefore applicable to differentiate RRMS from Ab-NMOSD patients, regardless of lesion burden, inflammatory activity or disease duration, especially when it is known that a clinico-radiological paradox exists in MS.

While the above is useful for diagnostic purposes, it would be particularly informative to explore the metabolic perturbations arising from an acute, isolated MS lesion in individuals, independent of the contributions from other quiescent lesions or neurodegenerative processes, as the identified metabolic signature and candidate metabolites could be used as prognostic biomarkers for disease activity and to better understand the pathogenesis of a lesion. However, identifying this lesional signature is hugely challenging in practice as a significant proportion of patients presenting with CIS already has evidence of prior CNS inflammatory events on MRI (Fisniku et al., 2008), which once again, highlights the clinico-radiological paradox in MS. Furthermore, in CIS patients in whom there are no other MRI lesions except for the symptomatic one, a clinical diagnosis of MS cannot be established at that time point (Thompson et al., 2018). In such cases, a sample for metabolomics needs to be taken at this 'CIS' time point but detailed follow-up would be required to confirm the diagnosis of MS and it would be unclear whether any other pathology might have been present. In view of these challenges, clinically relevant animal models of MS represent an important and attractive platform to better understand the local and systemic metabolic changes that arise from an

evolving neuroinflammatory lesion, from its initiation through to the chronic active phase. Indeed, to understand whether it is even possible to detect an 'echo' of a single, focal MS-like CNS lesion in the periphery or at what stage it might become possible to detect this 'echo' is an important matter that needs to be addressed in order to shed light on the nature and source of the metabolic change that has been observed in individuals with MS.

An MS model that has been utilised in previous metabolomics studies, many of which have already been described in the Introduction to this thesis, is the EAE model. A number of variations to this model exist although it principally involves the peripheral immunisation with myelin-based proteins/peptides (i.e. MBP, MOG, PLP), followed by the administration of CFA and pertussis toxin, generating a severe inflammatory response directed against host myelin/CNS components as evidenced by the presence of numerous, diffuse inflammatory lesions throughout the CNS, predominantly in the spinal cord (Constantinescu et al., 2011). EAE lesions typically show an inflammatory infiltrate consisting of macrophages with a preponderance of CD4 positive T cells, whereas CD8 positive T cells dominate in human MS (Huseby et al., 2012). Mechanistically, EAE is an attractive animal model to study CNS autoimmunity, however, several features make it less suitable to study metabolic changes arising solely from neuroinflammation. Firstly, the lesions generated in EAE are random with regards to their topography and size. This is an important consideration since the spinal cord, optic nerve, and brain regions have different cellular and structural make up, hence it is conceivable that damage to these regions (as with varying lesion sizes within the same regions) are likely to generate different metabolic profiles. Indeed, it has been shown using mass spectroscopy that different regions within the brain exhibit region-specific metabolic signatures, with the olfactory bulb and cerebellum having the most distinct metabolic profiles, while the cortical parenchyma and hippocampus were more similar in their metabolome (Choi et al., 2018). In other words, the metabolic profile of one EAE animal to the next is likely to be inconsistent, even within the same experiment. Furthermore, the lack of a pre-specific, defined CNS locale from which

histological sections are obtained precludes representative histological quantification and correlation with the metabolic alterations. A similar concern is also present in EAE models investigating NfL concentrations as the random involvement of various CNS structures implies that different amounts of NfL are released. Secondly, the EAE model is associated with overt clinical signs, typically appearing at day 9 to 14 (Bittner et al., 2014), ranging from weight loss, mild paralysis to complete paralysis and sometimes even death. These confound the metabolomics picture due to the aberrant metabolic contributions as a consequence of paralysis, weight loss, food deprivation and dehydration.

In view of the potential confounds associated with the EAE metabolome, an animal model of MS with a focal, single, well-defined CNS lesion with no overt clinical signs would be much better suited to study the metabolite profile. The delayed-type hypersensitivity (DTH) model was first described by Matyszak and Perry (Matyszak et al., 1995), in which a focal, cell-mediated lesion is generated, first by stereotaxic intracranial injection of heat-inactivated bacillus Calmette-Guerin (BCG) followed by peripheral sensitisation via intradermal injection of an emulsion made from inactivated BCG and CFA. The initial innate immune response to intracranial BCG implantation results in little neuroinflammation likely due to CNS-specific immune characteristics (i.e. decreased antigenic presentation, down-modulation of the inflammatory response by immunoregulatory molecules and neuropeptides) (Bechmann et al., 2014), that confers the brain's 'immune privilege'. The innate response to the intracranial BCG implantation is characterised by the initial presence of activated microglia and macrophages, but these display resting state morphology by week 4 and there is minimal presence of T cells at all time points (Matyszak et al., 1995), and the initial challenge is fully resolved three to four weeks after intracranial BCG injection. However, if peripheral sensitisation is performed, a robust peripheral immune response is initiated and is argued to involve the following processes: peripheral antigen presenting cells (APC) phagocytose and present the BCG antigens to T cells, which, in turn, become activated. Activated T cells then cross the intact BBB where they

encounter the implanted cognate BCG antigens presented by CNS resident APC (the microglia). The release of cytokines and chemokines recruits peripheral inflammatory cells (monocytes/macrophages) and activates resident microglia and astrocytes at the site of the initial intracranial BCG implantation. The histology of the DTH lesion recapitulates that seen in human MS: an inflammatory infiltrate consisting of T cells, macrophages, activated microglia, reactive astrocytes with perivascular cuffing and BBB breakdown as well as tissue damage as evidenced by demyelination and axonal loss (Broom et al., 2005; Matyszak et al., 1995; Newman et al., 2001). The acute DTH lesion is considered to be fully established between days 12 – 19, however chronic inflammation persists (i.e. chronic active), and BBB breakdown is still present albeit less marked (detectable by GAD enhanced MRI) at day 31 after peripheral immunisation (Broom et al., 2005; Newman et al., 2001). Indeed, the DTH lesion never fully resolves and its salient features are still detectable five months post-immunisation (Matyszak et al., 1995). Despite these features of neuroinflammation and tissue damage, it has to be noted that the DTH model does not produce any observable motor or behavioural disturbances (Anthony, Sibson, et al., 2014), eradicating the concern of aberrant metabolic contributions from paralysis, food and water deprivation.

4.1.1 Aims of Chapter 4

The aims of this chapter are:

1. To investigate the serum and CSF metabolic profile of the DTH lesion at different experiment time points, from acute lesion initiation to the chronic active phase relative to appropriate controls, and to relate the magnitude of metabolic alterations to the underlying neuroinflammatory and neurodegenerative histopathological changes.
2. To identify the principal serum and CSF discriminatory metabolites distinguishing diseased from control animals, and to explore if these lesion-specific metabolites correlate with the histological markers of neuroinflammation and tissue damage.

3. To explore CSF and serum NfL concentrations across the evolution of the DTH lesion, and to determine if NfL correlate with histological markers of neuroinflammation and neurodegeneration.

The experimental work presented within this chapter represents the first attempt to characterise the metabolomics profile and NfL concentrations in a focal animal model of MS.

The DPhil candidate's individual contributions to this work include: (1) design and conceptualisation of the study, (2) performance of all animal experiments, (3) acquisition of metabolomics and histological data, (4) analysis of all data as well as their interpretation. This chapter is currently being written up as a manuscript, with the DPhil candidate as first author.

4.2 Methods

An overview of the different experimental groups as well as the experimental time points is shown in **Figure 4.1**.

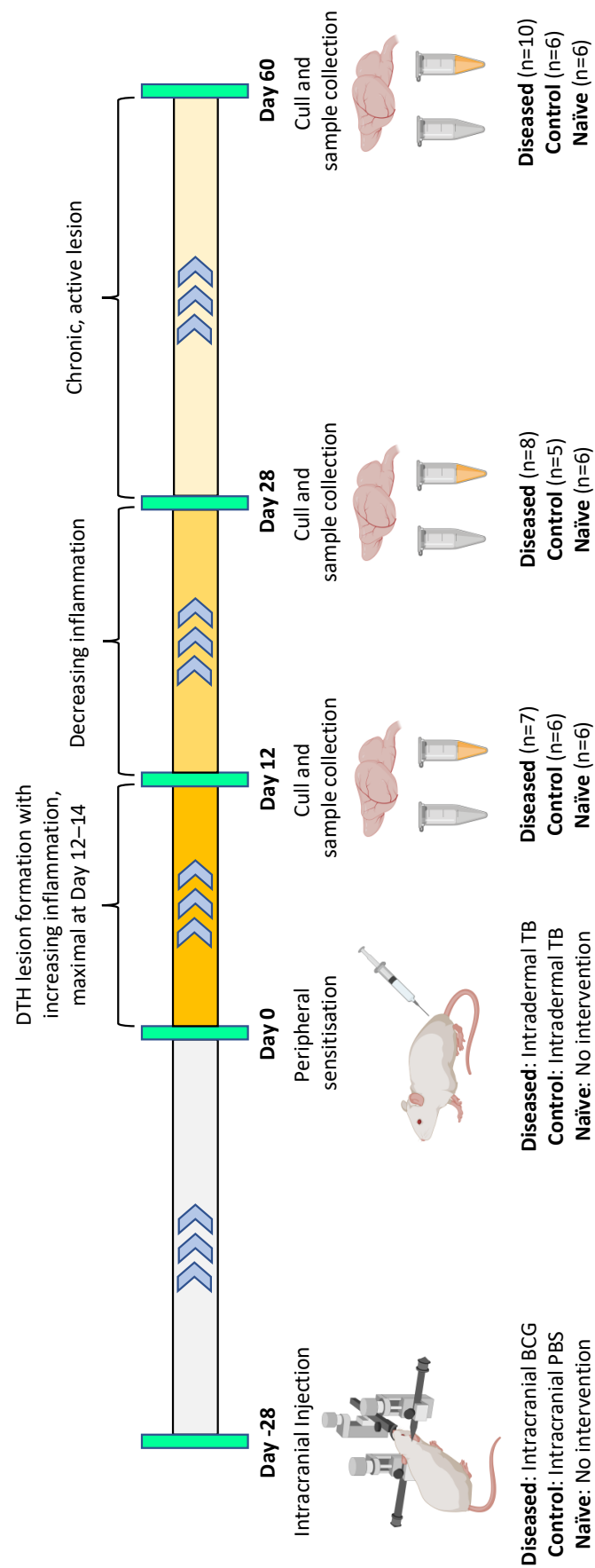


Figure 4.1: Overview of the different experimental groups and experimental time points in the study.

4.2.1 Animals

Four-weeks old male wild-type Lewis rats, weighing ~100 g were obtained from Charles River UK Ltd (Margate, UK) and housed in standard 12:12 diurnal/nocturnal light cycles with ad libitum access to food and water. Experiments were approved by the University of Oxford's Animal Welfare and Ethical Review Board (AWERB) under the project license number P996B4A4E. All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986. Efforts were made to ensure compliance with the ARRIVE guidelines at all times (Kilkenny et al., 2010). Animals were randomly assigned to naïve, control, and diseased (focal DTH lesion) groups and were culled at day 12, 28, and 60 after peripheral sensitisation.

4.2.2 Induction of the focal DTH lesion

The methodology employed to initiate the DTH lesion has been extensively reported elsewhere (Matyszak et al., 1995). Modifications to these published procedures, as used in the current study, are described in detail below.

4.2.2.1 Stereotaxic injection of heat-killed BCG (day -28)

Heat-killed BCG (a kind gift from Dr Rachel Tanner, The Jenner Institute, University of Oxford) was reconstituted in sterile phosphate-buffered saline (PBS) to achieve a concentration of 1×10^5 organisms/ μL . Animals were placed in an induction chamber for anaesthetic induction with a mixture of 3% isoflurane/oxygen at 2 L/min, shaved and mounted on a stereotaxic frame (Kopf® Instruments, California, USA) with 2% isoflurane/oxygen at 2 L/min as anaesthetic maintenance during surgery. Under an operating microscope, local anaesthesia was administered, and a small midline incision was made in the scalp. A burr hole was then drilled through the skull at 1 mm anterior and 3 mm lateral to bregma to expose the cortical surface. Two microlitres of heat-killed BCG (i.e. 2×10^5 organisms) was then injected slowly over 5 minutes via a finely drawn glass

microcapillary (Drummond Scientific Co., Pennsylvania, USA) into the left striatum at a depth of 4 mm from the cortical surface. The implantation site is illustrated in **Figure 4.2 A**. For control animals, an equivalent volume of sterile PBS was injected, while for naïve animals, no stereotaxic surgery was performed. After the intracranial injection was performed, the incision was closed with PDS™ II 6-0 sutures (Ethicon Inc., Cornelia, USA) and the animals were transferred into a warming chamber to allow recovery from anaesthesia.

4.2.2.2 Peripheral sensitisation with heat-killed mycobacteria tuberculosis (day 0)

Twenty-eight days after the intracranial injection, when the initial innate immune response to intracranial BCG has subsided, peripheral sensitisation with heat-killed mycobacteria tuberculosis (TB) was performed to provoke an immune cell-mediated response for the formation of focal DTH lesions at the site of BCG implantation. An emulsion containing CFA (Sigma-Aldrich Ltd, Dorset, UK), augmented with additional heat-killed mycobacteria TB (BD Difco™, Berkshire, UK) was prepared, giving a final TB concentration of 3.5 mg/mL. Under isoflurane-induced anaesthesia, animals in the diseased and control groups received 50 µL intradermal TB into each of the hind limbs (i.e. total of 0.35 mg of TB in 100 µL for each animal). Naïve animals did not receive any peripheral challenge. Post-injection wounds were taken care of as appropriate and housing conditions were similar for all animals regardless of treatment condition.

4.2.3 Culling of animals and sample collection (day 12, 28, 60)

Animals were culled on day 12, 28, and 60 after peripheral sensitisation. Animals were anaesthetised with a mixture of 3% isoflurane/oxygen at 2 L/min and shaved. A hollow cylinder was used to hyperflex the neck to allow for maximal access to the cisterna magna, via which ~150 µL of clean CSF was drawn using a 25 G needle attached to a 1 mL syringe (both from Terumo, Nottingham, UK). CSF samples were then placed in cryotubes (Crystal clear™, STARLAB UK Ltd), centrifuged at 15,000 g for 10 minutes at room temperature, aliquoted and frozen immediately at -80 °C in cryotubes till further

use. CSF samples that had visible blood-contamination were not used for metabolomics analysis. After CSF sampling, cardiac puncture was performed using a 21 G needle attached to a 5 mL syringe (both from Terumo, Nottingham, UK). Upon blood collection, the needle was detached (to prevent haemolysis) and blood was transferred to a red-top serum tube (BD™ 367837). This was left to stand at room temperature for 30 minutes after which blood was centrifuged at 1,300 g at room temperature for ten minutes to obtain serum. Serum was then aliquoted and frozen immediately at -80 °C in cryotubes till further use.

After CSF and blood collection, a U-shaped incision was made over the abdomen and chest wall to expose the thorax, followed by a small incision to the right atrium. Animals were transcardially perfused with 0.9% heparinised saline followed by 200 mL of 4% paraformaldehyde (both from Sigma-Aldrich Ltd, Dorset, UK). Brains were harvested, post-fixed in 4% paraformaldehyde overnight and immersed in 30% sucrose (Fisher Scientific, Loughborough, UK) for cryoprotection till the brains sunk. The brains were embedded in OCT compound (VWR Chemicals, Lutterworth, UK), then gently frozen in cold (-40 °C) isopentane (Fisher Scientific, Loughborough, UK) and stored at -20 °C till further use.

4.2.4 Immunohistochemistry

Embedded brains were sectioned at 10 µm thickness using a cryostat (Leica Biosystems Inc, Illinois, USA), mounted on gelatinised glass slides, dried overnight at room temperature and stored at -20 °C till further use. On the day of immunostaining, slides were dried for 45 minutes at 37 °C followed by a brief immersion in PBS. Heat-induced antigen retrieval was performed (90 °C sodium citrate buffer, pH 6.0) for 10 minutes. This was allowed to cool followed by quenching with 1% hydrogen peroxide (Sigma-Aldrich, Dorset, UK) in methanol (Fisher Scientific, Loughborough, UK) for 5 min with gentle agitation to suppress endogenous peroxidase activity. After several cycles of washing with PBS/0.1% PBST, sections were incubated with 10% goat serum in PBS/0.1% PBST for 1 hour at room

temperature to prevent cross-reactivity with endogenous proteins in the tissue. This was followed by incubation with primary antibodies diluted in 1% goat serum and PBS/0.1% PBST at 4 °C overnight. The primary antibodies used are detailed in **Table 4.1**.

Table 4.1: Primary antibodies used in the study.

Antibody	Manufacturer/code	Host species	Dilution	Target cell/structure
anti-IBA1	Abcam/ab178846	Rabbit	1:2000	Microglia and macrophage
anti-GFAP	Dako/Z0334	Rabbit	1:2000	Astrocyte
anti-MBP	Abcam/ab40390	Rabbit	1:1000	Myelin
anti-NfH	Abcam/ab8135	Rabbit	1:1000	Axon

Sections were then incubated with species-specific biotinylated secondary antibodies (Vectorlabs, Peterborough, UK) in 1% goat serum and PBS/0.1% PBST at room temperature for 2 hours and followed by incubation with Vectastain® Elite ABC HRP kit (Vectorlabs, Peterborough, UK) for 1 hour, with PBS/0.1% PBST washing in between. Antibody binding was visualised by an avidin-biotin peroxidase method using 3,3'-Diaminobenzidine (DAB) tetra-hydrochloride hydrate (Sigma-Aldrich, Dorset, UK) as a chromogen. After rinsing in acidified water, the sections were counterstained in 0.1% Cresyl Violet solution, progressively dehydrated with graded ethanol solutions (80%, 95%, 100%, 100%), and cleared in xylene for 5 minutes twice. As the last step, slides were mounted with DPX mounting media (Fisher Scientific, Loughborough, UK) and left to dry for 48 hours.

4.2.5 Histological quantification

Histological quantification was performed by an assessor blinded to the samples' treatments and culling time points.

4.2.5.1 Cell density quantification

Neuroinflammation induced by the focal DTH lesion was quantified by counting the number of IBA1 (microglia/macrophages) and GFAP (astrocytes) cells in Cresyl Violet counterstained sections using

five non-overlapping fields at 20 x magnification from regions adjacent and medial to the BCG implantation site (**Figure 4.2 C**). Images were captured (Nikon light microscope), imported into ImageJ and cells manually counted using the 'Cell Counter' function (Schindelin et al., 2012). Final cell density was expressed as mean number of cells/mm² calculated from the five fields.

4.2.5.2 Area quantification

Area of myelin and axonal loss was measured by the region devoid of MBP and NfH staining respectively within the striatum. Sections were scanned using a whole slide image scanner connected to a microscope (Nikon light microscope) at 20 x magnification and the area calculated using ImageJ (Schindelin et al., 2012), expressed in mm².

4.2.6 NMR sample preparation

Rat CSF and serum NMR sample preparation was performed as described in the Material and Methods section, with the exception of the ultracentrifugation step.

4.2.7 NMR spectra acquisition

All ¹H NMR experiments for metabolomics were performed in-house at Oxford using a 700-MHz Bruker AVIII spectrometer. The CPMG relaxation editing pulse sequence was employed for serum spectra acquisition while the NOESY presaturation sequence was used for CSF spectra acquisition. Technical specifications of the NMR experiments and details of data handling can be found in the Materials and Methods. In all, 194 serum metabolite 'bins' and 122 CSF 'bins' were available for multivariate statistical analysis.

4.2.8 CSF and serum NfL determination

CSF and serum NfL levels were measured using the Simoa® assay (Quanterix, Massachusetts, USA) performed at the University of Basel, in collaboration with Dr Jens Kuhle. Assay techniques and

principles have been previously described (Disanto et al., 2017). All laboratory personnel were unaware of the treatment status of the samples.

4.2.9 Multivariate statistical analysis

OPLS-DA with a 'leave one out internal cross-validation' approach was used to interrogate the spectral data to identify CSF and serum metabolic differences between the diseased and control animals. Details of this statistical approach are as described in the Materials and Methods.

4.2.10 Univariate statistical analysis

Univariate analysis of all other data was performed using GraphPad Prism (version 6, California, USA). Comparative analyses between groups were performed using one-way ANOVA with Tukey's pair-wise corrections. Two-way ANOVA was used to compare across treatment groups and time points, with Sidak's test for multiple comparison corrections. Pearson's correlation was used to explore correlations between histological markers with individual metabolites and with NFL. Two-tailed p values of <0.05 were considered statistically significant. Data was presented as mean $\pm$ SEM unless stated otherwise.

4.3 Results

4.3.1 Characterisation of the focal DTH model

Following peripheral challenge, a focal inflammatory lesion centred at the site of BCG implantation was observed in all diseased animals (**Figure 4.2 B and C**). The lesion had a central necrotic core, surrounding by an intense rim of IBA1 staining. Beyond this rim, an outer region consisting of a dense inflammatory infiltrate was observed. The five non-overlapping fields for cell density quantification were taken from the edge of this inflammatory infiltrate, on the medial side of the lesion (**Figure 4.2 C**). Blood vessels within this inflammatory infiltrate showed dilatation with

perivascular cuffing (i.e. accumulation of immune cells around the vessel wall). No lesion was found in the contralateral (i.e. right) hemisphere of the diseased group as well as in the control and naïve animals.

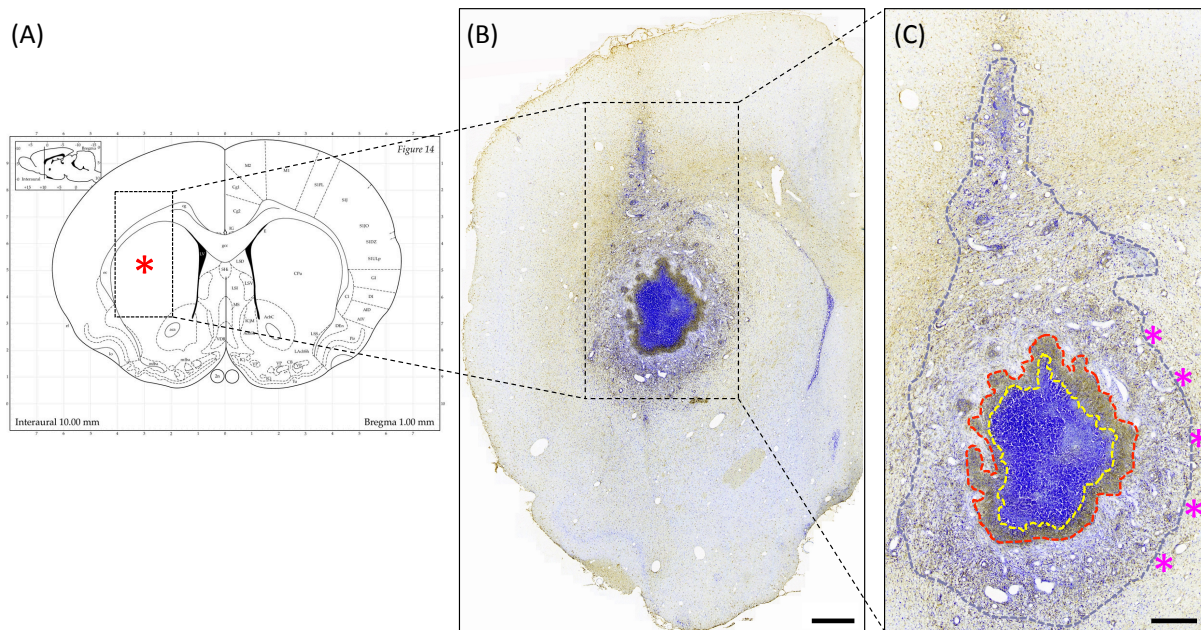


Figure 4.2: Focal DTH lesion. **(A)** Stereotaxic coordinates of the intra-striatal BCG injection (marked by the red asterisk) with reference to the bregma: 1 mm anterior, 3 mm lateral, 4 mm depth. Modified from Paxinos and Watson, *The rat brain in stereotaxic coordinates*, compact 7th edition. **(B)** Day 12 focal DTH lesion stained for IBA1 with Cresyl Violet counter-staining. Scale bar = 200 μ m. **(C)** On higher magnification, the lesion composed of a necrotic core (bordered by the yellow dashed line) with deep Cresyl Violet staining, surrounded by a rim of intense IBA1 staining (between the yellow and red dashed lines) and an outer region of extensive inflammatory infiltrate (between the grey and red dashed lines). Perivascular cuffing and dilatation of vessels were noted within this inflammatory infiltrate. This region also demonstrated a gradient of decreasing cellularity with increasing distance away from the lesion core. Five non-overlapping representative fields (indicated by the pink asterisks) at the medial edge of this dense infiltrate were selected for IBA1 and GFAP cell density quantification. Scale bar = 100 μ m.

4.3.1.1 Weight of animals

It is important to note that the first clinical manifestation of the classical EAE model prior to paralysis is weight loss, which is likely due to presence of high levels of circulating pro-inflammatory cytokines. In this study, the final weight of each animal was recorded at the time of culling, before CSF collection. There were no weight differences between the three treatment groups at each time

point (**Figure 4.3**). This was consistent with the observation made in this study, as well as with previous reports, that the focal DTH lesion induced at the chosen CNS locale did not produce any observable motor or behavioural disturbances (Anthony, Sibson, et al., 2014), in compliance with the ARRIVE guidelines (Kilkenny et al., 2010). In particular, the tail was noted to be rigid and there was no sign of gait disturbances at all time points.

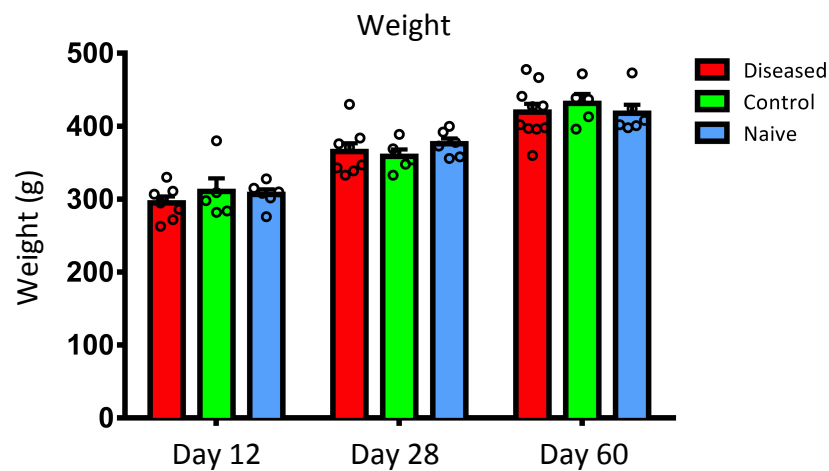


Figure 4.3: Weight of all animals at all three time points. No weight differences were observed between the treatment groups at each time point on two-way ANOVA.

4.3.1.2 Quantification of microglia/macrophage cell density

Anti-IBA1 antibody was used to immunostain for IBA1 expression of microglia/macrophages. Two-way ANOVA revealed that diseased animals had higher density of IBA1 cell staining compared to control animals at all time points (**Figure 4.4 A**). The greatest density was observed at day 12 when there is active lesion formation and BBB breakdown, with a significant decrease in staining density at day 28 and at day 60, during which there is BBB restoration. To compare within diseased animals across different time points while accounting for the basal density of IBA1 positive cells within controls, the cell densities of diseased animals were normalised to the mean cell density of the controls at each time point. Expectedly, the highest fold change was seen at day 12 and there was no difference in staining density noted between day 28 and day 60 (**Figure 4.4 B**). These observations

suggests that microglia/macrophage recruitment to the DTH lesion is the most intense at day 12, compatible with previous descriptions (Matyszak et al., 1995).

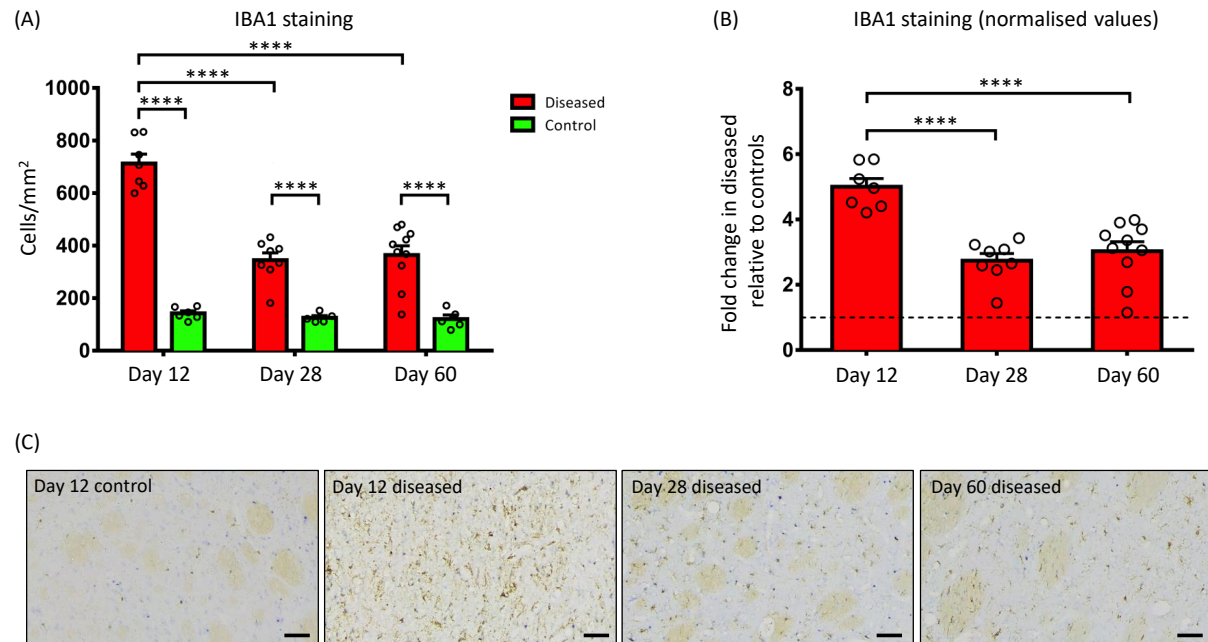


Figure 4.4: Microglia/macrophage cell densities across treatment groups and time points. **(A)** The mean density of IBA1 positive cells was greater in diseased animals compared to controls at all experimental time points, with the peak microglia/macrophage density noted at day 12. **** indicates $p < 0.0001$ on Sidak's post-hoc test after two-way ANOVA. **(B)** Fold change of microglia/macrophage density within diseased animals, relative to the mean density of the controls at each time point (indicated by the horizontal dashed line at 1.0). **** indicates $p < 0.0001$ on Tukey's post-hoc test after one-way ANOVA. **(C)** Representative images of IBA1 staining in day 12 control and in diseased animals at all three time points. Scale bars = 50 µm.

4.3.1.3 Quantification of astrocytic cell density

Anti-GFAP antibody was used to detect GFAP expression on astrocytes. Two-way ANOVA showed that the density of GFAP positive cells was higher in diseased animals compared to controls at all time points (**Figure 4.5 A**). Although statistically non-significant, it was noted that there was a trend of increasing astrocytic density within the controls with time. It is now increasingly recognised that there is a development of neuroinflammatory A1-like phenotype in astrocytes associated with aging, indeed, GFAP expression increases with age and in reactive astrocytes (Palmer et al., 2018). Therefore, the slight increase in GFAP staining within controls over time could be due to a function

of increasing age in an area of previous injury (control animals had stereotaxic injection of saline). To factor in this observation, the cell densities of diseased animals were normalised to the mean cell density of the controls at each time point. This normalisation strategy revealed that the fold change of astrocytic density was the greatest at day 12, similar to that observed in IBA1 staining, and decreases with time with a nadir at day 60 (**Figure 4.5 B**). Taking these findings in totality, this implies that reactive astrocytosis is the most intense at day 12.

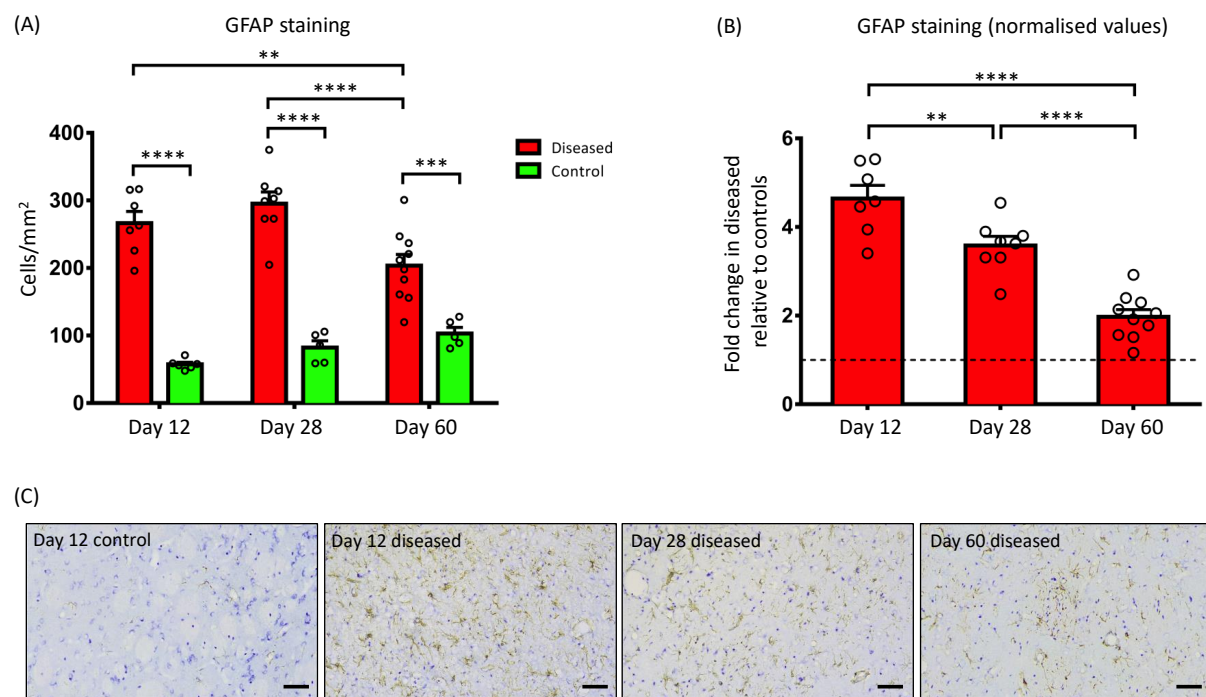


Figure 4.5: Astrocytic cell densities across treatment groups and time points. **(A)** The mean density of GFAP positive cells was higher in diseased animals compared to controls at all experimental time points. **** indicates $p < 0.0001$ and ** $p < 0.01$ on Sidak's post-hoc test after two-way ANOVA. **(B)** Fold change of astrocytic density within diseased animals, normalised to the mean density of the controls at each time point. Horizontal dashed line at 1.0 indicates the mean density of controls. **** indicates $p < 0.0001$, ** $p < 0.01$ on Tukey's post-hoc test after one-way ANOVA. **(C)** Representative images of GFAP staining in day 12 control and in diseased animals at the three time points. Scale bars = 50 μm .

4.3.1.4 Quantification of myelin loss

Anti-MBP antibody was used to probe for MBP expression on myelin. On one-way ANOVA, the area of myelin loss was the lowest at day 12, rose at day 28 with no further increase at day 60 (**Figure 4.6 A**). This implies that there was no further demyelination (or indeed remyelination) between day 28

and day 60 in a chronic active DTH lesion during which the BBB is gradually restored. There was no evidence of demyelination in control animals (as indeed in naïve animals), therefore this dataset did not require normalisation.

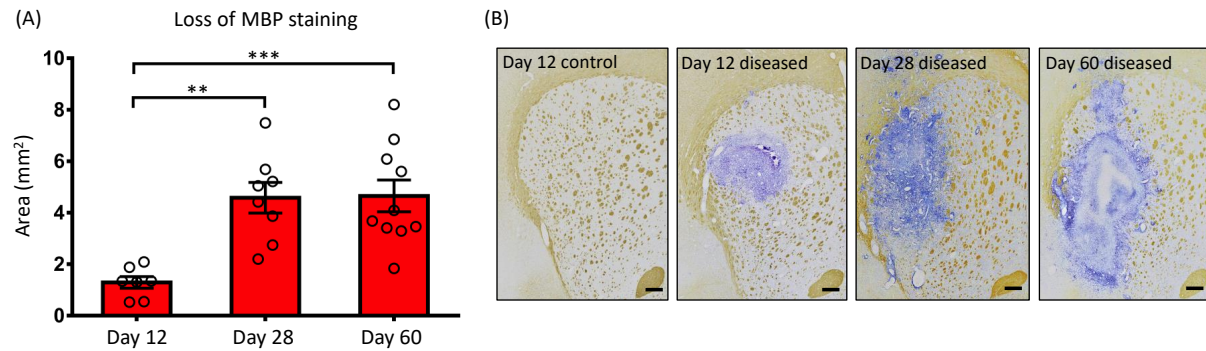


Figure 4.6: Area of demyelination within diseased animals. **(A)** Myelin loss was the least pronounced at day 12 and increased at day 28 with a plateau at day 60. ***indicates $p < 0.001$, ** $p < 0.01$ on Tukey's post-hoc test after one-way ANOVA. **(B)** Representative images of MBP staining in day 12 control and in diseased animals at all time points, showing a loss of MBP staining extending from the DTH lesion core to the outer inflammatory infiltrate layer. The region of Cresyl Violet counterstaining reflected the hypercellularity arising from the inflammatory infiltrate and within it, loss of MBP staining was observed. Scale bars = 50 μm .

4.3.1.5 Quantification of axonal loss

Anti-NfH antibody was used to assess axonal loss by detecting NfH expression of axons. Akin to MBP staining, the area of axonal loss within diseased animals was the lowest at day 12 and peaked at days 28 and 60 (**Figure 4.7 A**). There was a slight reduction in axonal loss at day 60 as compared to day 28 but this was not statistically significant. These findings suggest that maximal axonal loss occurs by day 28 and there may be some but limited axonal regeneration (or axonal sprouting) at day 60 (but the seeming reduction in NfH area loss may also reflect brain atrophy which is difficult to quantify accurately and meaningfully). There was no evidence of axonal loss in control animals or in naïve animals, therefore this dataset was not normalised.

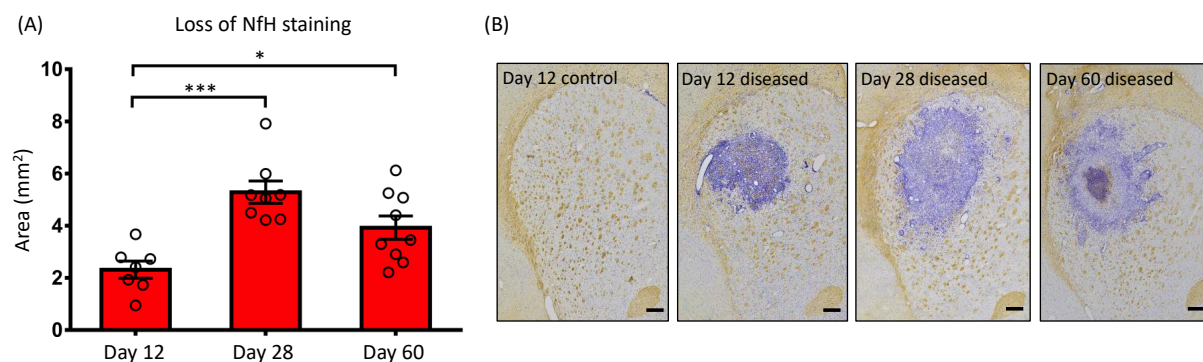


Figure 4.7: Area of axonal loss within diseased animals. **(A)** Axonal loss in diseased animals was the lowest at day 12 and was increased at days 28 and 60. ***indicates $p < 0.001$, * $p < 0.05$ on Tukey's post-hoc test after one-way ANOVA. **(B)** Representative images of NfH staining in day 12 control and in diseased animals at all time points, demonstrating a loss of NfH staining involving the lesion core and in the outer inflammatory infiltrate region. The region of Cresyl Violet counterstaining reflected the hypercellularity arising from the inflammatory infiltrate and within it, loss of NfH staining with axonal disruption was observed. Scale bars = 50 µm.

4.3.2 Serum metabolomics – Distinguishing diseased and control animals

To identify if serum metabolic perturbations are present due to the presence of a focal DTH lesion, OPLS-DA (with a leave-one-out strategy) was used to construct discriminatory models using CPMG spectral data to distinguish diseased and control animals at each experimental time point.

4.3.2.1 Day 12 time point

The representative OPLS-DA scores plot showed an excellent separation between diseased and control animals (**Figure 4.8 A**). The mean predictive accuracy for the ensemble of the OPLS-DA models comparing diseased vs. control animals was significantly higher than the mean predictive accuracy of the ensemble created by random class assignments (mean ± SD, 80.6 ± 10.7% vs. 45.9 ± 20.6%; $p < 0.0001$) (**Figure 4.8 B**), validating the serum metabolic differences observed between diseased and control animals during acute DTH lesion formation.

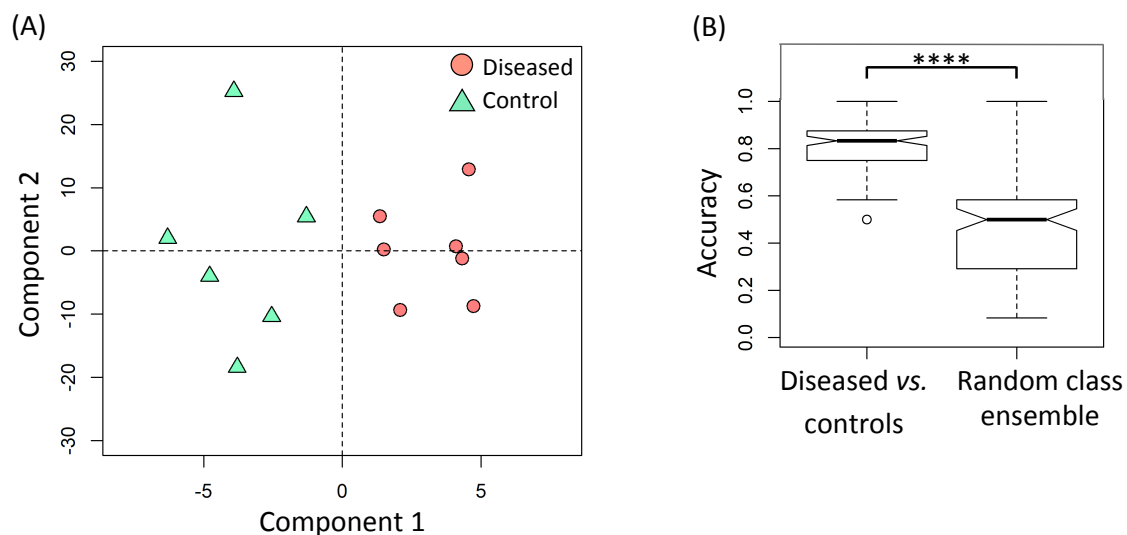


Figure 4.8: OPLS-DA models generated from CPMG spectral data of diseased vs. control animals. (A) Representative OPLS-DA scores plot (diseased = red circles, controls = green triangles). (B) Box plots of predictive accuracies from the OPLS-DA models of diseased vs. control animals, against random class assignment. ****indicates $p < 0.0001$ by Kolmogorov-Smirnov test.

4.3.2.2 Day 28 and day 60 time points

To explore if serum metabolic perturbations are also present during the chronic active phase of the DTH lesion, OPLS-DA models were constructed using CPMG spectral data for diseased vs. control animals at day 28 and at day 60. The mean predictive accuracy for the ensemble of the OPLS-DA models at day 28 was significantly higher than the mean predictive accuracy of the random class ensemble (mean $\pm$ SD, $66.6 \pm 14.9\%$ vs. $45.7 \pm 19.1\%$; $p < 0.0001$) while the mean accuracy observed at day 60 was $72.7 \pm 12.5\%$ for diseased vs. controls against $44.4 \pm 17.7\%$ for the random ensemble ($p < 0.0001$). These results imply that there are serum metabolic differences between diseased and control animals at these time points.

The fold change in the predictive accuracies (normalised to the accuracy of random chance, i.e. 50%) of diseased vs. control animals at each time point are shown in **Figure 4.9**, highlighting that the maximal serum metabolic perturbation within diseased animals (as compared to control animals) occurred at day 12 when there is maximal neuroinflammatory intensity.

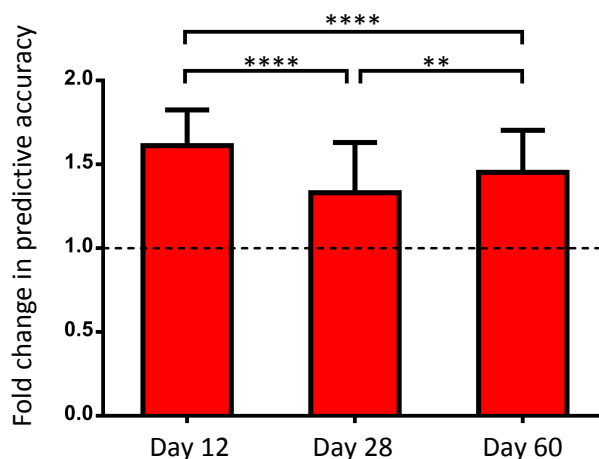


Figure 4.9: Fold change in predictive accuracies of the OPLS-DA models of diseased vs. controls at each time point, normalised to random chance. Fold change of random chance is indicated by the dashed horizontal line at 1.0. **** indicates $p < 0.0001$, ** $p < 0.01$ by post-hoc Tukey's correction. Error bars indicate SD.

4.3.3 Serum metabolomics – Identifying the top discriminatory metabolites and correlating these with histological markers of neuroinflammation and neurodegeneration

4.3.3.1 Identifying the top serum discriminatory metabolites

To identify the top discriminatory metabolites that enable diseased and controls to be distinguished at day 12 when there is the greatest metabolic separation, VIP scores from the OPLS-DA models were generated. The VIP ranking plot is shown in Error! Reference source not found.. The top two metabolites were identified to be allantoin and cytidine by 2D NMR (**Figure 4.11**), and details of their contributing spectral bins are shown in **Table 4.2**.

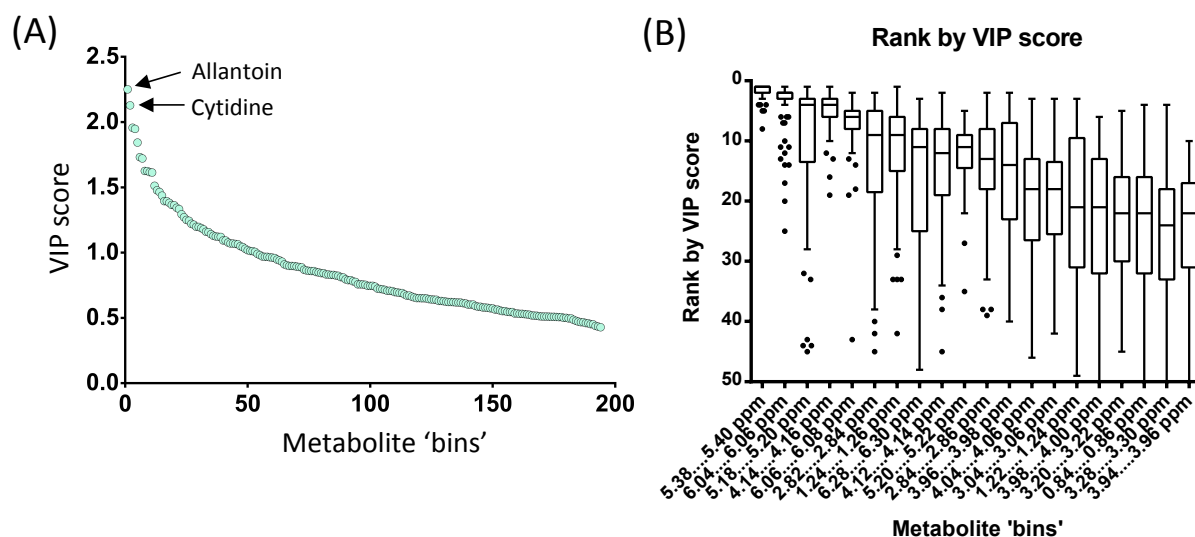


Figure 4.10: Top discriminatory serum metabolites. **(A)** VIP score ranking plot obtained from the OPLS-DA models of diseased vs. controls at day 12. **(B)** Boxplot of VIP rankings of the top 20 metabolites across models. Ranking was chosen, instead of raw VIP scores, as this takes into consideration the importance of other metabolites within each model. The high rankings of the top ranked metabolite (allantoin) remained stable/reproducible across models. Allantoin only has one 'bin' in the NMR spectra (5.38....5.40 ppm). The rankings of the second ranked metabolite, cytidine ('bin' 6.04....6.06 ppm), were also reproducible (the lowest rank was 25), albeit less so compared to allantoin. Cytidine also has another 'bin' (6.06....6.08 ppm) in the NMR spectra, which was the fifth ranked 'bin'. This 'bin' also showed stable rankings with a narrow distribution. The consistency of the high rankings of the top metabolites suggests that each OPLS-DA model is identifying the same metabolites as being discriminatory.

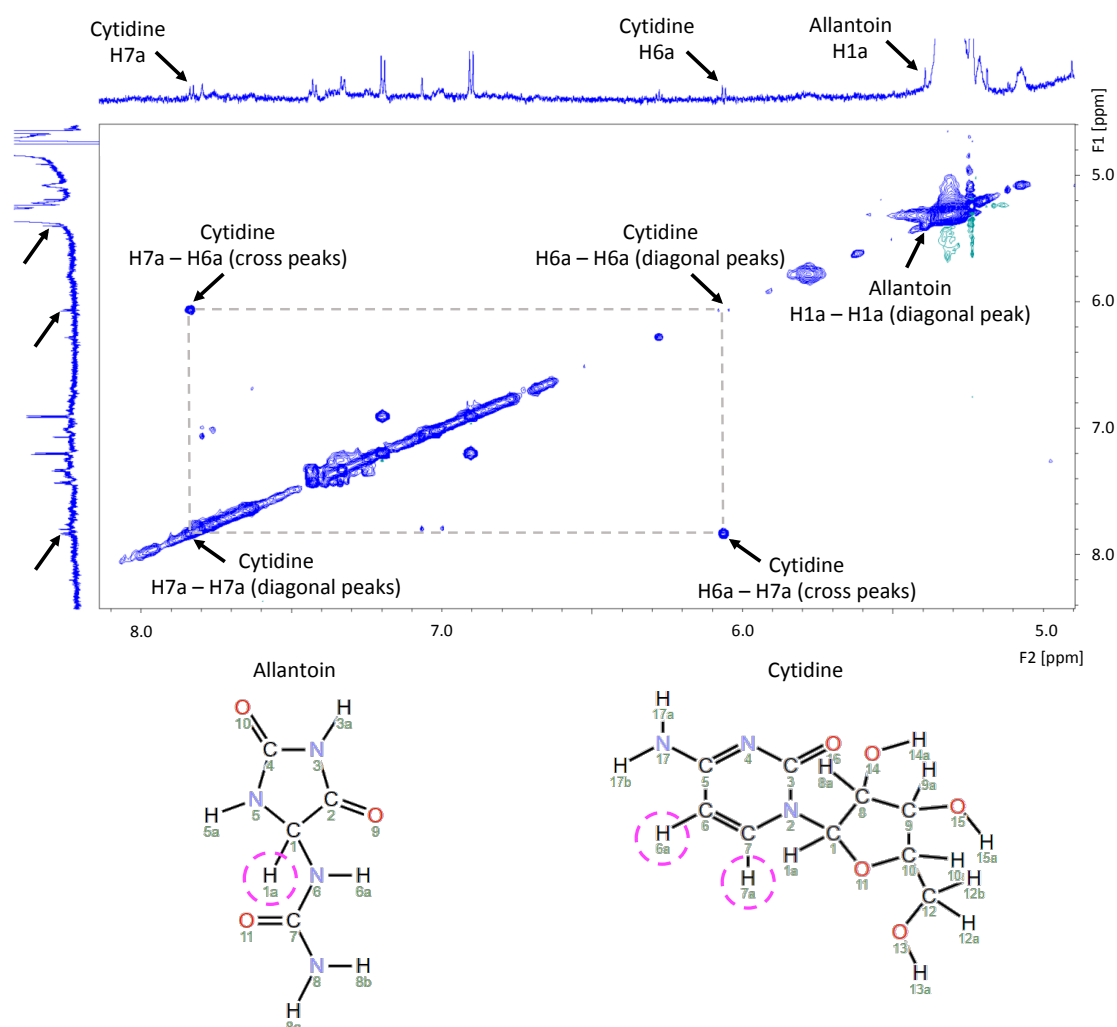


Figure 4.11: 2D TOCSY NMR experiment for allantoin and cytidine assignment. Allantoin has a single NMR resonance (singlet) centred at 5.39 ppm, as reflected by a single diagonal peak. Cytidine has two NMR resonances in this region of the NMR spectrum, one centred at 6.06 ppm (doublet) and the other at 7.83 ppm (doublet). Pulse excitation of the H6a nucleus leads to magnetisation transfer to the H7a nucleus, resulting in a cross peak at 7.83 ppm, and vice versa. Chemical structures of the compounds were obtained from HMDB (Wishart et al., 2018).

Table 4.2: The top two metabolites differentiating diseased and control animals at day 12.

Top 2 discriminatory metabolites	Chemical shift of contributing spectral 'bins' (VIP score, VIP rank)
Allantoin	5.38.....5.40 ppm (2.25, 1)
Cytidine	6.04.....6.06 ppm (2.13, 2) 6.06.....6.08 ppm (1.84, 5)

4.3.3.2 Allantoin and cytidine concentrations at experimental time points

Using appropriate controls in this study has permitted the identification of metabolite changes owing to the presence of the focal DTH lesion, beyond the contribution of the peripheral intradermal lesion. However, the potential interaction between the peripheral and CNS lesions with resultant metabolic perturbations does remain a consideration. To demonstrate that the alterations in allantoin and cytidine concentrations are solely due to the presence of the CNS DTH lesion, one-way ANOVA of both metabolites at day 12 across all three treatment groups were performed (**Figure 4.12**). The results revealed that the concentrations of both allantoin and cytidine were not just lower in diseased compared to control animals, but to naïve animals as well.

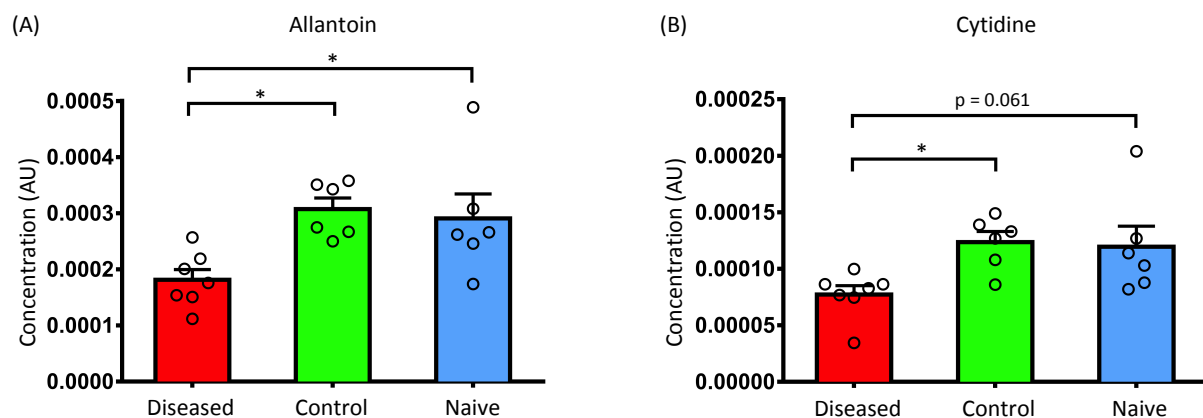


Figure 4.12: Allantoin and cytidine concentrations at day 12. Diseased animals had the lowest concentrations of allantoin and cytidine, compared to both control and naïve animals, indicating that these changes are truly specific to the presence of a CNS DTH lesion. *indicates $p < 0.05$ on Tukey's post-hoc test after one-way ANOVA.

To explore how allantoin and cytidine concentrations change over the time course of the DTH lesion, the concentrations of both metabolites within diseased animals were first normalised to basal levels within control animals at each time point. One-way ANOVA performed across the specific time points within diseased animals revealed the both allantoin and cytidine concentrations increased with time back to levels seen in controls (**Figure 4.13**).

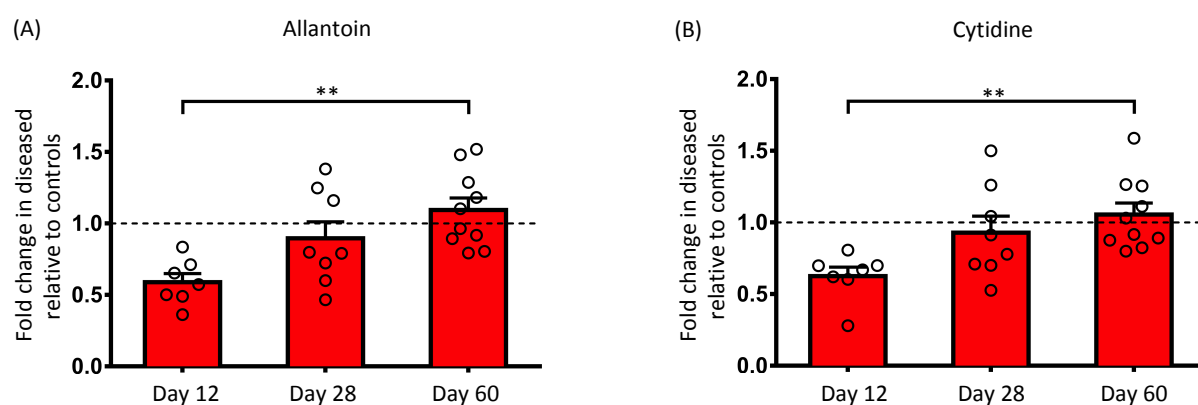


Figure 4.13: Fold change in allantoin and cytidine concentrations in diseased relative to control animals at each time point. Fold change in controls is indicated by the horizontal dashed line at 1.0. **indicates $p < 0.01$ by post-hoc Tukey's correction.

4.3.3.3 Correlation of allantoin and cytidine with histological markers

To investigate if allantoin and cytidine concentrations can reflect the underlying neuroinflammatory and neurodegenerative processes within the DTH lesion, correlations of both metabolites with histological markers were explored (**Figure 4.14**). For variables whereby a fold change (diseased relative to control at each time point) can be determined, i.e. microglia/macrophage cell density, astrocyte density, allantoin concentration and cytidine concentration, the fold change data was used for correlation analyses as this takes into consideration the basal levels within controls, thus reflecting the actual contribution by the DTH lesion. As there was no myelin or axonal loss observed for control animals, the absolute values in diseased animals were used for these parameters.

Allantoin concentration was inversely correlated with astrocyte cell density, with a Pearson's correlation coefficient of -0.562, indicating moderate correlation. Cytidine concentration was correlated with all four histological markers, albeit with lower correlation coefficients ranging from -0.424 to -0.464, indicating weak to moderate correlations.

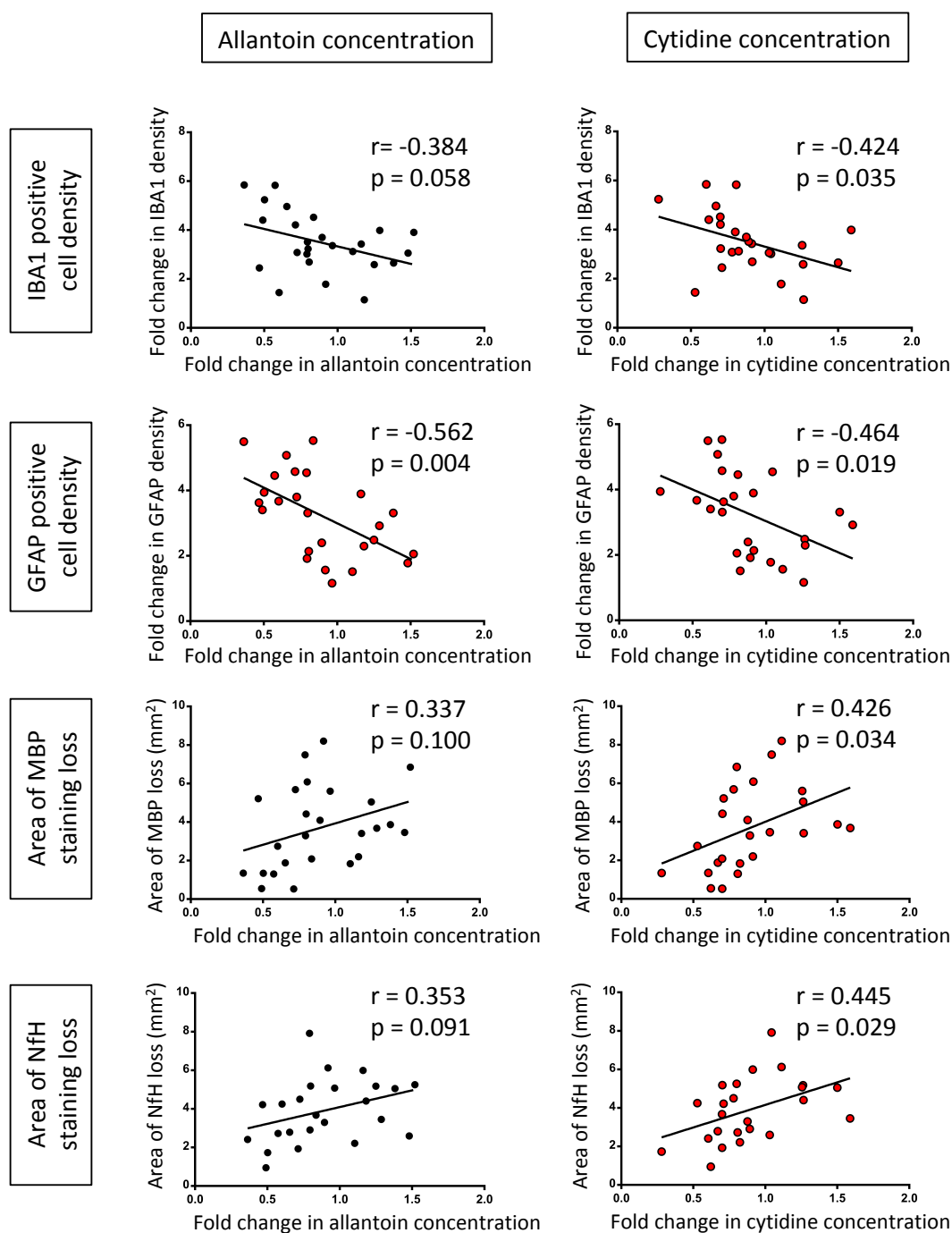


Figure 4.14: Correlation matrix showing correlation indices between allantoin and cytidine with histological markers. Statistically significant correlations are indicated by red symbols. No multiple comparison testing was performed. r = Pearson's correlation coefficient.

4.3.4 CSF metabolomics – Distinguishing diseased and control animals

The results from the above sections showed that serum metabolic perturbations are present in diseased animals (as compared to controls) at all experimental time points, but the greatest

deviation was at the earliest day 12 time point. The top discriminatory metabolites identified from the day 12 time point was found to be DTH lesion specific (despite being in serum), and measurements over time showed an increasing trend back to levels seen in controls with time, and correlated with histological markers of neuroinflammation and tissue damage.

A similar analytical strategy was employed to explore if metabolic changes in the CSF due to the presence of a focal DTH lesion were also present at each experimental time point, and whether specific CSF metabolites can be informative of the underlying neuroinflammatory and neurodegenerative processes. As mentioned in the Methods within this chapter, any CSF samples that had visible blood contamination were excluded from the CSF metabolomics study as this can introduce aberrant NMR resonances in the CSF NOESY spectra as illustrated in **Figure 4.15**. There were no CSF samples with aberrant NMR resonances that had not already been noted following visual inspection for blood contamination during CSF collection. Indeed, only one CSF sample (a day 12 control) had to be excluded from analysis.

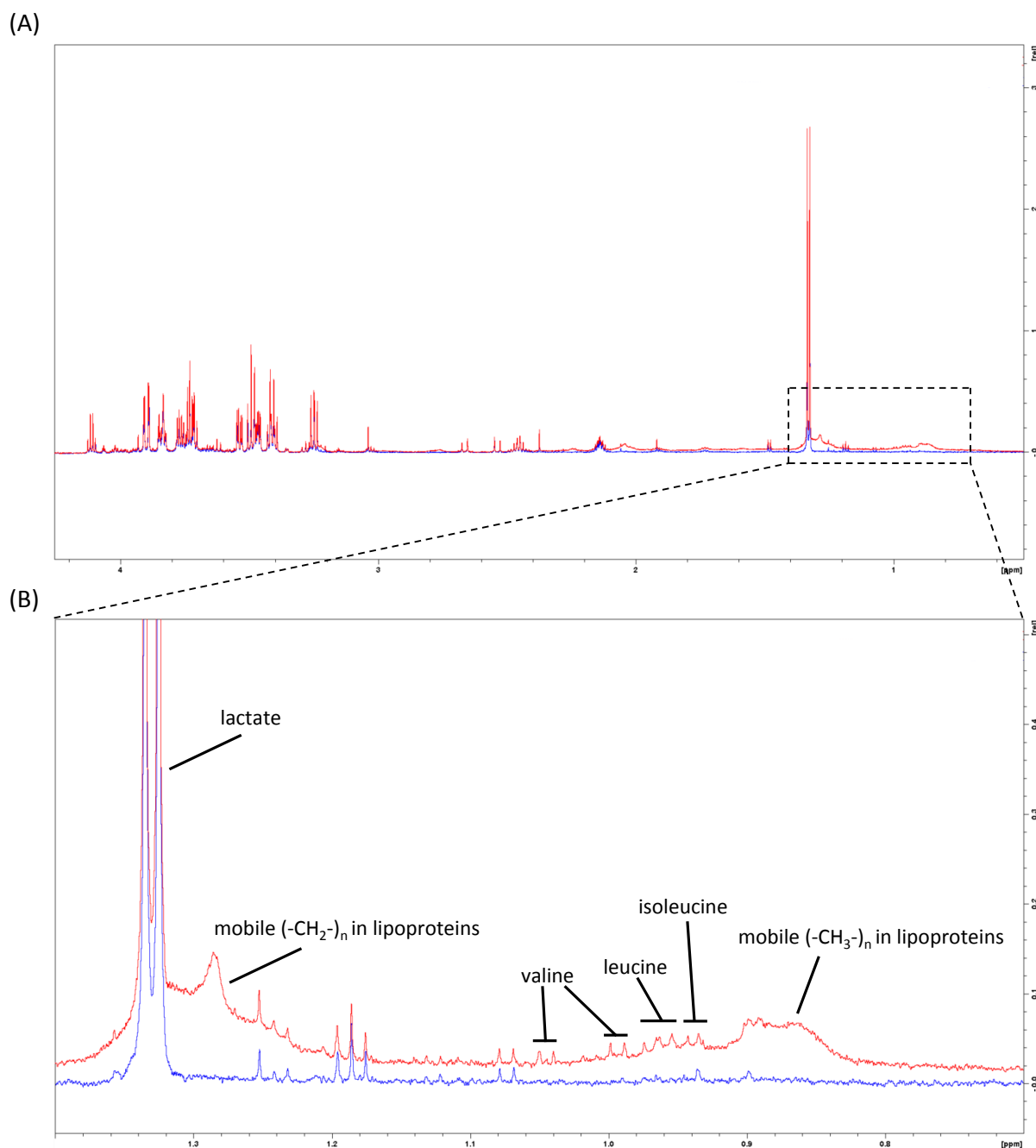


Figure 4.15: Comparison of clean vs. blood contaminated CSF NMR spectra. (A) 1D NOESY spectra (chemical shift shown: 0.60 ppm – 4.20 ppm) demonstrating clean CSF (in blue) and blood-contaminated CSF (in red). This revealed aberrant NMR resonances contributed by lipoproteins and macromolecules (e.g. albumin) in blood, resulting in a broad signal with an elevated baseline (boxed region, chemical shift shown: 0.70 ppm – 1.40 ppm). (B) Zoom-in view of this boxed region showed additional resonances arising from the mobile methyl (CH_3) and methylene (CH_2) groups within lipoproteins, as well as from the branched chain amino acids (i.e. isoleucine, leucine and valine).

4.3.4.1 Day 12 time point

OPLS-DA models were constructed using CSF NOESY spectral data for diseased vs. control animals at day 12. The representative OPLS-DA scores plot revealed the presence of an excellent separation between diseased and control animals (**Figure 4.16 A**). The mean predictive accuracy for the ensemble of the OPLS-DA models of diseased vs. control animals was significantly higher than the mean predictive accuracy of the ensemble created by random class assignments (mean $\pm$ SD, 69.3 $\pm$ 13.5% vs. 42.9 $\pm$ 19.3%; $p < 0.0001$) (**Figure 4.16 B**), validating the CSF metabolic differences observed between diseased and control animals during this phase of DTH lesion formation.

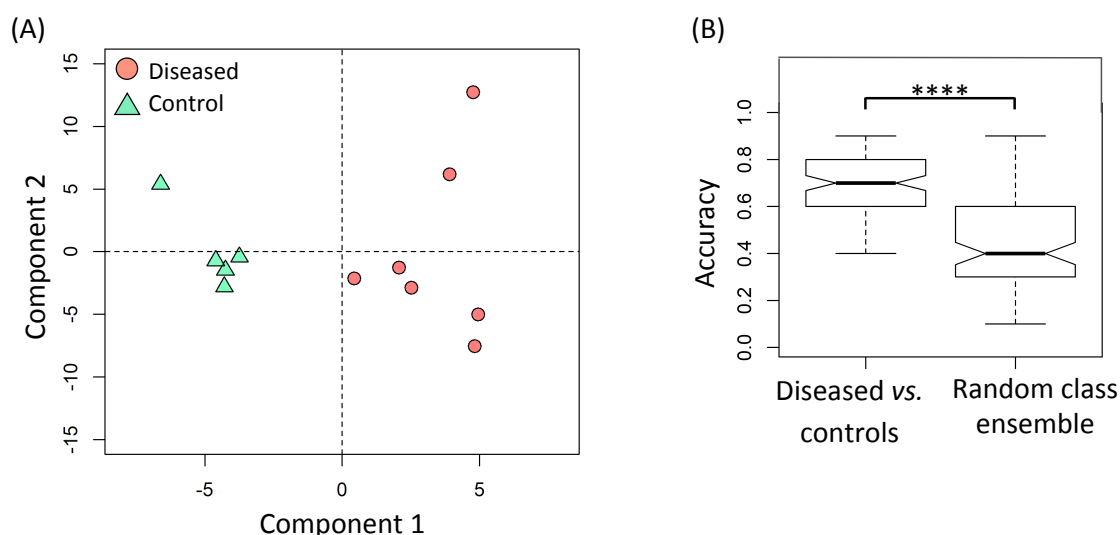


Figure 4.16: OPLS-DA models generated from CSF NOESY spectral data of diseased vs. control animals. (A) Representative OPLS-DA scores plot (diseased = red circles, controls = green triangles). (B) Box plots of predictive accuracies from the OPLS-DA models of diseased vs. control animals, against random class assignment. ****indicates $p < 0.0001$ by Kolmogorov-Smirnov test.

4.3.4.2 Day 28 and day 60 time points

The mean predictive accuracy for the ensemble of the OPLS-DA models at day 28 was higher than the mean predictive accuracy of the random class ensemble (mean $\pm$ SD, 60.7 $\pm$ 14.0% vs. 44.0 $\pm$ 20.8%; $p < 0.0001$) while the mean accuracy observed at day 60 was 50.6 $\pm$ 12.3% for diseased vs. controls against 44.3 $\pm$ 18.4% for the random ensemble ($p = 0.0008$). These results suggest that

although there were still metabolic differences within the CSF between diseased and control animals at these latter time points, they were less pronounced as those observed at day 12.

The fold change in the predictive accuracies (normalised to accuracy of random chance, i.e. 50%) of diseased vs. control animals at each time point are shown in **Figure 4.17**, illustrating that the magnitude of CSF metabolic changes in diseased animals (as compared to controls) decreased with time.

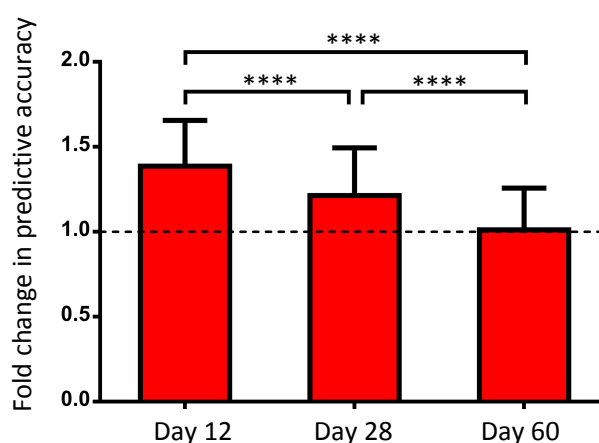


Figure 4.17: Fold change in predictive accuracies of the OPLS-DA models of diseased vs. controls at each time point, normalised to random chance. The fold change of random chance is 1.0 as indicated by the dashed horizontal line. **** indicates $p < 0.0001$ by post-hoc Tukey's correction. Error bars indicate SD.

4.3.5 CSF metabolomics – Identifying top discriminatory metabolites as biomarkers of neuroinflammation and neurodegeneration

4.3.5.1 Identifying the top discriminatory metabolites

VIP scores from the OPLS-DA models from day 12 were generated to identify the top discriminatory metabolites separating diseased and controls at day 12 when there was the greatest CSF metabolic alteration. The VIP ranking plot is shown in **Figure 4.18**. Glutamine and glucose were identified as the top two metabolites and their contributing spectral bins are detailed in **Table 4.3**.

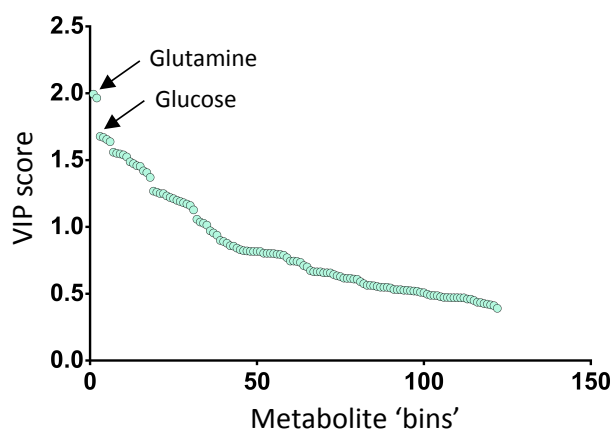


Figure 4.18: VIP score ranking plot obtained from the OPLS-DA models of diseased vs. controls at day 12.

Table 4.3: The top two CSF metabolites differentiating diseased and control animals at day 12. *As multiple 'bins' are attributable to glucose, only those with a VIP rank of <10 are shown.

Top 2 discriminatory metabolites	Chemical shift of contributing spectral 'bins' (VIP score, VIP rank)
Glutamine	2.48....2.50 ppm (1.99, 1) 2.40....2.42 ppm (1.96, 2)
Glucose*	3.70....3.72 ppm (1.68, 3) 3.74....3.76 ppm (1.66, 5) 3.44....3.46 ppm (1.64, 6) 3.82...3.84 ppm (1.55, 8)

4.3.5.2 Glutamine and glucose concentrations at experimental time points

To demonstrate that changes in glutamine and glucose levels are due to the presence of the DTH lesion alone, one-way ANOVA of both metabolites at day 12 across all three treatment groups were performed (**Figure 4.19 A and B**). Consistent with the observations in serum metabolites, the concentration of glutamine was lower in diseased animals, compared to control and naïve animals. The mean concentration of glucose within diseased was observed to be numerically lower than naïve animals, however this did not reach statistical significance ($p = 0.110$).

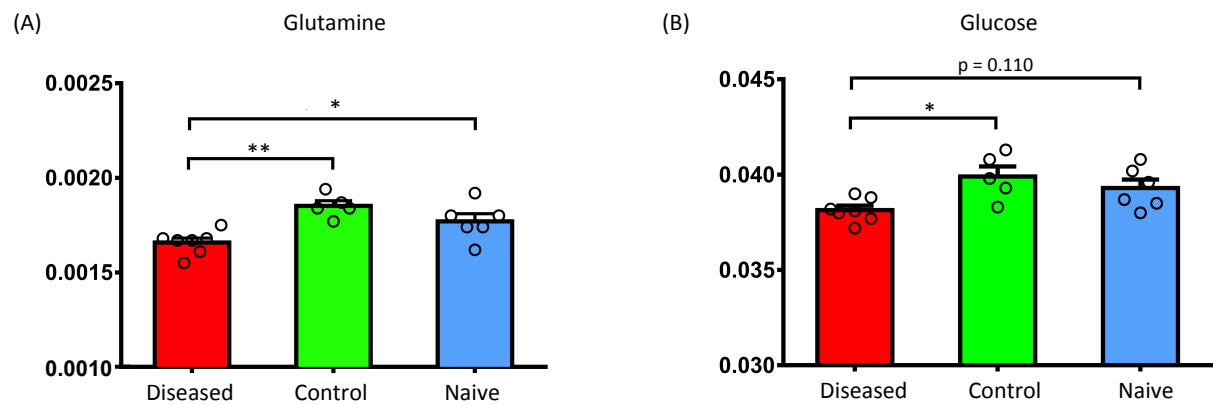


Figure 4.19: Glutamine and glucose concentrations at day 12. Diseased animals had the lowest concentrations of glutamine and glucose, compared to both control and naïve animal. **indicates $p < 0.01$, * $p < 0.05$ on Tukey's post-hoc test after one-way ANOVA.

The concentrations of both glutamine and glucose in diseased animals were then normalised to those in controls at each time point to explore how their concentrations change over the experimental time course. One-way ANOVA performed across the time points within diseased animals showed that glutamine concentrations increased with time to levels seen in controls while glucose concentration was the lowest at day 12 with a peak at day 28 (Figure 4.20).

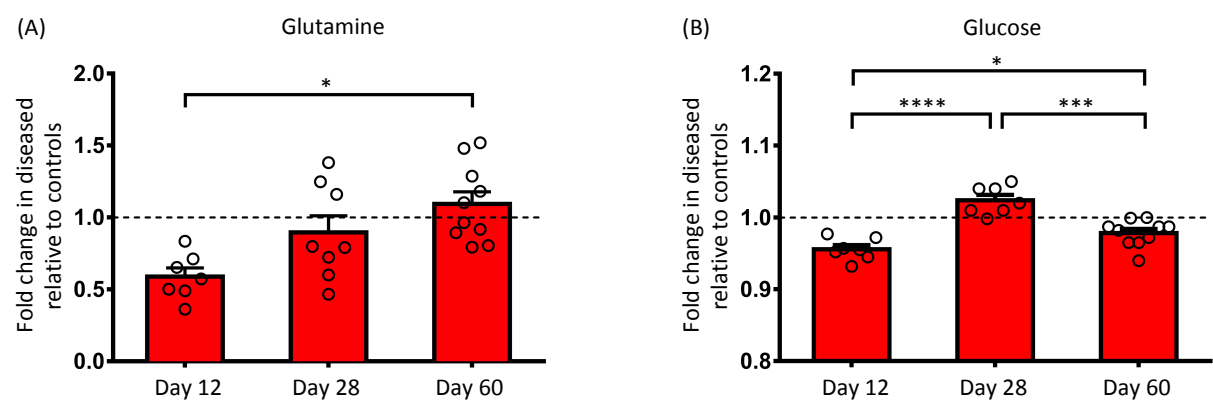


Figure 4.20: Fold change in glutamine and glucose concentrations in diseased relative to controls at each time point. Dashed horizontal line at 1.0 indicates fold change in control animals. ****indicates $p < 0.0001$, *** $p < 0.001$, * $p < 0.05$ by post-hoc Tukey's correction after one-way ANOVA.

4.3.5.3 Correlation of glutamine and glucose with histological markers

Correlations of both metabolites with histological markers were performed to explore if glutamine and glucose can reflect the neuroinflammatory and neurodegenerative changes within the DTH lesion throughout the experimental time course (**Figure 4.21**). As before, for parameters whereby a fold change (diseased relative to controls at each time point) can be determined, this was used for correlation analyses.

Glutamine concentration was inversely correlated with astrocyte cell density (Pearson's correlation coefficient of -0.528, indicating moderate correlation) and positively correlated with area of myelin and axonal loss, albeit weakly. Glucose concentration was positively correlated with area of axonal loss, with a Pearson's correlation coefficient of 0.667 indicating moderate to strong correlation.

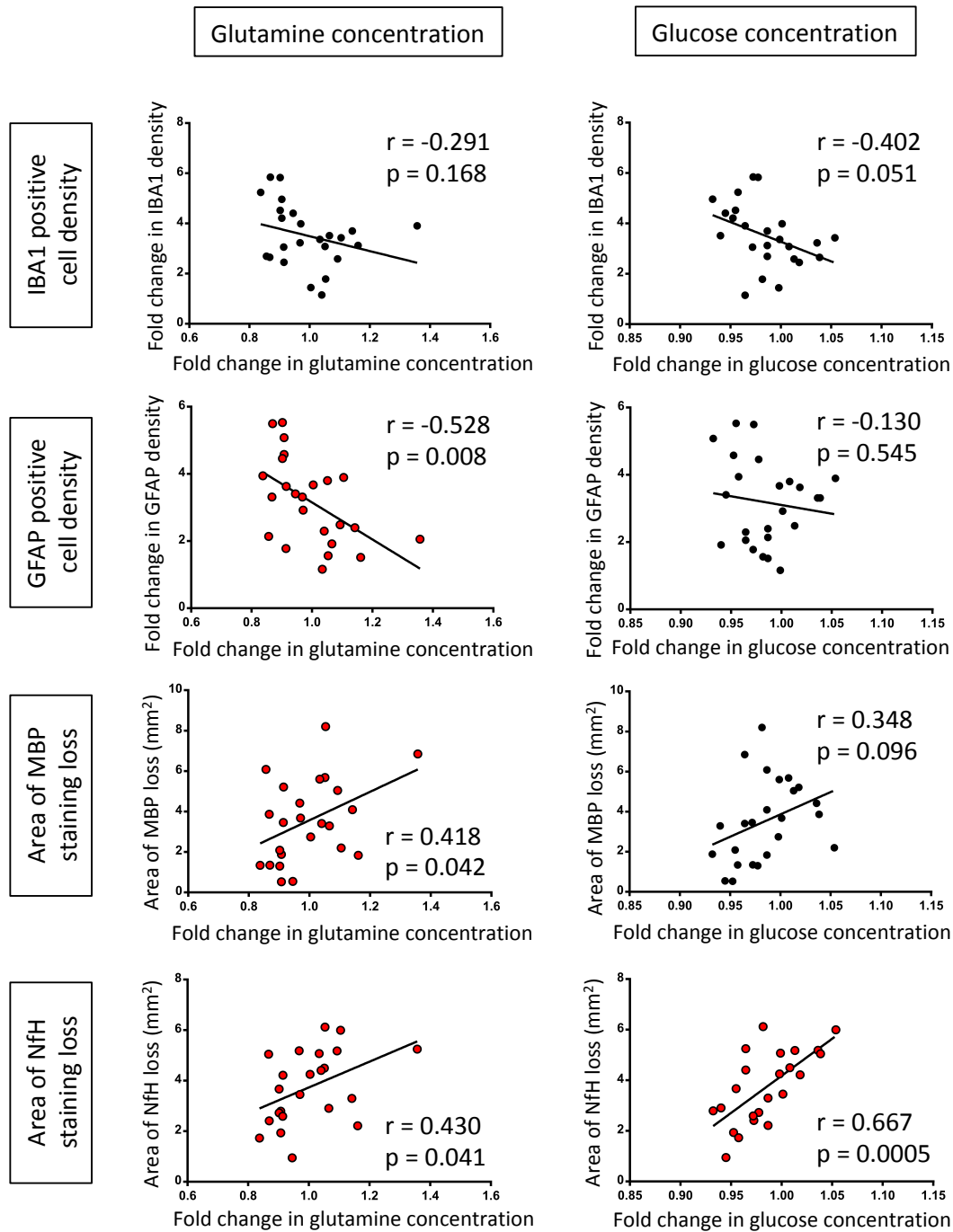


Figure 4.21: Correlation matrix showing correlation indices between glutamine and glucose with histological markers. Statistically significant correlations are indicated by red symbols. No multiple comparison testing was performed. r = Pearson's correlation coefficient.

4.3.6 Exploring the dynamics of CSF and serum NfL concentrations in the DTH model of MS

As mentioned in the Introduction to this chapter, although characterisation of NfL levels has been performed in EAE models of MS, these may not be suitable as the NfL detected in CSF and in blood

originates from randomly and diffusely involved CNS regions which can have differential expression of NfL. In other words, the differential involvement of CNS regions from one EAE animal to the next precludes a consistent measure of NfL in these models. In comparison, the DTH model generates a focal lesion in a defined CNS region, allowing for a consistent NfL readout across all diseased animals. The sections below represent the first attempt to characterise NfL concentrations in a focal animal model of MS.

4.3.6.1 CSF and serum NfL concentrations in control and naïve animals

To explore if peripheral sensitisation with TB alone causes changes in CSF and serum NfL levels, two-way ANOVA of NfL concentration stratified by treatment status and time point was performed (**Figure 4.22**). There were no differences in CSF and serum NfL concentrations between control and naïve animals across all experimental time points, consistent with the histological observation that peripheral sensitisation alone does not induce axonal damage.

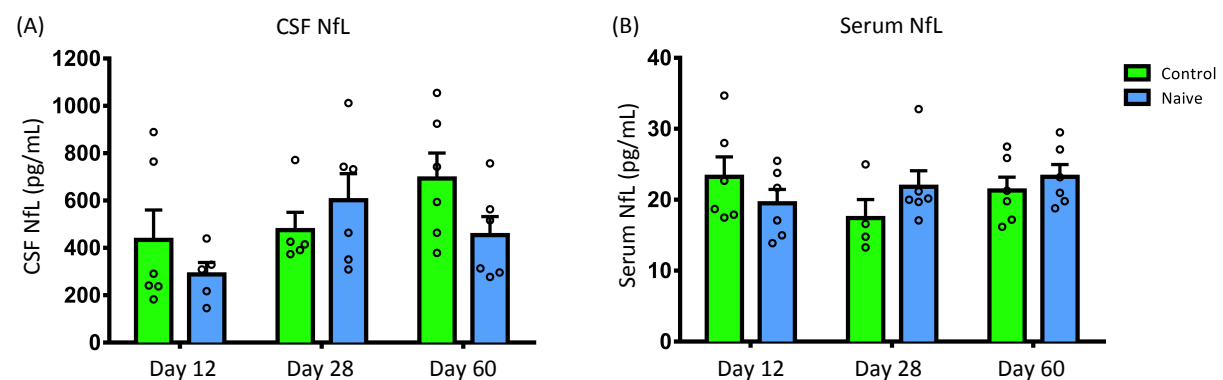


Figure 4.22: CSF and serum NfL concentrations in control and naïve animals. No differences in NfL concentrations were observed between the two groups in both (A) CSF and (B) serum.

4.3.6.2 Characterising CSF and serum NfL concentrations in diseased animals

To determine if NfL can distinguish between diseased vs. control animals at each time point, two-way ANOVA of NfL concentrations was first performed (**Figure 4.23 A and C**). The NfL concentrations in diseased animals were then normalised to the mean NfL concentrations of control animals at each

time point (to account for basal NfL levels in controls) followed by one-way ANOVA to investigate the dynamics of NfL concentrations throughout the experimental time course (**Figure 4.23 B and D**). There was no difference in CSF or serum NfL concentrations between diseased and control animals at day 12 (i.e. during active DTH lesion formation with axonal loss). At day 28, both CSF and serum NfL concentrations were higher in diseased animals while there was again no differences observed at day 60. This corresponds to the normalised NfL concentrations noted within diseased animals, which increased and peaked at day 28, with a subsequent decreased at day 60 (although the decline in CSF NfL was not statistically significant when comparing between day 28 and day 60). These findings suggest that NfL: (1) is a delayed marker of lesion formation and could be a surrogate marker for tissue damage, particularly for the neurodegenerative changes that occur later, and (2) is a dynamic marker in that it increases and then decreases with time.

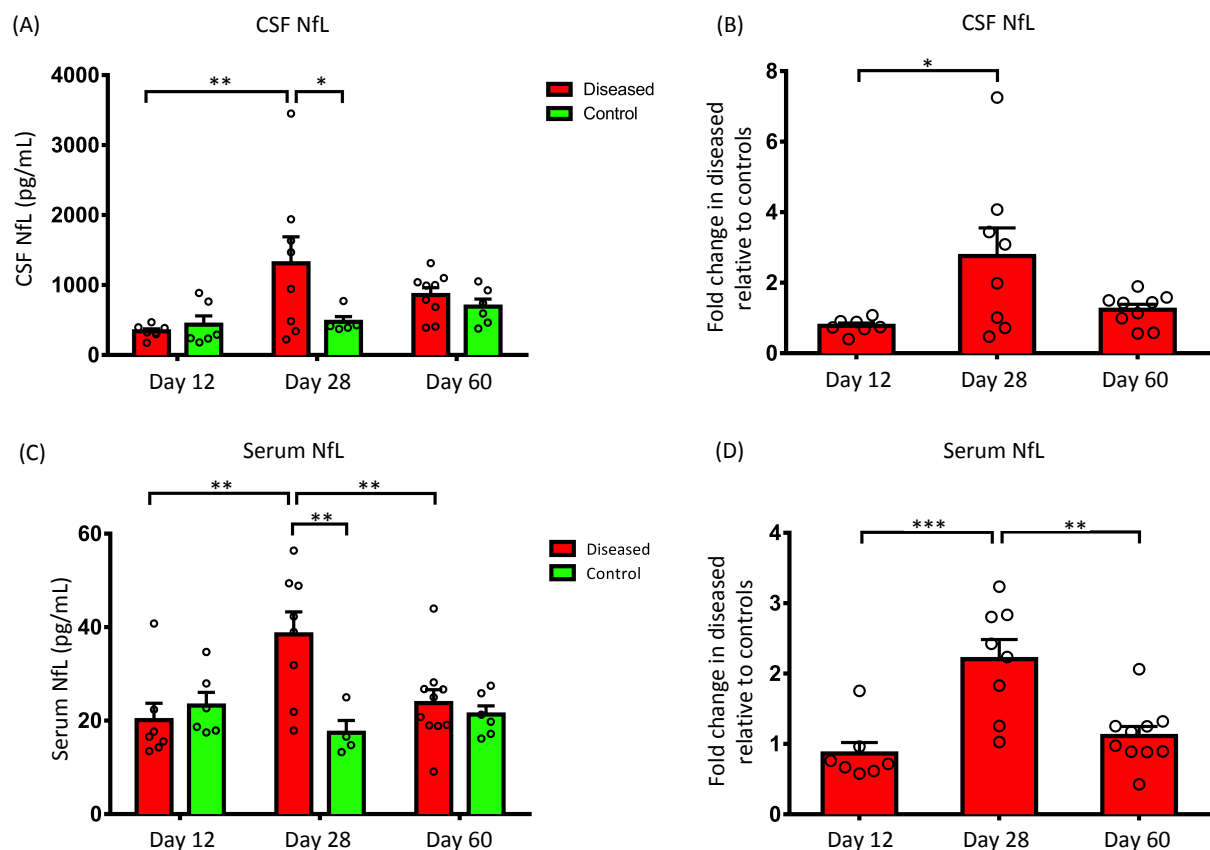


Figure 4.23: CSF and serum NfL concentrations between diseased and control animals, and within diseased animals at each time point. (A) CSF NfL was only able to differentiate the two treatment groups at day 28, even when there was already axonal loss demonstrated on histology at day 12. (B) Expectedly, the peak of CSF NfL concentration was observed at day 28 although the pair-wise comparison between day 28 and day 60 was not statistically significant. (C) A similar observation was noted for serum NfL whereby the distinction between diseased and control animals can only be made at day 28, (D) with the highest concentration observed at day 28. ***indicates $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ by Sidak's post-hoc correction in panels (A) and (C), and Tukey's post-hoc correction in panels (B) and (D).

4.3.6.3 Correlation of CSF and serum NfL with histological markers

Correlations of CSF and serum NfL concentrations with histological markers were performed to determine if NfL can be surrogate markers for neuroinflammation or tissue damage associated with the DTH lesion throughout the experimental time course (**Figure 4.24**). The fold change in NfL concentration (diseased relative to control at each time point) was used for correlation analyses to account for basal levels within controls. There was a weak positive correlation between CSF NfL concentration and area of myelin loss, while a moderate positive correlation ($r = 0.494$) was observed for serum NfL concentration and demyelination. Interestingly, there was no correlation between CSF NfL concentration and area of axonal loss, although there was a moderate to strong correlation ($r = 0.641$) of serum NfL concentration with axonal loss. Although NfL is thought to represent axonal damage, it was observed that serum NfL concentration correlated with both area of myelin loss and axonal. This was likely because the region of demyelination associated closely (but not completely) with the region of axonal loss, thus it is not unexpected that the area of MBP loss correlated with serum NfL concentration, although a stronger correlation, and expectedly so, was seen with area of NfH loss.

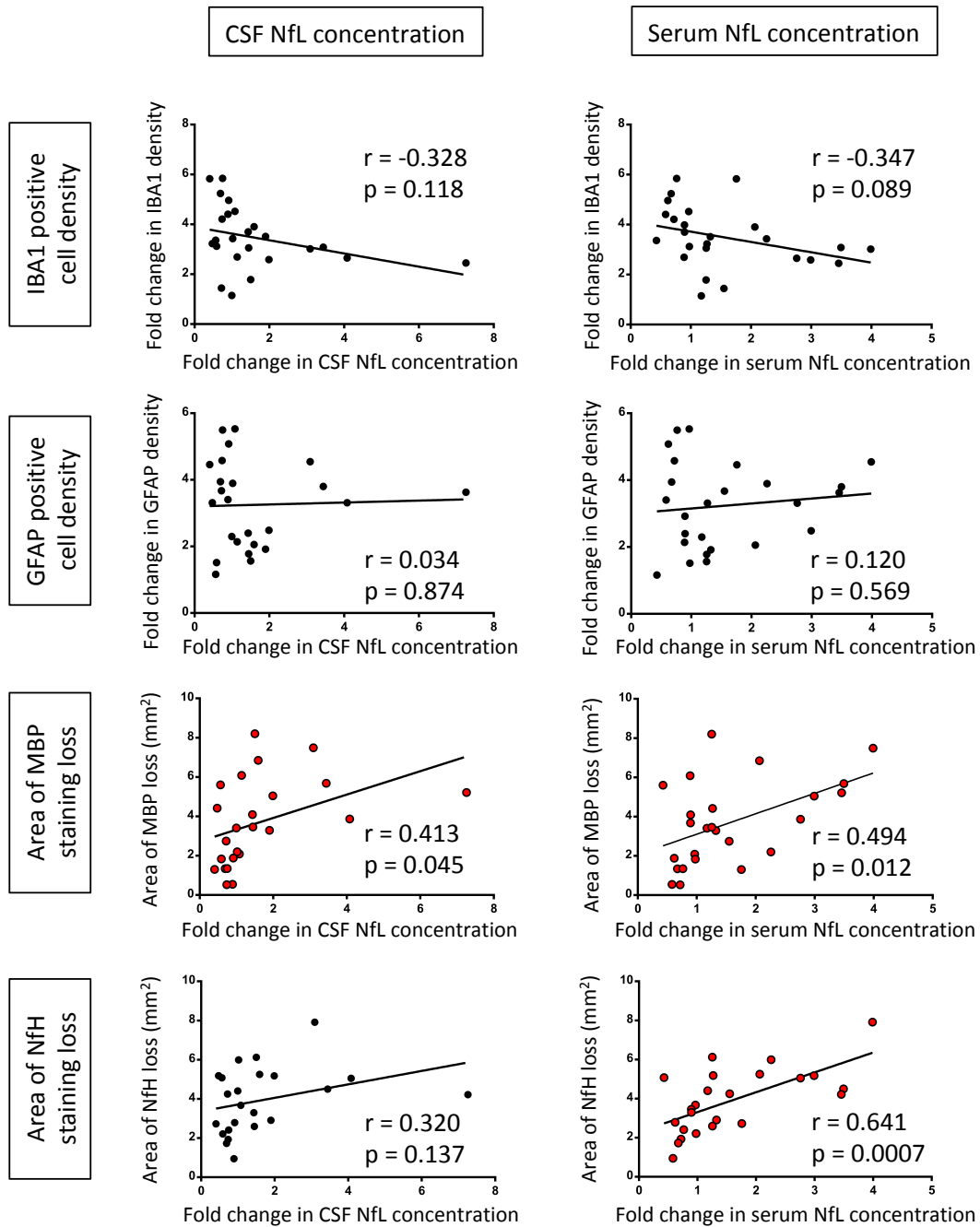


Figure 4.24: Correlation matrix showing correlation indices between CSF and serum NfL with histological markers. Statistically significant correlations are indicated by red symbols. No multiple comparison testing was performed. r = Pearson's correlation coefficient

4.3.7 Correlation of CSF and serum NfL

It is postulated that when axons are damaged, NfL is first released into the extracellular space/brain interstitial fluid, then into the CSF and finally into the blood (Khalil et al., 2018). Hence the concentration of CSF and serum NfL should be, in theory, correlated – so it comes as a surprise that

there was only a correlation between NfH loss (histologically) and serum NfL. In clinical studies in patients with MS, frontotemporal dementia, amyotrophic lateral sclerosis, HIV associated brain injury, moderate to strong correlations (correlation coefficients ranging from 0.53 to 0.89) have been reported (Disanto et al., 2017; Gisslen et al., 2016; Novakova et al., 2017; Wilke et al., 2016). Correlations of NfL concentrations in these two biofluid compartments were hence explored in this study (**Figure 4.25**).

Within all animals across all time points, a moderate to strong correlation was observed ($r = 0.642$), as was the case within diseased animals across all time points ($r = 0.666$) (**Figure 4.25 A and B**). However, there was no correlation noted within control and naïve animals (**Figure 4.25**). When diseased animals were stratified by time points, day 12 samples paradoxically showed a negative correlation, while moderate to strong correlations were observed at day 28 ($r = 0.698$) and day 60 ($r = 0.654$), albeit statistically non-significant because of low power (**Figure 4.25 C**). These observations suggest that correlations are only present if the CSF NfL concentration exceeds a high enough threshold. This is consistent with another study investigating plasma and CSF NfL levels in murine models of tauopathy, alpha-synucleinopathy and β -amyloidosis (Bacioglu et al., 2016). Strong correlations (correlation coefficients ranging from 0.86 to 0.94) were noted in diseased animals in which CSF NfL concentrations of more than 20,000 pg/mL were observed. However, in wild type animals whereby CSF NfL concentrations were mostly below 1000 pg/mL, the correlation was weaker with a correlation coefficient of 0.47.

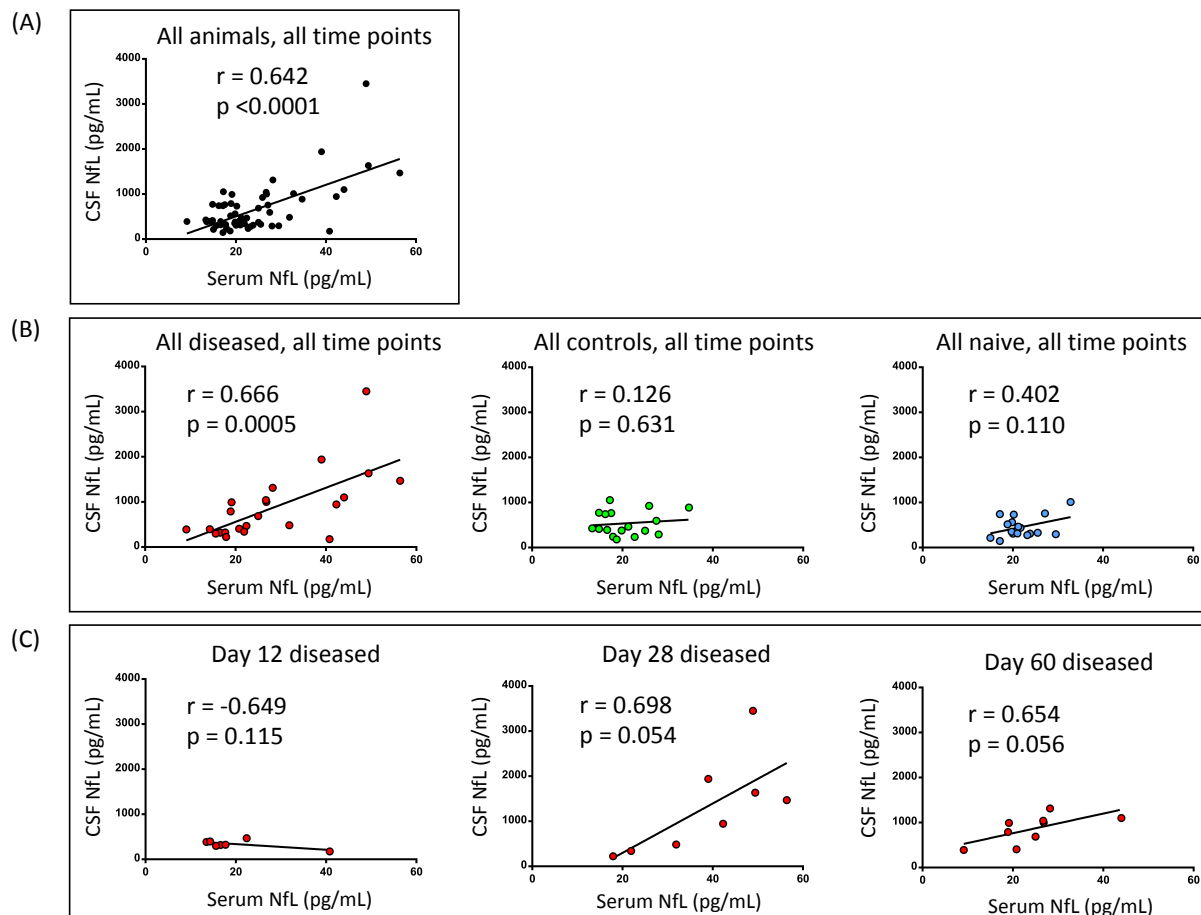


Figure 4.25: Correlation between CSF and serum NfL concentrations. **(A)** Within all animals regardless of treatment or time points. **(B)** Stratified by treatment groups regardless of time points. **(C)** Within diseased animals, stratified by time points.

4.4 Discussion

This chapter illustrates that: (1) using NMR metabolomics, serum and CSF metabolic perturbations from a focal DTH lesion are detectable at all experimental time points, (2) the magnitude of the metabolic perturbation is the greatest at day 12 in both serum and CSF and decreases with time, (3) the changes in concentration of the top discriminatory serum (allantoin and cytidine) and CSF (glutamine and glucose) metabolites at day 12 were specific for the presence of a DTH lesion, being the lowest at day 12 and increasing with time to levels seen in controls, and (4) the concentrations of these metabolites correlated with histological markers of neuroinflammation and tissue

damage/neurodegeneration, particularly the moderate to strong correlations of astrocytosis with serum allantoin and with CSF glutamine, as well as axonal loss with CSF glucose.

These observations suggest that CSF and serum metabolic changes occur early in the time course of the DTH lesion. Indeed, the maximal metabolic perturbation is at the earliest time point when there is active DTH lesion formation with intense neuroinflammation, rather than when the lesion size, axonal loss or demyelination were most extensive. It is hence not surprising that the top discriminatory metabolites at day 12 correlated with astrocyte density throughout the experimental time course. In comparison, CSF and serum NfL concentrations did not distinguish between diseased and control animals at this early time point. Indeed, NfL concentrations, be it in CSF or serum, did not correlate with any of the neuroinflammatory histological markers. The distinction between diseased and controls by CSF and serum NfL could be established at day 28 when the DTH lesion was in a chronic active phase although the levels taper off at day 60 in diseased animals to become identical to that seen in control animals. Serum NfL levels correlated with the extent of demyelination and axonal loss, but CSF NfL concentrations correlated only with area of myelin loss and not with area of axonal loss. This is likely due to the dynamic nature of NfL in that it peaks (at day 28) then decreases (at day 60) with time, whereas the extent of axonal loss on histology was no different at day 60 as compared to day 28. It is also likely that, the NfL 'substrate' is slowly exhausted as the lesion grows, in keeping with clinical studies that show that patients with progressive MS have lower levels of CSF NfL compared to RRMS patients in remission (Martin et al., 2019).

These findings indicate the NfL is a delayed marker of lesion formation as well as tissue damage as there is already evidence of axonal loss and demyelination at day 12. The reason for this is might relate to the dynamics of NfL transport and clearance, which, to date still remains poorly understood. There could be slow clearance of free NfL (released from damaged axons) from the brain interstitial fluid into the CSF, possibly via glymphatics or the intramural peri-arterial drainage pathway (Albargothy et al., 2018; Louveau et al., 2017). It has been proposed that BBB breakdown

could represent a potential exit route, but the fact that serum NfL was not elevated at day 12 (when there is BBB breakdown) and NfL concentration is 50 to 80 times lower in blood compared to CSF means that this is unlikely to be a major pathway. This is supported by a study showing that serum NfL concentrations do not correlate with measures of BBB permeability in humans and in mice (Kalm et al., 2017). Our findings also indicate that the correlation between CSF and serum NfL concentrations is only present if the CSF NfL concentration is highly elevated, presumably to allow sufficient amounts to leak into the blood compartment for an observable change in serum to be detected. These lines of evidence, albeit indirect, suggest that the main route of NfL elimination is from the brain interstitial fluid, to the CSF, then into the systemic compartment.

In addition, it was observed that there was variability in CSF and serum NfL levels even within controls and naïve animals, although no DTH lesion was generated and all other potential variations have been tightly controlled. Expectedly, the variation in NfL levels was more marked within DTH animals because in addition to the variation already observed in controls and naïve animals, there was superimposed variation due to the subtle differences in DTH lesion sizes. This is consistent with other animal studies whereby the levels of NfL also showed variability in experimental as well as in control animals (Bacioglu et al., 2016; Soylu-Kucharz et al., 2017). Using a molecular neuropathology perspective, the variation observed (within similarly treated animals) could be due to a few related factors: (1) the differential dynamics of NfL transport and clearance, (2) the differential rate of NfL protein degradation after axonal injury (in DTH animals) and (3) the NfL measurement technique. As mentioned in the preceding paragraph, the exact mechanisms responsible for NfL efflux from the brain interstitial fluid to CSF and then to serum are poorly understood and there is indeed sparse literature investigating this matter. Inter-individual/animal differences with regards to these transport mechanisms could explain the varying NfL levels in CSF and serum. Additionally, it is shown that neurofilament proteins are degraded by various proteolytic systems and the susceptibility to degradation is dependent on neurofilament assembly and phosphorylation status (Rao et al., 2012;

Yuan et al., 2017; Yuan et al., 2015). Therefore, upon NfL release from damaged axons, differential degradation of the NfL protein could generate a variety of NfL degradation products (conceivably of different sizes), with each product utilising a preferential efflux mechanism and hence potentially different rate of efflux. Furthermore, the different degradation products (from a single NfL protein) could conceivably be bound to the same capture antibody on the Simoa® assay, altering the quantitative readout.

The multivariate OPLS-DA approach allows for the comparison of CSF and serum metabolic signatures between diseased and control animals, and from these models, identify the top discriminatory metabolites. Serum allantoin and cytidine as well as CSF glutamine and glucose were identified as the principal metabolites differentiating diseased and control animals (all lower in diseased) at day 12. Beside establishing the relationship of their concentrations to histological markers of neuroinflammation and tissue damage, the study of their metabolic pathways also provides inference to the underlying pathophysiological processes associated with lesion formation, especially since in animals studies all other variables between treatment groups would have been controlled whereas in humans subjects it may be more difficult to ascribe the metabolic changes to specific metabolic pathways due to the cumulative lesion load likely consisting of both active and quiescent MS lesions.

Allantoin is the metabolic end product of purine catabolism in rodents and is excreted renally (**Figure 4.26**) (Briggs et al., 1977; Simoyi et al., 2003). In humans and higher primates, a mutation in the gene encoding urate oxidase, which converts uric acid to allantoin, causes the enzyme to be non-functional (Koch et al., 2006), thus uric acid is the end product of purine metabolism in these species. Three possible explanations could account for the low concentrations of allantoin observed in DTH rats: (1) increased renal excretion of allantoin, (2) decrease in urate oxidase activity and/or amount, or (3) low levels of metabolites upstream of allantoin. The first two reasons are unlikely as there is little biological explanation as to why the DTH model would cause renal dysfunction or

altered enzymatic function. On the other hand, it is intriguing to note that uric acid (the immediate metabolic precursor of allantoin) is a potent anti-oxidant in blood. It has been shown in a murine MBP-immunised EAE model that treatment with uric acid after the onset of clinical signs improved the clinical symptoms and prolonged survival (Hooper et al., 1998). Further work from the same group using the same murine model showed that uric acid treatment decreased BBB permeability and reduce inducible nitric oxide synthase (iNOS) mRNA expression in the spinal cord (Kean et al., 2000). Mechanistically, it has been shown that uric acid acts as a natural scavenger of peroxynitrite (and its derivatives) (Squadrito et al., 2000), a highly reactive nitrogen species which has been shown to cause lipid peroxidation, DNA strand breakage, protein damage via nitration of tyrosine residues and impair mitochondrial respiration (via damage to complex I and IV) within in vitro studies (Koch et al., 2006; Robinson et al., 2019). The presence of nitrotyrosine (as a consequence of tyrosine nitration by peroxynitrite) has also been demonstrated in brain tissues from MS patients (Cross et al., 1998). Therefore, the low allantoin concentrations observed in this study could be a consequence of low uric acid levels resulting from a consumptive process in which uric acid is utilised to scavenge peroxynitrite produced by activated microglial/macrophages, astrocytes and lymphocytes in the DTH lesion. The inverse correlation of allantoin concentration with astrocytic density observed in this study is consistent with this hypothesis. However, the chemical structure of uric acid renders it invisible to ^1H NMR, therefore its concentration was not available in this study to confirm this hypothesis. It is interesting to note that metabolomics studies in MOG-EAE models have revealed lower plasma and urine allantoin as compared to controls, consistent with that observed in this chapter (Poisson et al., 2015; Singh et al., 2019).

In humans, numerous studies (including a recent meta-analysis) have also reported lower uric acid levels in MS compared to controls (Wang et al., 2016), and a longitudinal two-year study reported a decrease in uric acid levels within the same MS individuals (Moccia et al., 2015). Interestingly, the occurrence of MS and gout within an individual is observed to be mutually exclusive (Hooper et al.,

1998). A small study (n = 16) of inosine (a precursor of uric acid) supplementation in RRMS patients showed that raised serum urate levels were associated with improved EDSS and decreased GAD-enhancing lesions, however four patients developed renal stones (Markowitz et al., 2009). There is now emerging interest in increasing blood urate levels to treat neurological conditions with randomised controlled trials (RCT) being conducted in Parkinson's disease and acute ischaemic stroke showing encouraging results with no safety concerns (Chamorro et al., 2014; Schwarzschild et al., 2019), thus a larger RCT in MS could be performed to establish its use in this context.

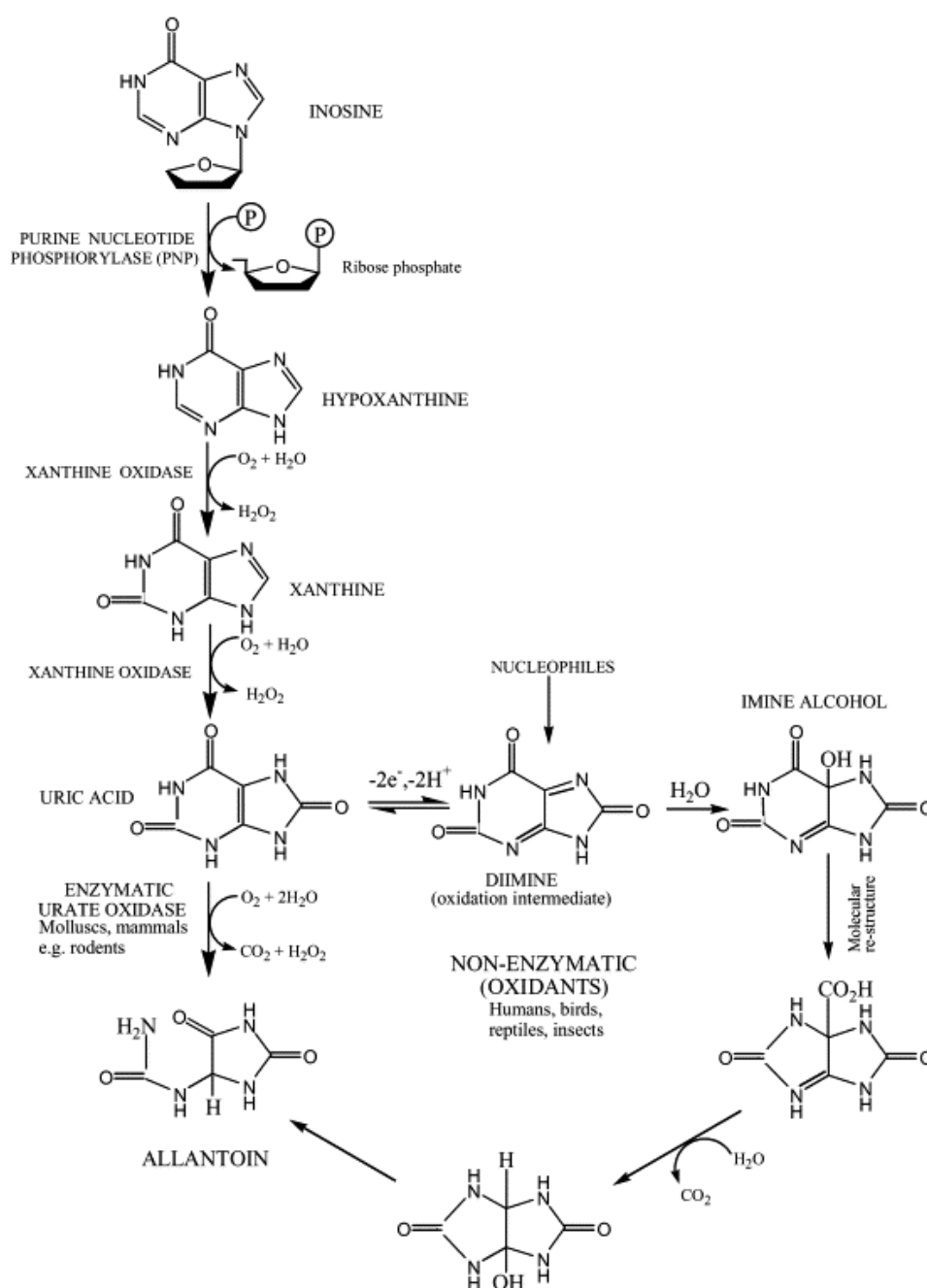


Figure 4.26: Inosine catabolism showing the end products of purine metabolism in humans (uric acid) and in rodents (allantoin). Reprinted from Simoyi et al (2003) with kind permission from the journal.

Cytidine is a pyrimidine nucleoside comprised of cytosine bound to a ribose ring via a beta-N₁-glycosidic bond. It is needed for RNA synthesis and is also a precursor for cytidine triphosphate (CTP) that is required for the production of phosphatidylcholine, which is a major component of the neuronal membrane and myelin (**Figure 4.27**) (Cansev, 2006; Siegel, 1999). Indeed, a study using the MOG-induced EAE model as well as the cuprizone model showed that supplementation with cytidine

diphospho (CDP)-choline enhanced remyelination and improved clinical scores (Skripuletz et al., 2015). Therefore, the low cytidine concentration observed in diseased animals could be plausibly due to: (1) increased incorporation into RNA to meet transcriptional demands for protein synthesis during acute neuroinflammation, and/or (2) increased utilisation to produce membrane and myelin components after damage, especially when it is known that cytidine can cross the BBB (Cansev, 2006). Hence, it is perhaps not unexpected that cytidine concentration correlated with both neuroinflammatory markers and with extent of demyelination.

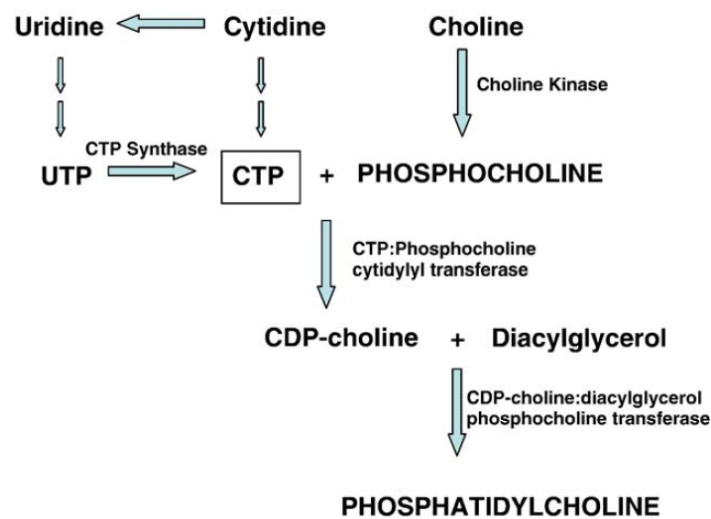


Figure 4.27: Synthesis of phosphatidylcholine from cytidine and choline. CDP-choline = cytidine diphosphocholine, CTP = cytidine triphosphate, UTP = uridine triphosphate. Reprinted from Cansev (2006) with kind permission from the journal.

Glutamine is a conditionally essential amino acid, meaning that mammals are able to synthesise it but during hypercatabolic states, requirements could exceed that produced endogenously (Watford, 2015). In the periphery, almost all glutamine is converted to glutamate, which is then used as a substrate in the TCA cycle (for ATP production) or for the synthesis of glucose and urea. As the CSF is in direct communication with the brain interstitial fluid (Louveau et al., 2017), lower CSF glutamine concentration in DTH rats could reflect lower levels in the brain interstitial fluid, and the same inference could be applied to the low CSF glucose concentration observed. Perturbations in these

two CSF metabolites point to an energy deficient state, caused by the disruption of metabolic coupling between neurons and glial cells (see section 1.4.1.3 in the Introduction of the thesis) during the induction of the DTH lesion. The low glucose state is likely due to: (1) increased glucose utilisation by neurons as the major substrate to fuel ATP production as a result of reduced lactate shuttling from astrocytes, (2) reduction of oxidative phosphorylation due to mitochondrial failure with a corresponding increase in anaerobic glycolysis in neurons, and (3) increased glucose uptake by reactive astrocytes, activated microglia and immune cells to meet energy demands required to mount an inflammatory response. This is in keeping with the observations made in the CSF of MS patients in a ^1H NMR study (Kim et al., 2017). Low glutamine concentrations could be caused by reduced glutamine efflux by reactive astrocytes and the inverse correlation of glutamine concentration with astrocytic density observed is in line with this hypothesis. Low glutamine levels could also result from its increased conversion to glutamate (i.e. glutaminolysis), which is then converted to α -ketoglutarate to be used a substrate in the TCA cycle. The findings are consistent with ^1H NMR and mass spectrometry studies showing the CSF glutamine levels are lower in MBP-EAE Lewis rats (Blanchet et al., 2011; Noga et al., 2012), compared to controls not immunised with MBP. Of note, in another ongoing collaboration between the Oxford and Basel groups, lower CSF glutamine level was observed in CDMS patients compared to clinically relevant non-MS controls (*manuscript in preparation*). In this study, CSF metabolomics was performed on 41 CDMS patients and 64 non-MS controls. The mean predictive accuracy of the OPLS-DA models distinguishing CDMS from non-MS controls was $70.3 \pm 3.9\%$ (mean accuracy of random ensemble was $51.1 \pm 5.8\%$). ROC analysis of the discriminatory metabolites (identified by VIP ranking) showed that CSF glutamine had the greatest discriminatory value (AUC = 0.76) in distinguishing CDMS from non-MS controls.

4.4.1 Chapter summary

This chapter illustrates that serum and CSF metabolic perturbations from a focal DTH lesion are detectable at all experimental time points, being the greatest at the earliest time point when there is

marked neuroinflammation. The identified serum and CSF metabolites were specific for the presence of a DTH lesion, normalised with time away from lesion initiation, and correlated with histological markers of neuroinflammation. In contrast, both serum and CSF NfL levels rose only at day 28, with serum NfL, but not CSF NfL, correlating well with axonal loss. Taking the above observations in totality and within the context of human MS, metabolomics and NfL may be utilised in a complementary manner – metabolomics could potentially be used as an early tool to detect patients with newly developing neuroinflammatory lesions while NfL could be a later marker in which the rise and subsequent fall in its concentrations indicate ‘resolution’ from these neuroinflammatory events. The next chapter will explore the blood metabolomics signatures of inflammatory events in MS and explore if specific metabolites can inform on disease activity across and within patients. The role of serum NfL in this context will also be explored, in a complementary as well as in a comparative fashion, with respect to serum metabolomics.

Chapter 5: Global and targeted metabolomics identify metabolite biomarkers of clinical relapses in MS

5.1 Introduction

Chapter 4 of this thesis revealed that serum and CSF metabolomics could distinguish rats with DTH brain lesions from controls and that the maximal metabolic perturbation occurred at the earliest time point (i.e. day 12) when there is active DTH lesion formation with intense neuroinflammation. The magnitude of the metabolic perturbations became less pronounced as the DTH lesion evolved into a chronic active lesion. The top discriminatory serum and CSF metabolites from the day 12 OPLS-DA models correlated with histological measures of neuroinflammation and inference into their respective metabolic pathways confirmed the pathological relevance of these metabolites. Taking these observations into account, it would be pertinent to explore if metabolic perturbations are also present in MS patients during periods of intense neuroinflammation, above and beyond their basal levels, the clinical correlates of which are episodes of new or worsening neurological symptoms and signs – relapses.

At least 80 – 85% of MS patients experience clinical relapses during the course of their disease (Compston et al., 2008; Steinman, 2014). From a clinical perspective, relapses represent an important component in the management of MS with regards to: (1) MS diagnosis (CIS vs. CDMS), (2) classification of clinical course (RRMS vs. PPMS during initial diagnosis, and RRMS vs. SPMS in the transitional phase), and (3) therapeutics decision-making (intensive treatments for ‘highly-active’ MS vs. less intensive treatments for ‘inactive’ MS). From a patient perspective, relapses represent a worsening of existing function with the uncertainty of incomplete recovery resulting in residual disability. It has been shown that there is clear association of relapses with disability worsening in the short-term (Hirst et al., 2008; Keegan et al., 2002), and there is evidence, albeit weaker, to suggest that high relapse rates early in the disease are predictive of long-term disability (Goodin et

al., 2016; Scalfari et al., 2010). Within MS clinical research, relapse-related measures are still one of the primary endpoints in recent major phase III RCT for DMT (Coles et al., 2012; Giovannoni et al., 2010; Hauser et al., 2017; Leist et al., 2014). Given that the current treatment paradigm is still largely guided by the presence of relapses, accurate determination of relapses is hugely important, especially when it is known that pseudo-relapses, which are transient neurophysiological disturbances due to infection, fever, heat-exposure or exercise, can cloud the clinical picture (Mills et al., 2017).

To date, the patient's history and neurological examination, as compared to previous examinations, are the principal methods used to establish relapses. This is often supplemented by MRI whereby the presence of GAD-enhancing or new/enlarging T₂ lesion/s within a CNS locale consistent with the neurological deficits offers unequivocal support for a clinical relapse. However, in clinical practice, a clinico–radiological discordance at time of relapse (although this paradox can exist at any point of the MS disease course) is often encountered. The presence of new clinical deficits without MRI correlates could be due to the poor sensitivity of conventional MRI to detect small lesions, particularly in the spinal cord, cortical grey matter and optic nerve (Chard et al., 2017; Hickman, 2007; Kearney et al., 2015). With regards to the use of biofluid markers to identify relapses, none have been validated as yet although some appear to show promise, particularly NfL. As mentioned in the Introduction of this thesis, a recent meta-analysis has showed that CSF NfL is elevated in MS patients during relapses compared to patients in remission (Martin et al., 2019). However, these analyses were performed at group level and there is little data on how long the NfL elevation persists within patients, especially given the long half-lives of neurofilaments (in neuronal cultures) and reports of persistently elevated NfL after traumatic brain injury (Shahim et al., 2017; Wallace et al., 2018; Yuan et al., 2009). Furthermore, it is shown in Chapter 4 that both serum and CSF NfL are delayed markers of DTH lesion formation, and they correlated with histological markers of tissue damage rather than neuroinflammation.

As metabolomics can detect neuroinflammation early as shown in Chapter 4, it is conceivable that metabolomics can serve as a biomarker of clinical relapses and at the same time provide inference to the underlying pathophysiological processes.

5.1.1 Aims of Chapter 5

The aims of this chapter are:

1. To elucidate, at a group level, if global metabolic perturbations are present in MS patients during relapses and if present, to determine the duration of this metabolic derangement.
2. To identify the top discriminatory metabolites distinguishing patients in relapse and patients who are 'inactive', and to quantify these discriminatory metabolites using a targeted metabolomics platform.
3. To validate these discriminatory metabolites as individualised and responsive biomarkers of relapses within patients who have paired relapse-remission samples.
4. To compare the discriminatory ability of the identified metabolite biomarkers against serum NfL for distinguishing in relapse vs. stable/remission status, across patients and within individuals.

As mentioned in the Methods, Section 2.3, all patients involved in this study were recruited under a new prospective cohort (i.e. MET cohort) established by the DPhil candidate. The candidate's individual contributions to this work include: (1) design and conceptualisation of the study, (2) recruitment of patients and blood sample collection, (3) acquisition of metabolomics and clinical data, (4) analysis of all data as well as their interpretation. This chapter is currently being written up as a manuscript, with the DPhil candidate as first author.

5.2 Methods

5.2.1 Patients

RRMS patients (under the MET cohort collection) were prospectively recruited from the John Radcliffe Hospital, Oxford University Hospital Trust. All patients fulfilled the 2017 revisions to the McDonald criteria for MS (Thompson et al., 2018). Patients suspected to be having a relapse were first triaged by an experienced MS nurse via phone consultation and those suspected to have a relapse were then seen at the 'acute relapse' clinic. Relapse status was established by MS neurologists and defined clinically (without the need for additional MRI confirmation) in accordance to the McDonald criteria, i.e. a monophasic clinical episode with patient-reported symptoms and objective findings typical of MS, reflecting a focal or multifocal inflammatory demyelinating event in the CNS, developing acutely or subacutely, with a duration of at least 24 hours, with or without recovery, and in the absence of fever or infection (Thompson et al., 2018). Patients with pseudo-relapses were excluded. This was performed by systemic review of infective symptoms, temperature measurement and urine dipstick assessment to rule out urinary tract infections. Detailed information on smoking, alcohol intake, time from last meal and type of food for last meal were also collected at the time of recruitment.

Patients were divided into four groups according to the interval between their last relapse to blood sampling: (1) in relapse, defined as <1 month from the onset of relapse; (2) last relapse (LR) $\geq$ 1 month to <6 months ago; (3) LR $\geq$ 6 months to <24 months ago; and (4) LR $\geq$ 24 months ago. These groups are henceforth referred to as 'In R', 'LR 1 – 6 M', 'LR 6 – 24 M' and 'LR $\geq$ 24 M' respectively.

5.2.2 Blood collection, serum processing and NMR sample preparation

The *optimised* protocol, as described in Material and Methods, was used for pre-analytical sample handling. Two hundred and fifty microlitres of serum was used for global metabolomics while 750uL of serum from the same blood sample was used for targeted metabolomics.

5.2.3 Global metabolomics – CPMG spectra data acquisition

All ^1H NMR experiments for global metabolomics were performed in-house at the Department of Chemistry, University of Oxford using a 700-MHz Bruker AVIII spectrometer, with the CPMG relaxation editing pulse sequence for spectra acquisition. Technical specifications of the NMR experiments and details of data handling are as described in Materials and Methods. In all, 191 metabolite ‘bins’ were available for multivariate statistical analysis.

5.2.4 Targeted metabolomics - AXINON® lipoFIT® spectra data acquisition

Targeted metabolomics was performed with the AXINON® lipoFIT® system at numares AG, Regensburg, Germany, using a 500-MHz Bruker NMR spectrometer with the NOESY pulse sequence for spectra acquisition. This test system deconvolutes the broad methyl lipoprotein resonance of the ^1H NMR NOESY spectrum into its constituent parts, allowing for the direct measurement and quantification of the cholesterol content, number of particles, and mean particle diameter of each lipoprotein subpopulation (numares, 2020a). Lipoprotein groups measured include VLDL, LDL, IDL, and HDL, with each group further divided into large and small subpopulations. Additionally, the AXINON® lipoFIT® system provides absolute quantification of glucose as well as metabolites located close to the ^1H NMR lipoprotein resonances, as the resonances of these metabolites overlap with those arising from lipoproteins. These metabolites include lactate, glucose, alanine, as well as the BCAA – isoleucine, leucine and valine. In all, 29 variables from targeted metabolomics were available for multivariate statistical analysis.

5.2.5 Serum NfL level determination

Serum NfL levels were measured using the Simoa® assay (Quanterix, Massachusetts, USA) performed at the University of Basel, in collaboration with Dr Jens Kuhle. Assay techniques and principles have been previously described (Disanto et al., 2017). All laboratory personnel were blinded to the assignment of patient groups.

5.2.6 Multivariate statistical analysis

OPLS-DA was used to interrogate the CPMG (global metabolomics) and AXINON® lipoFIT® (targeted metabolomics) data sets to identify metabolic differences between the groups of patients as defined above. Details of the OPLS-DA approach are as described in Materials and Methods.

5.2.7 Univariate statistical analysis

Univariate analysis of demographic, clinical, and metabolite data was performed using STATA software (release 14, Texas, USA) and GraphPad Prism (version 6, California, USA). Comparative analyses between patient groups were performed using one-way ANOVA or Kruskal Wallis test as appropriate for continuous variables, with pair-wise post-hoc corrections using Bonferroni and Dunn tests respectively. Chi-square or Fisher exact tests were used for categorical variables as appropriate, with Bonferroni correction when comparing ≥ 3 groups. Pearson's or Spearman's correlation was used to explore correlations depending on data normality. Two-tailed p values of <0.05 were considered statistically significant and data was presented as mean $\pm$ SD unless stated otherwise.

5.3 Results

5.3.1 Clinical characteristics of study population

A total of two hundred and one RRMS patients were included in this study: In R (n = 38), LR 1 – 6 M (n = 28), LR 6 – 24 M (n = 34), and LR ≥24 M (n = 101). Demographic and clinical characteristics are shown in **Table 5.1**.

Table 5.1: Demographic and clinical details of study population. *p* values within the right most column indicates differences across the four groups of patients. Symbols indicate *p* <0.05 for pair-wise comparison after post-hoc correction. *In R vs. LR ≥24 M; †LR 1 – 6 M vs. LR ≥24 M; ‡In R vs. LR 1 – 6 M; §In R vs. LR 6 – 24 M; ¶LR 6 – 24 M vs. LR ≥24 M.

	In R (n = 38)	LR 1 – 6 M (n = 28)	LR 6 – 24 M (n = 34)	LR ≥24 M (n = 101)	p value across groups
Age in years, mean (SD)	38.3 (9.5)*	38.7 (7.0)†	43.5 (9.7)	44.2 (9.9)*†	0.002
Female, No. (%)	27 (71.1)	23 (82.1)	22 (64.7)	73 (72.3)	0.503
White ethnicity, No. (%)	37 (97.4)	25 (89.3)	31 (91.2)	92 (91.1)	0.589
Recent/current steroid use, No. (%)	5 (13.2)*	0 (0.0)	1 (2.9)	1 (1.0)*	0.011
DMT use, No. (%)	18 (47.4)*	12 (42.9)†	20 (58.8)	75 (74.3)*†	0.002
Alemtuzumab	1 (5.6)	1 (8.3)	1 (5.0)	2 (2.7)	
Dimethyl fumarate	6 (33.3)	2 (16.7)	6 (30.0)	13 (17.3)	
Fingolimod	2 (11.1)	2 (16.7)	2 (10.0)	10 (13.3)	
Glatiramer acetate	6 (33.3)	5 (41.7)	7 (35.0)	25 (33.3)	
Interferons	1 (5.6)	0 (0.0)	3 (15.0)	18 (24.0)	
Natalizumab	1 (5.6)	2 (16.7)	1 (5.0)	5 (6.7)	
Teriflunomide	1 (5.6)	0 (0.0)	0 (0.0)	2 (2.7)	
EDSS, median (range)	3.3 (1 – 7)* ‡ §	2.5 (1 – 6.5)¶	2.3 (0 – 8.5)¶	2.0 (0 – 7)*	<0.001
Disease duration in years, median (range)	11.1 (0.73 – 28.7)	7.5 (0.19 – 28.3)†	4.4 (0.54 – 28.5)¶	12.1 (2.3 – 47.3)† ¶	<0.001
No comorbidities, No. (%)	16 (42.1)	10 (35.7)	8 (23.5)	42 (41.6)	0.271

Presence of new T ₂ lesion/s within last 1 year, referenced to a baseline scan done ≤1 year apart, No. (%)	5/12 (41.7)	7/8 (87.5) [†]	4/9 (44.4)	3/22 (13.6) [†]	0.002
Presence of GAD-enhancing lesion/s within last 1 year, No. (%)	5/16 (31.3)	7/11 (63.6) [†]	4/12 (33.3)	1/21 (4.8) [†]	0.003
BMI, median (range)	26.5 (20 – 49)	25.0 (19 – 38.7)	27.0 (19.8 – 57.4)	24.8 (15 – 42)	0.081
Current smoker, No. (%)	5 (13.2)	5 (17.9)	3 (8.8)	12 (11.9)	0.751
Alcohol intake in units/week, median (range)	0 (0 – 16)	1.5 (0 – 35)	0 (0 – 18)	1 (0 – 24)	0.394
Time from last meal in hours, median (range)	3.7 (1.3 – 18.5)	3.6 (0.8 – 20.9)	3.6 (0.4 – 16.3)	3.3 (0.9 – 16.7)	0.319

In R as well as LR 1 – 6 M patients were younger and had lower proportions of DMT use, compared to LR ≥24 M patients. More In R patients had steroid use (all five completed their steroids within the past one week before blood sampling) compared to LR ≥24 M patients. As expected, the EDSS of In R patients were higher compared to the other three groups. A higher proportion of LR 1 – 6 M patients had evidence of MRI activity (new T₂ and/or GAD enhancing lesion/s) compared to LR ≥24 M patients. This likely reflects pragmatic clinical practices as clinicians are more likely to perform MRI in patients after being seen in the ‘acute relapse’ clinic, with the MRI done a few weeks later, whilst clinicians are less likely to perform frequent scans and GAD-enhancing scans in LR ≥24 M patients.

5.3.2 Global metabolomics (CPMG spectral data)

5.3.2.1 In R vs. LR ≥24 M patients

To identify global metabolic signatures and perturbations reflective of clinical relapses, OPLS-DA was used to construct discriminatory models using CPMG spectral data to distinguish between In R and

LR ≥ 24 M patients. The representative OPLS-DA scores plot showed a moderate separation between In R and LR ≥ 24 M patients (**Figure 5.1 A**). The mean predictive accuracy for the ensemble of the OPLS-DA models of In R vs. LR ≥ 24 M patients was significantly higher than the mean predictive accuracy of the ensemble created by random class assignments (mean $\pm$ SD, $62.6 \pm 4.8\%$ vs. $50.9 \pm 8.2\%$; $p < 0.0001$) (**Figure 5.1 B**), validating the metabolic differences seen between the two groups of patients.

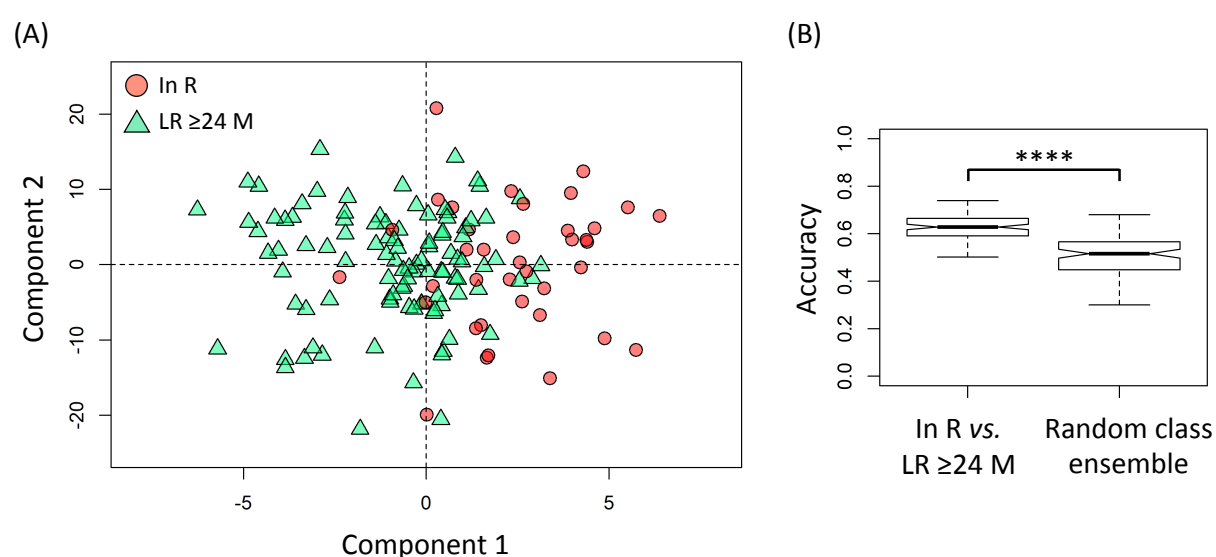


Figure 5.1: OPLS-DA models generated from CPMG data of In R vs. LR ≥ 24 M patients. **(A)** Representative OPLS-DA scores plot (In R = red circles, LR ≥ 24 M = green triangles). **(B)** Box plots of predictive accuracies from the OPLS-DA models of In R vs. LR ≥ 24 M patients, against random class assignment. ****indicates $p < 0.0001$ by Kolmogorov-Smirnov test.

5.3.2.2 LR 1 – 6 M vs. LR ≥ 24 M patients, and LR 6 – 24 M vs. LR ≥ 24 M patients

To explore how long global metabolic perturbations persist after relapses, OPLS-DA models were constructed using CPMG spectral data for LR 1 – 6 M as well as for LR 6 – 24 M patients against the reference comparator, i.e. LR ≥ 24 M patients. The mean predictive accuracy for the ensemble of the OPLS-DA models for LR 1 – 6 M vs. LR ≥ 24 M patients was significantly higher than the mean predictive accuracy of the random class ensemble (mean $\pm$ SD, $61.2 \pm 7.5\%$ vs. $48.8 \pm 8.5\%$; $p < 0.0001$). In contrast, the mean accuracy of the OPLS-DA models for LR 6 – 24 M vs. LR ≥ 24 M

patients was not different from the mean accuracy of the random class ensemble (mean $\pm$ SD, 50.5 $\pm$ 6.7% vs. 50.6 $\pm$ 7.8%; $p = 0.971$).

The fold change in the predictive accuracy (normalised to the accuracy of random chance, i.e. 50%) of each patient group, using LR ≥ 2 years patients as reference comparator, is shown in **Figure 5.2**. Taking these findings in totality, this implies that global metabolic perturbations last for at least 6 months after a clinical relapse.

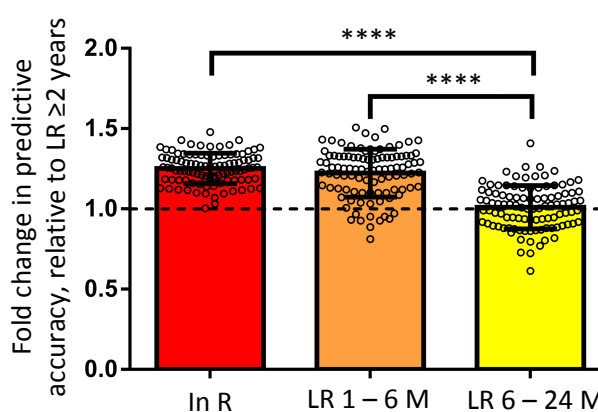


Figure 5.2: Fold change in predictive accuracies of the OPLS-DA models of the different patient groups with respect to the reference comparator, i.e. LR ≥ 24 M patients. The fold change of random chance is 1.0 as indicated by the dashed horizontal line. **** indicates $p < 0.0001$ by post-hoc Bonferroni correction.

5.3.2.3 Identifying discriminatory metabolites from the In R vs. LR ≥ 24 M OPLS-DA models

To identify the top discriminatory metabolites (and potential metabolite biomarkers of relapse) from the In R vs. LR ≥ 24 M OPLS-DA models, VIP scores were generated. The VIP score cut-off at 1.35 was determined by identifying a ‘drop-off’ on the VIP ranking plot (**Figure 5.3**). Metabolites with VIP scores above this cut-off are detailed in **Table 5.2**. These consisted predominantly of lipoproteins, amino acids and glucose. As most (i.e. two thirds) of these discriminatory metabolites were detected by the AXINON® lipoFIT® system, targeted metabolomics was performed next. Although targeted metabolomics covers a smaller region of the NMR spectra, it allows for detailed lipoproteins, amino acids and glucose analyses, as well as absolute quantification of these metabolites. The additional

metabolites measured by the AXINON® lipoFIT® system that were not identified to be highly discriminatory by global metabolomics included alanine, isoleucine, lactate and valine.

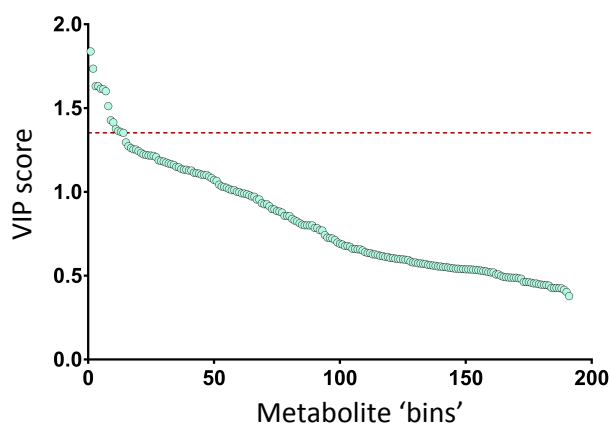


Figure 5.3: VIP score ranking plot obtained from the OPLS-DA models of *In R* vs. *LR* ≥ 24 M patients. The dashed red line indicates the VIP score threshold of 1.35, before a ‘drop-off’ in VIP score.

Table 5.2: Top discriminatory metabolites from global (CPMG) metabolomics distinguishing *In R* vs. *LR* ≥ 24 M patients. *indicates metabolites measured and quantified by AXINON® lipoFIT® system (targeted metabolomics).

Discriminatory metabolites	Chemical shift of contributing spectral ‘bins’ (VIP score, VIP rank)
Mobile $(-\text{CH}_3)_n$ HDL/LDL*	0.84....0.86 ppm (1.36, 12) 0.86....0.88 ppm (1.62, 5)
Leucine*	0.96....0.98 ppm (1.36, 13)
Mobile $(-\text{CH}_2)_n$ LDL*	1.22....1.24 ppm (1.43, 9) 1.24....1.26 ppm (1.74, 2)
Lysine	1.40....1.42 ppm (1.61, 6) 1.42....1.44 ppm (1.51, 8)
βCH_2^*	1.62....1.64 ppm (1.63, 3)
$/=\text{CH}-\text{CH}_2-\text{CH}_2^*$	1.96....1.98 ppm (1.63, 4) 1.98....2.00 ppm (1.84, 1)
Asparagine	2.84....2.86 ppm (1.41, 10)
Glucose*	3.88....3.90 ppm (1.38, 11)
Phenylalanine (meta-)	7.42....7.44 ppm (1.60, 7)

5.3.3 Targeted metabolomics (AXINON® lipoFIT® spectral data)

5.3.3.1 In R vs. LR $\geq$ 24 M patients

To identify if there were metabolomics perturbations in relapses using a targeted metabolomics approach, OPLS-DA was employed to interrogate the AXINON® lipoFIT® parameters obtained from In R and LR $\geq$ 24 M patients. The mean predictive accuracy for the ensemble of the OPLS-DA models of In R vs. LR $\geq$ 24 M patients was significantly higher than the mean predictive accuracy of the ensemble created by random class assignments (mean $\pm$ SD, 58.1 $\pm$ 5.5% vs. 50.5 $\pm$ 7.0%; $p < 0.0001$) (**Figure 5.4**), confirming the presence of targeted metabolic differences between the two groups of patients. The decrease in predictive accuracy as well as the less obvious separation of the patient groups on the OPLS-DA scores plot is likely due to lesser amount of data available within a targeted region of the NMR spectra, as compared to the OPLS-DA models generated by global metabolomics.

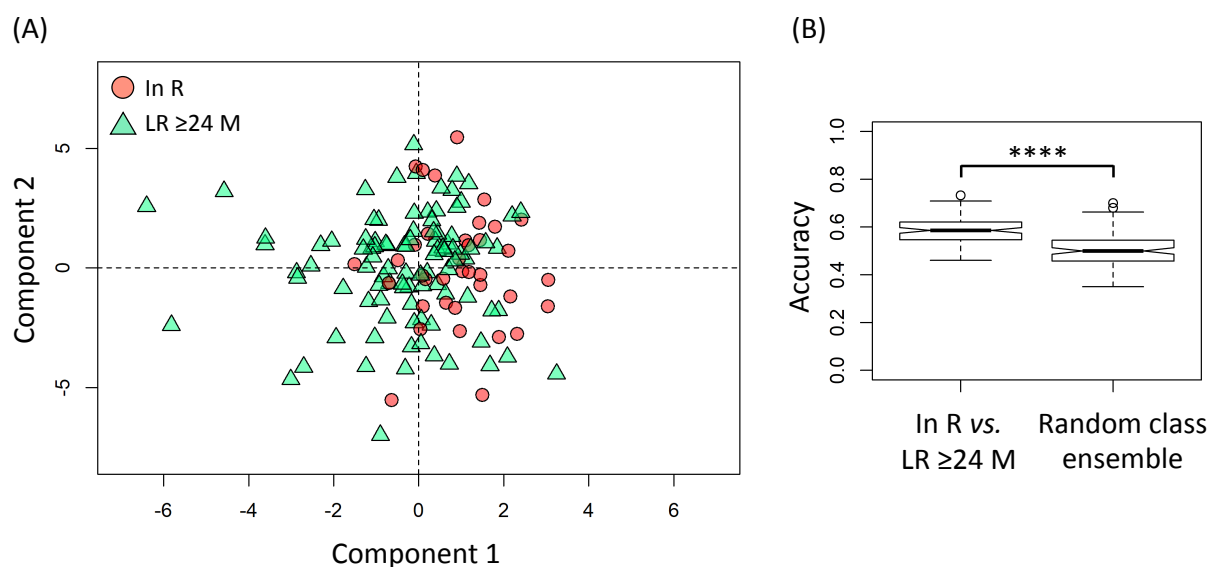


Figure 5.4: OPLS-DA models generated from AXINON® lipoFIT® data of In R vs. LR $\geq$ 24 M patients. (A) Representative OPLS-DA scores plot (In R = red circles, LR $\geq$ 24 M = green triangles). (B) Box plots of predictive accuracies from the OPLS-DA models of In R vs. LR $\geq$ 24 M patients, against random class assignment. **** indicates $p < 0.0001$ by Kolmogorov-Smirnov test.

5.3.3.2 Identifying discriminatory metabolites from the In R vs. LR ≥ 24 M OPLS-DA model

VIP scores from the In R and LR ≥ 24 M OPLS-DA models were generated to elucidate the principal discriminatory metabolites from targeted metabolomics. The VIP ranking plot revealed isoleucine and leucine (both are BCAA) as the two most important metabolites (**Figure 5.5**), with VIP scores of 2.05 and 2.01 respectively.

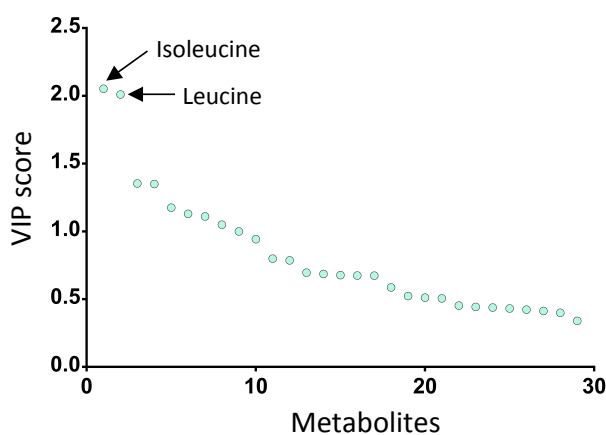


Figure 5.5: VIP score ranking plot obtained from the OPLS-DA models of IR vs. LR ≥ 2 years patients identifies isoleucine and leucine as the top two discriminatory metabolites.

Isoleucine was not identified as one of the principal discriminatory metabolites on global metabolomics although it was the top discriminatory metabolite in targeted metabolomics. This is likely due to the fact that its resonances (a triplet centred at 0.941 ppm arising from three ^1H nuclei and a doublet centred at 1.012 ppm also arising from three ^1H nuclei) overlap with the broad methyl lipoprotein signal on the CPMG spectra, therefore masking its signal substantially (**Figure 5.6**). In comparison, while the resonance from leucine (a triplet centred at 0.963 ppm) also overlaps with the broad lipoprotein signal, its integral value is greater as this comprise of signals arising from six ^1H nuclei, hence enhancing its detection (**Figure 5.6**). Of note, the top lipoprotein parameter from the AXINON[®] lipoFIT[®] was LDL-s (mean diameter of LDL particles) which was ranked third based on its VIP score of 1.35.

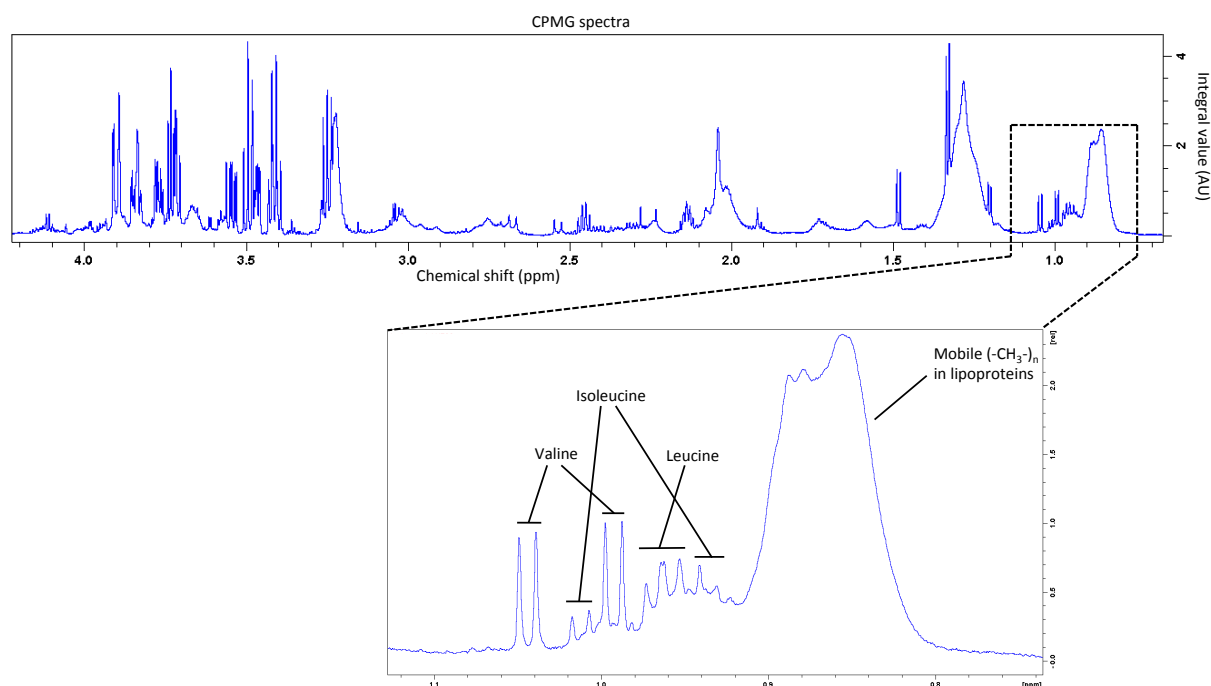


Figure 5.6: BCAA resonances in a CPMG spectrum. The zoom in panel shows that the spectral resonances from isoleucine overlap substantially with the broad methyl lipoprotein resonance, thus masking its signal substantially. Leucine resonances also overlap with this broad lipoprotein signal; however, its integral value is greater (as compared to isoleucine) as this is derived from six ^1H nuclei (note the taller and broader signals compared to isoleucine), thus the signal is more apparent despite the masking effect of the broad lipoprotein signal.

5.3.4 Exploring potential metabolite biomarkers of clinical relapses

From the previous sections, using the OPLS-DA analytical approach, global metabolic perturbations can be observed during relapses and up to six months after relapses. The top discriminatory metabolites from targeted (isoleucine and leucine) and global metabolomics (metabolites listed in **Table 5.2** not marked by an asterisk, i.e. not covered by targeted metabolomics) distinguishing In R vs. LR ≥ 24 M patients were shortlisted as potential metabolite biomarkers of clinical relapses. One-way ANOVA was performed for each of these shortlisted metabolite across the four patient groups, using LR ≥ 24 M patients as the reference group. The workflow for choosing the metabolites for ANOVA is illustrated in **Figure 5.7**. Of note, discriminatory metabolomics from global metabolomics (i.e. lipoproteins, leucine and glucose) that are already covered in-depth by targeted metabolomics (AXINON[®] lipoFIT[®] system) were not included in this analysis.

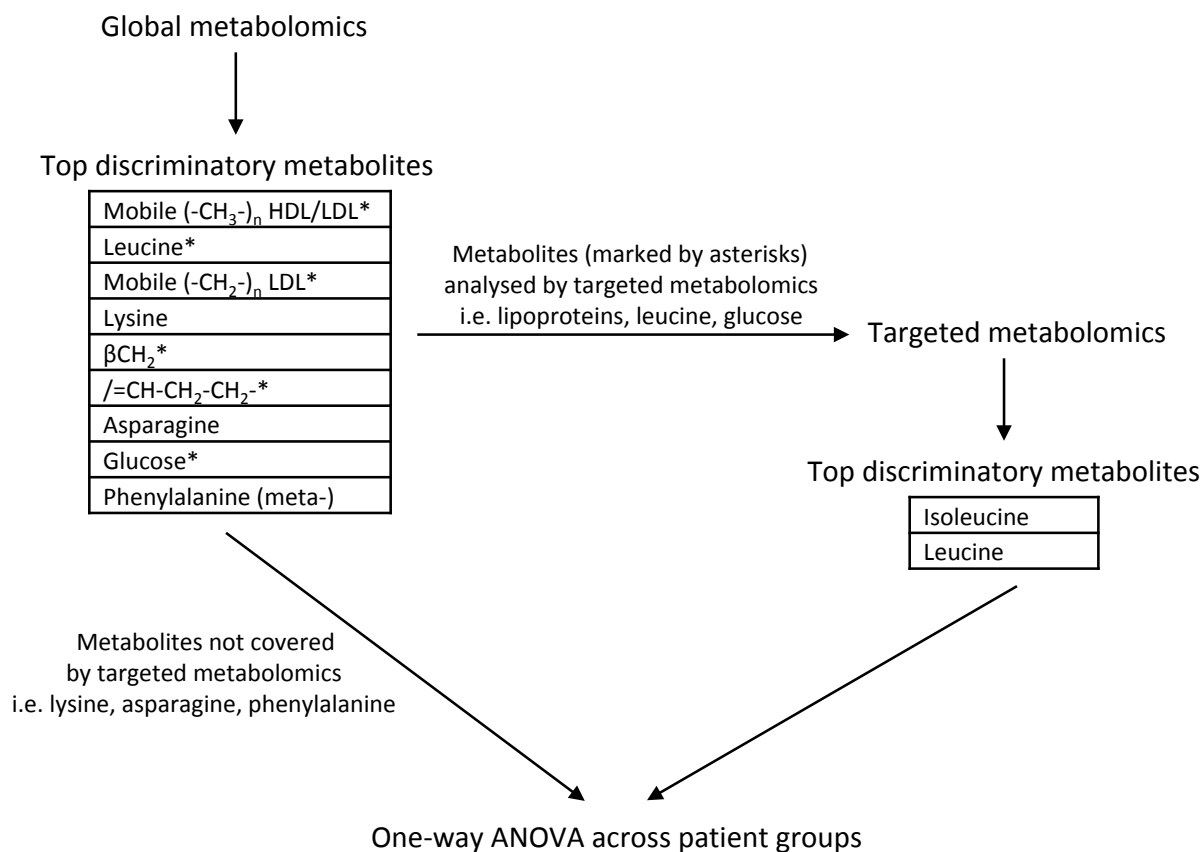


Figure 5.7: Workflow illustrating the selection of metabolites for ANOVA. *indicates metabolites detected by targeted metabolomics.

Lysine and asparagine (from global metabolomics), as well as isoleucine and leucine (from targeted metabolomics) were significant on one-way ANOVA. Both lysine and asparagine were higher in In R vs. LR ≥ 24 M patients, and showed a decreasing trend over time (**Figure 5.8 A and B**). The converse was observed for isoleucine and leucine: lower levels in In R compared to LR ≥ 24 M patients and increasing with time away from relapse (**Figure 5.8 C and D**). Taking these observations in totality, this suggests that lysine, asparagine, isoleucine and leucine are potential metabolite biomarkers of clinical relapses.

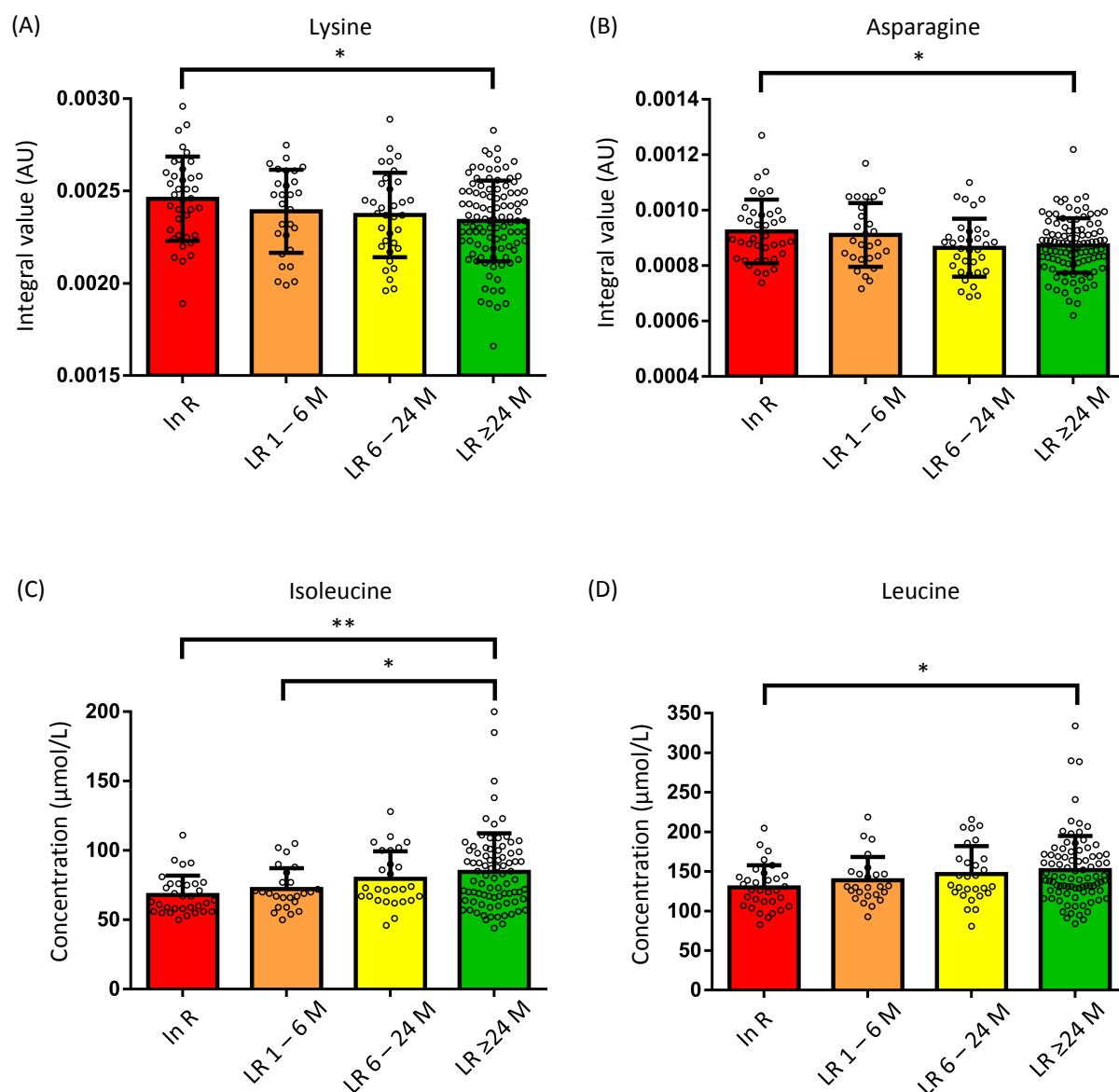


Figure 5.8: Significant metabolites on one-way ANOVA. **(A and B)** Lysine and asparagine levels were higher within In R patients compared to LR ≥24 M patients and decreased with time away from relapse. **(C and D)** In contrast, isoleucine and leucine concentrations were observed to be lower during relapses and increased with time away from relapse. **indicates $p < 0.01$ and *indicates $p < 0.05$ by post-hoc Holm-Sidak's test, with LR ≥24 M patients as the reference comparator.

On ROC analysis to distinguish between In R and LR ≥24 M patients, the univariate AUC of the four metabolite biomarkers ranged from 0.610 to 0.697 (**Figure 5.9 A – D**). Next, to explore if the combination of the four metabolites could enable higher discriminatory ability, the four metabolites were used as independent variables in a multivariable logistic regression model followed by ROC analysis. This resulted in an improved AUC of 0.758 (**Figure 5.9 E**).

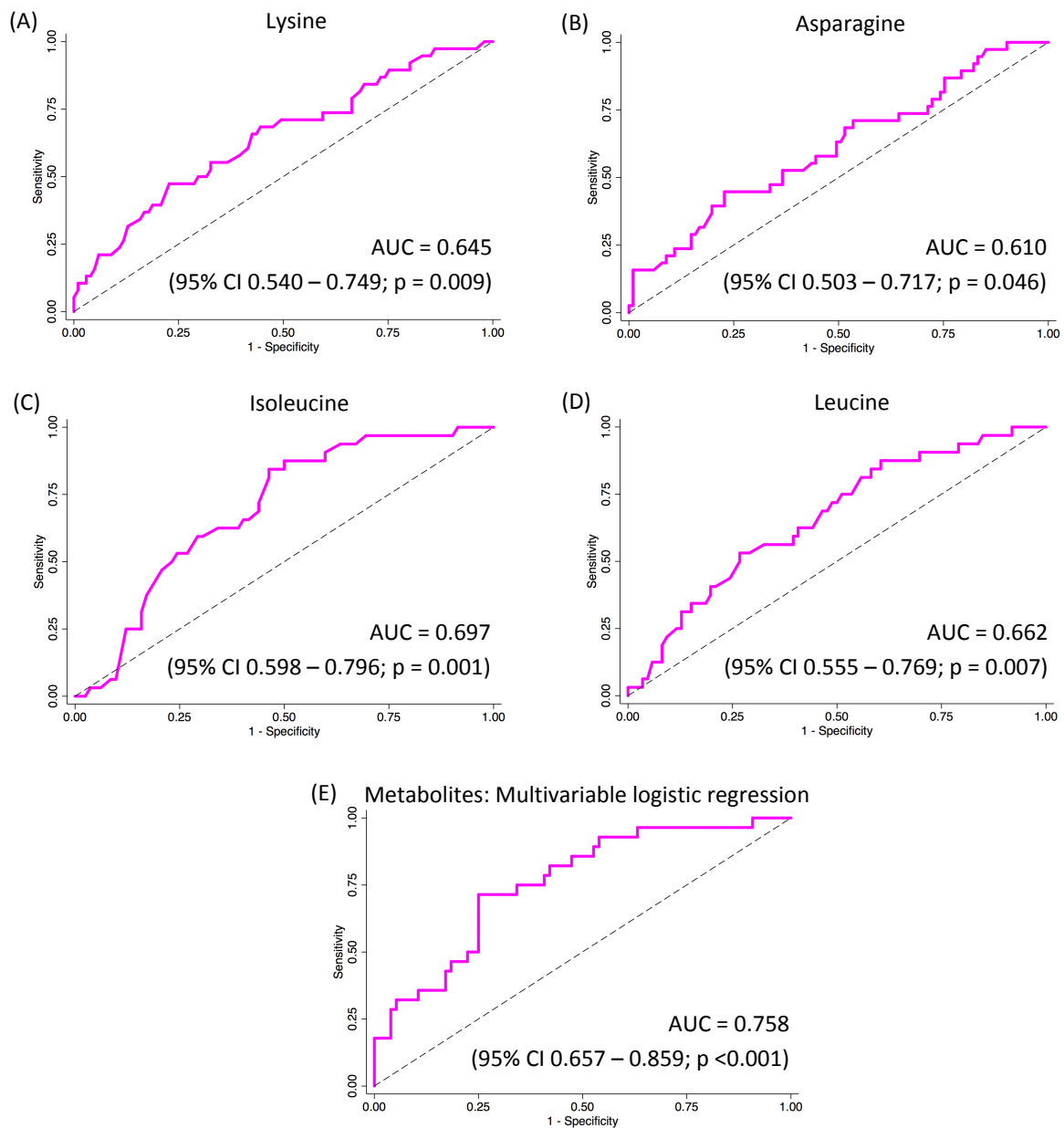


Figure 5.9: ROC analysis of the four metabolite biomarkers (A – D) individually and (E) in combination.

5.3.5 Exploring serum NfL as a potential biomarker of clinical relapses

The previous sections demonstrated that using both global and targeted metabolomics approaches, four metabolites were identified that could serve as potential biomarkers of relapse. As mentioned in the chapter Introduction, serum NfL appears to be a promising biomarker to inform on MS inflammatory activity and is reported to be elevated in clinical relapses, thus its diagnostic performance in this cohort of patients was explored. One-way ANOVA of serum NfL levels, using LR

≥24 M patients as the reference group, showed that In R patients had higher levels of serum NfL compared to LR ≥24 M patients, although this appeared to be driven by outliers with very elevated levels of serum NfL (**Figure 5.10 A**). Indeed, when the three highest serum NfL values (i.e. outliers) from the In R group were removed, the one-way ANOVA was no longer statistically significant ($p = 0.054$) and the post-hoc Holm-Sidak's test comparing In R vs. LR ≥24 M was also not statistically significant ($p = 0.061$). ROC analysis of all data points showed an AUC of 0.575 for serum NfL in distinguishing between In R vs. LR ≥24 M patients (**Figure 5.10 B**).

Next, it was explored if the ability to distinguish In R and LR ≥24 M patients could be increased by combining 4 metabolites and NfL. A multivariable logistic regression model was constructed followed ROC analysis (**Figure 5.10 C**), showing a slightly increased AUC of 0.789, as compared to using the combination of the four metabolites alone.

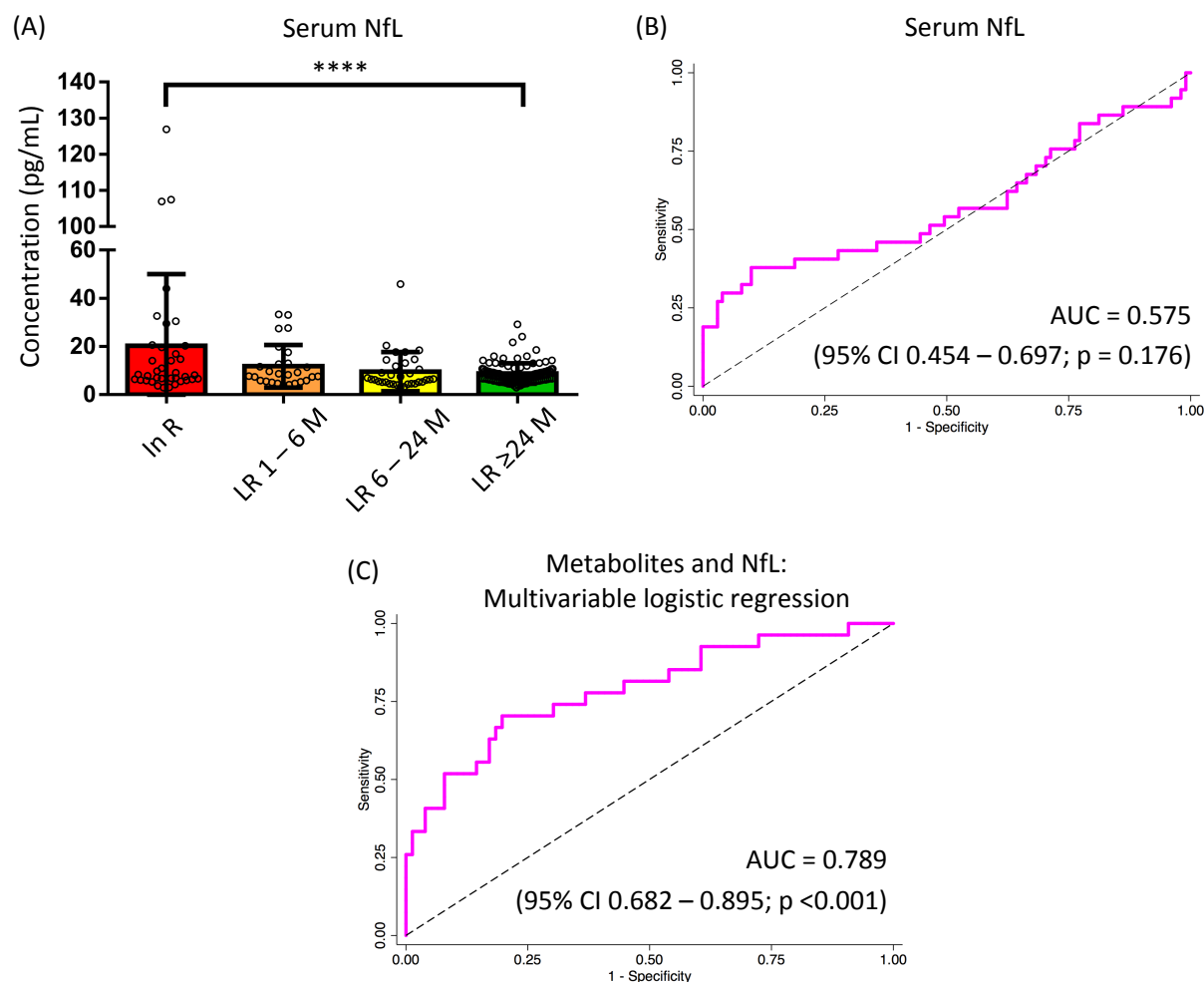


Figure 5.10: Discriminatory ability of serum NfL levels across the patient groups. **(A)** One-way ANOVA showed that In R patients had higher levels of serum NfL compared to LR ≥24 M patients. **** indicates $p < 0.0001$ by post-hoc Holm-Sidak's test, with LR ≥24 M patients as the reference comparator. **(B)** ROC analysis of serum NfL. **(C)** ROC analysis using a combination of lysine, asparagine, isoleucine, leucine and NfL.

5.3.6 Metabolites as individualised, responsive biomarkers of relapses

From the previous sections, lysine, asparagine, isoleucine and leucine were identified to be promising metabolite biomarkers of relapses. However, for a biomarker to be clinically useful, it must be: (1) applicable in an individualised manner (i.e. within an individual patient), and (2) be responsive enough such that the change in its levels between diseased states must be observable within a clinically useful time frame.

To this end, nine patients (under the MET cohort collection) who had paired relapse-remission samples (i.e. relapse first followed by remission), and with the remission sample collected within 6

months of relapse onset, were identified. The clinical characteristics of these nine patients are shown in **Table 5.3**. Of note, none of these patients received steroids at the time of relapse blood sampling.

Table 5.3: Clinical characteristics of the nine patients with paired relapse-remission samples, with data collected at the relapse time point. *Median EDSS at remission sampling was 2.5 (range 1.0 – 6.5).

	Patients with paired relapse-remission (n = 9)
Age in years, mean (SD)	40.2 (7.1)
Female, No. (%)	6 (66.7)
White, No. (%)	9 (100.0)
Recent/current steroid use, No. (%)	0 (0.0)
DMT use, No (%)	4 (44.4)
Alemtuzumab	1 (25.0)
Glatiramer acetate	2 (50.0)
Interferons	1 (25.0)
EDSS, median (range)	4.0 (2.0 – 6.5)*
Disease duration in years, median (range)	14.8 (1.9 – 23.5)
No comorbidities, No. (%)	4 (44.4)
BMI, median (range)	30.3 (24.2 – 46.4)
Interval between relapse onset and remission sampling in months, median (range)	3.6 (2.3 – 5.6)
Presence of new T ₂ lesion/s within last 1 year, referenced to baseline scan done ≤1 year apart, No. (%)	Not known as none had earlier baseline reference scan done ≤1 year
Presence of GAD enhancing lesion/s within last 1 year, No. (%)	1/5 (20)
Presence of new T ₂ lesion/s within next 6 months, referenced to baseline scan ≤1 year apart, No. (%)	2/4 (50)
Presence of GAD enhancing lesion/s within next 6 months, No. (%)	3/6 (50)

5.3.6.1 Univariate analysis

On paired t-test, all four metabolites showed significant differences in their levels during relapse and in remission (**Figure 5.11 A – D**), and the direction of change was consistent with that observed when comparing at group level across the four groups of patients (**Figure 5.8 A – D**). There was no

difference in serum NfL levels within this 6-month time frame (**Figure 5.11 E**). Six out of the eight patients (one patient had missing data for the relapse sample and was excluded from analysis) had lower serum NfL levels during remission while two had higher levels. It was observed that the change in serum NfL levels was gradual and there were considerable inter-individual differences in NfL levels during relapses, consistent with observations from group comparisons (**Figure 5.8**).

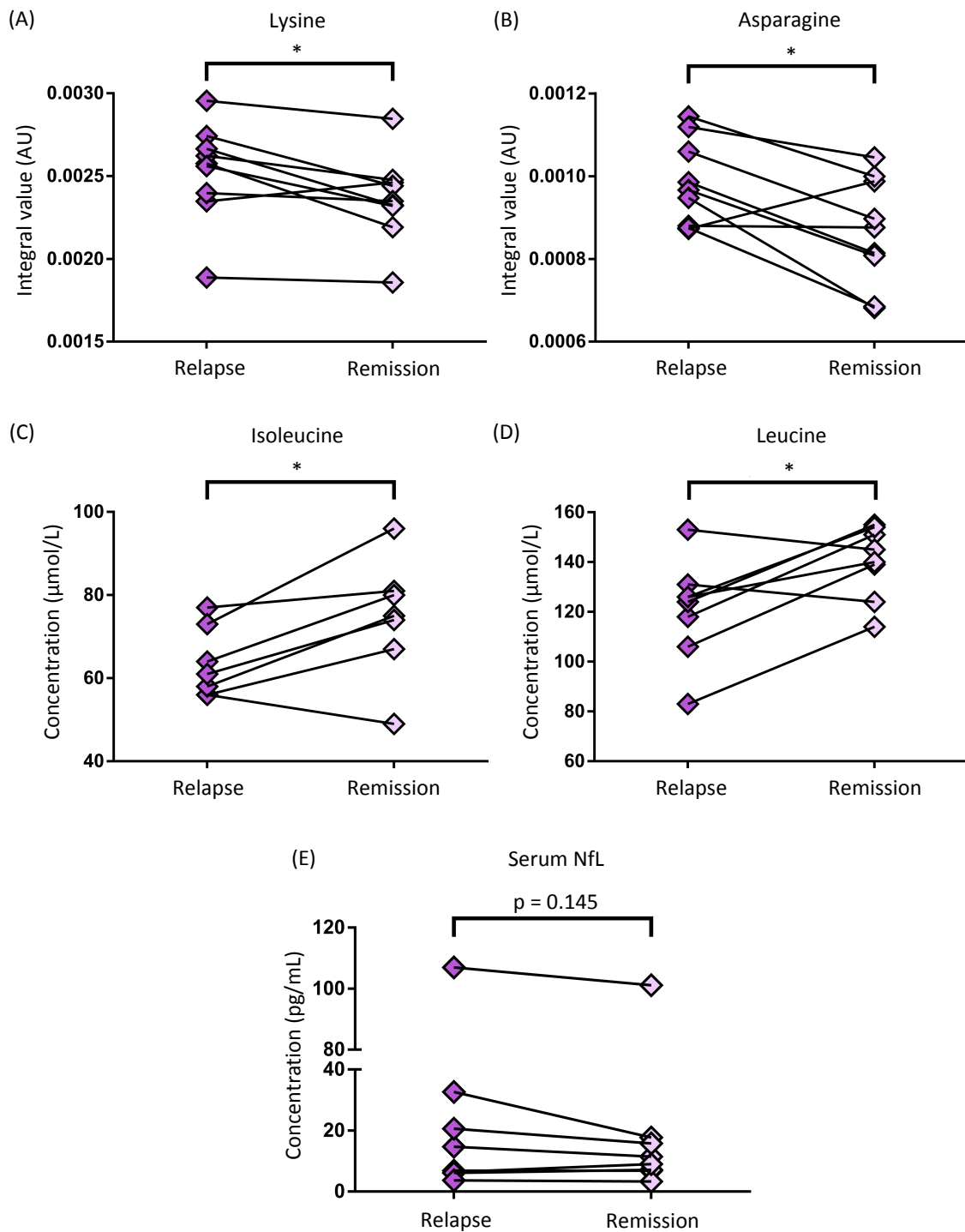


Figure 5.11: Paired relapse-remission levels for the (A – D) four metabolite biomarkers, and for (E) serum NfL. *indicates $p < 0.05$ on paired t-test.

5.3.6.2 Multivariate analysis

Five out of 9 of these patients had complete concordance in the direction of change for all four metabolites. For the remaining four patients, three had discordance in one metabolite while one had

disagreement for two metabolites. Given these observations, multivariate approaches were employed to determine if a combination of these four metabolites (i.e. a metabolic constellation), rather than individual metabolites, could be used as a composite biomarker. Using the four metabolites as independent variables in a multivariable logistic regression approach followed by ROC analysis, an AUC of 0.911 was achieved (**Figure 5.12 A**). The addition of serum NfL into this multivariable regression model resulted in slight decrease in AUC to 0.896 (**Figure 5.12 B**)

To further extend this concept of a composite metabolic biomarker, a vector-based OPLS-DA approach was employed. A representative OPLS-DA scores plot (generated using 7-fold internal cross-validation) of In R vs. LR $\geq$ 24 M patients was first constructed (**Figure 5.12 C**) using the same data used to generate the scores plot in **Figure 5.1 A**, however excluding the relapse data of the nine patients who had paired relapse-remission samples. This was done to allow the data (from these nine patients) to be used as an independent predictive set. The x-axis of the OPLS-DA scores plot (**Figure 5.12 C**), i.e. along the first component, can be thought to represent inflammation, with a leftward vector signifying decreasing inflammatory activity. Data from the paired relapse-remission samples were then inserted into this OPLS-DA scores plot as a predictive set (**Figure 5.12 D**). All remission samples, except one, moved in a leftward direction (along the first component) with respect to their paired relapse sample, consistent with the vector representing decreasing inflammation.

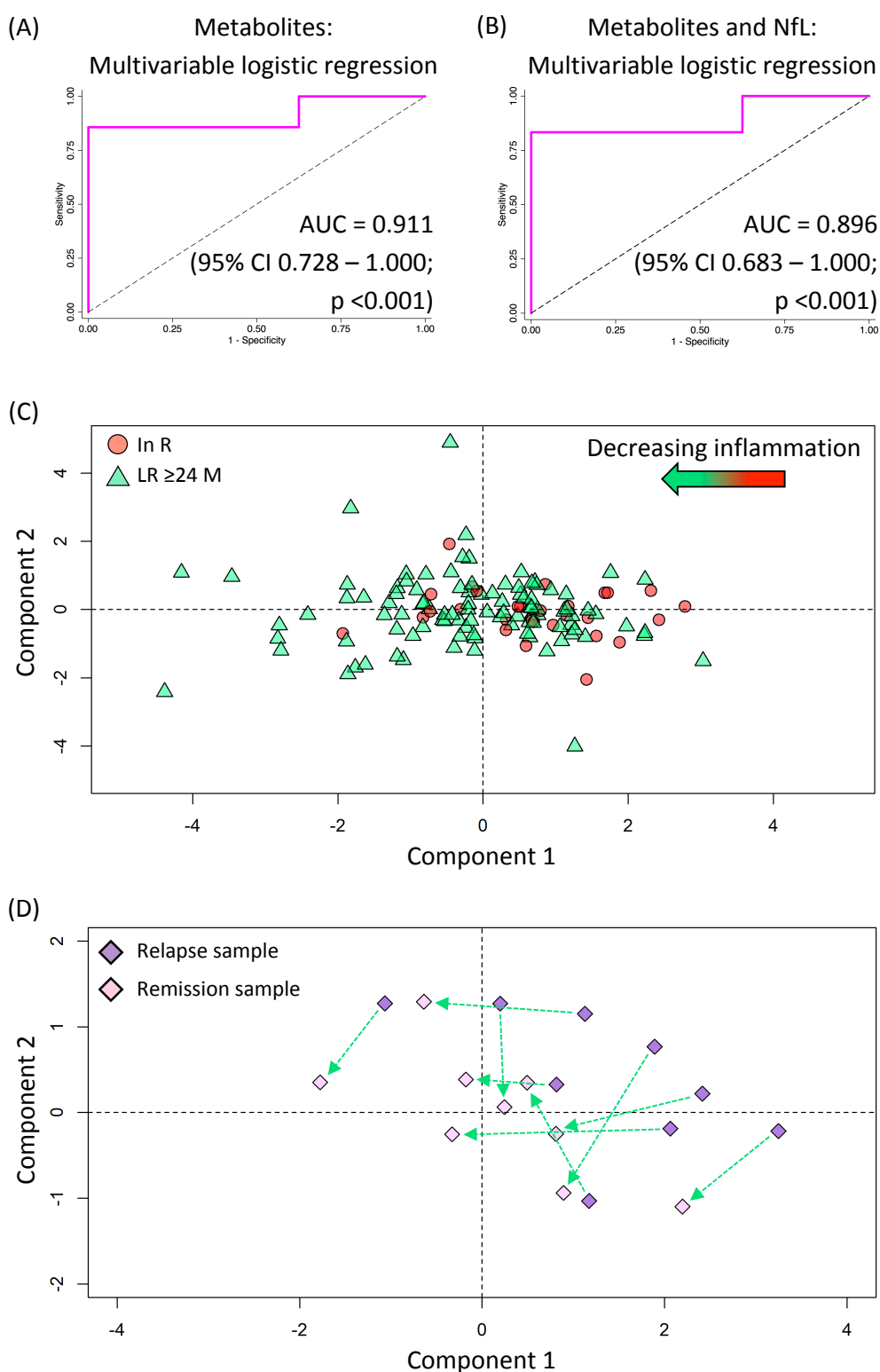


Figure 5.12: Multivariate approaches to combine the four metabolites as a composite biomarker. **(A)** ROC analysis after multivariable logistic regression using the four metabolites. **(B)** Multivariable ROC analysis with the addition of serum NfL to the four metabolites. **(C)** OPLS-DA scores plot of In R vs. LR ≥24 M patients (In R = red circles, LR ≥24 M = green triangles), minus the in relapse data points of the nine patients with paired relapse-remission samples. **(D)** Insertion of data from paired relapse-remission samples into the OPLS-DA scores plot as a predictive set (relapse = purple diamonds, remission = light pink diamonds). Green arrows indicate paired samples.

5.3.7 Isoleucine and leucine as potential predictive biomarkers of relapses

To explore if the identified metabolites can be predictive biomarkers of relapses, three patients (from the MET cohort) who had remission samples prior to relapse samples were identified. The mean interval between remission and relapse was 14.1 months. Two of the three patients had higher isoleucine levels during remission compared to in relapse, while the levels remained the same for one patient (**Figure 5.13 A**). For leucine, two patients showed higher levels during remission (**Figure 5.13 B**). The leucine measurement in remission for one patient was not available and was thus excluded from analysis. The direction of change was consistent with that described in the previous sections. For lysine and asparagine however, this was not observed (i.e. lower levels during remission). For serum NfL, two patients showed lower levels in remission while one had higher level in remission (**Figure 5.13 C**). This observation could mean that isoleucine and leucine are reflective of the state of immune activation, while lysine and asparagine could be the consequence a relapse.

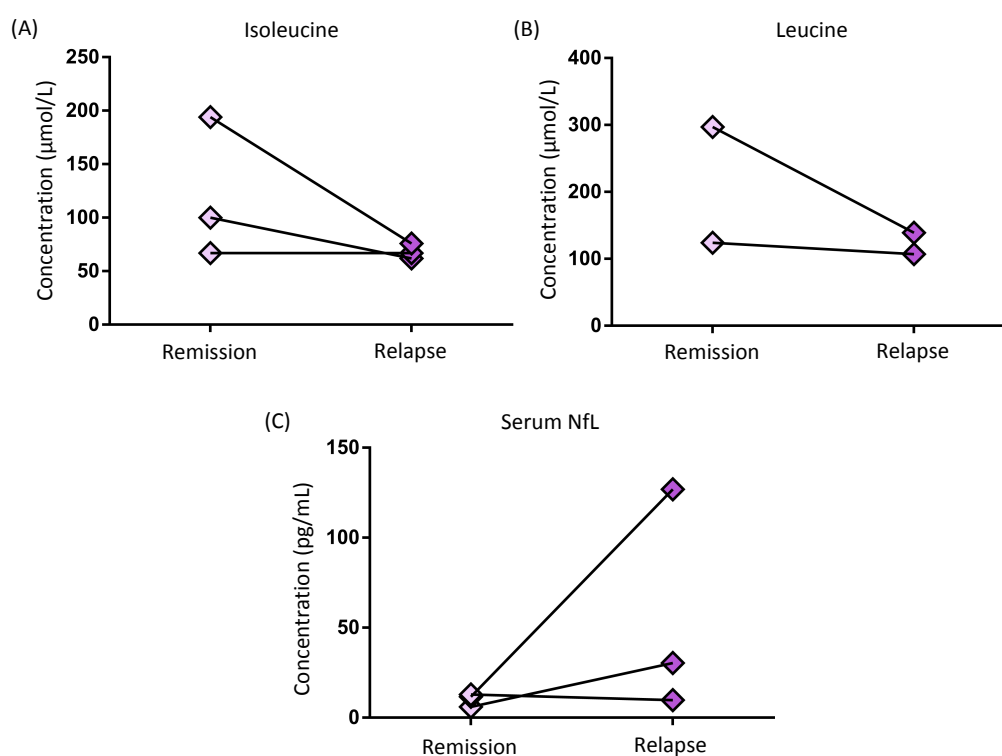


Figure 5.13: Paired remission-relapse metabolites and serum NfL levels as predictive biomarkers of future relapses (A) Isoleucine, (B) leucine, and (C) serum NfL.

5.3.8 Addressing potential confounders for the identified metabolite biomarkers

As illustrated in **Table 5.1**, several baseline characteristics were different across the four patient groups; notably age, steroid use, and DMT use were dissimilar between In R vs. LR $\geq$ 24 M patients on post-hoc analysis. Two approaches were used to address these potential confounders: (1) for quantitative variables, correlation of the potential confounding variable with each of the four identified metabolite biomarkers was performed, and (2) for categorical variables, the levels of the four metabolites were explored stratified by the potential confounding variable (i.e. steroid use and DMT use).

There were no correlations between any of the four metabolite biomarkers with age, EDSS and disease duration (the highest R^2 was 0.113 between age and lysine) across the entire cohort of patients ($n = 201$). There were no differences in any of the metabolite concentrations stratified by steroid use in the whole cohort (7 steroid users, 194 non-steroid users) and indeed within In R patients (5 steroid users, 33 non-steroid users). For DMT use, there were higher isoleucine levels in DMT users (mean $\pm$ SD, 82.3 ± 25.6 $\mu\text{mol/L}$ vs. 72.8 ± 18.7 $\mu\text{mol/L}$; $p = 0.011$) across the entire cohort (125 DMT users, 76 non-DMT users). However, no differences in isoleucine levels were observed between DMT users and non-users within In R patients (18 DMT users, 20 non-DMT users) ($p = 0.120$) as well as within LR $\geq$ 24 M patients (75 DMT users, 26 non-DMT users) ($p = 0.304$). There were also no significant associations/correlations of the discriminatory metabolites with smoking status, alcohol intake, and time from last meal.

5.3.9 Association of metabolite biomarkers with MRI indices of inflammatory activity

The association of the identified metabolite biomarkers with MRI indices of inflammation, i.e. the presence of new T_2 lesion/s (referenced to baseline scan done ≤ 1 year apart) as well as the presence of GAD-enhancing lesion/s within the last 1 year, were explored. There were no differences in the metabolite levels stratified by the presence of new T_2 lesion/s. Interestingly, lysine (mean $\pm$ SD, 25.5

$\times 10^{-4} \pm 2.1 \times 10^{-4}$ AU vs. $23.7 \times 10^{-4} \pm 2.4 \times 10^{-4}$ AU; $p = 0.008$) and asparagine (mean $\pm$ SD, $9.6 \times 10^{-4} \pm 1.2 \times 10^{-4}$ AU vs. $8.9 \times 10^{-4} \pm 1.2 \times 10^{-4}$ AU; $p = 0.048$) levels were significantly higher in patients who had GAD-enhancing lesions. However, this association was not observed for isoleucine ($p = 0.074$) and leucine ($p = 0.231$).

5.4 Discussion

This chapter illustrates that: (1) metabolic perturbations are present in patients during relapses using both global and targeted metabolomics, as compared to patients with no relapses for the past two years, (2) the four discriminatory metabolites that were significant on ANOVA (lysine, asparagine, isoleucine and leucine) across the different patient groups showed a consistent trend (either increasing or decreasing) with time away from relapse, and importantly, (3) these metabolites are informative in an individualised manner within a clinically useful time frame. Taking these observations in totality, this suggests that the identified metabolites could be useful biomarkers of clinical relapses.

The metabolic perturbation seen in MS relapses is likely to be due to the summative effects of various immunopathological processes that occur during these inflammatory events: (1) activation of peripheral T cells and monocytes, and their subsequent access into the CNS, (2) activation of resident microglial and astrocytes, (3) initiation of injurious effector mechanisms leading to the production of ROS and mitochondrial stress, and (4) demyelination with possible axonal injury. Further details on how each of these processes can produce metabolic derangements are detailed in the Introduction of this thesis. It is important to note that it is perhaps not surprising, given that most of these pathophysiological processes occur within the CNS, that the metabolic perturbations can be detected in the serum. Two possible explanations can account for this observation: (1) CNS metabolites involved in these pathophysiological processes can cross the BBB (and indeed in the opposite direction) facilitated by increased permeability on a background of an inflamed BBB, and/or

(2) the metabolic perturbation is contributed mostly by peripheral processes, namely the activation of peripheral immune cells and the peripheral response to CNS injury which is mediated primarily by the liver (Anthony et al., 2012). The postulated roles of the identified metabolite biomarkers in these processes will now be discussed.

The BCAA isoleucine and leucine are observed to be low during relapses in this chapter. This is in agreement with other metabolomics studies (both on ^1H NMR and mass spectrometry), showing that BCAA are low in plasma and CSF of MS patients (Kasakin et al., 2019; Podlecka-Pietowska et al., 2019). Indeed, a ^1H NMR study demonstrated lower CSF isoleucine and valine in RRMS-relapse ($n = 20$) compared to RRMS-remission ($n = 30$) patients (Kim et al., 2017). BCAA are essential amino acids, meaning that they must be obtained from the diet. BCAA play a vital role in protein metabolism as building blocks of tissue protein and enhancing protein synthesis via the activation of the mammalian target of rapamycin (mTOR) signalling pathway (in the case of leucine) (Zhang et al., 2017). They are also involved in lipid and glucose metabolism, and have been implicated in a variety of physiological functions such as maintaining intestinal nutrient absorption, stimulating the development of mammary epithelial cells and regulating embryonic development (Zhang et al., 2017). Most relevant to the current discussion is their role in immune function. It has been shown that leucine, via its role as an activator of the mTOR complex 1, is involved in T cell activation (Ananieva et al., 2016). A heightened immune system, as seen in MS, is characterised by a marked increase in demand for substrates in activated immune cells, especially those required for energy and protein production (Pearce et al., 2013). Human immune cells express branched-chain aminotransferase and branched-chain α -ketoacid dehydrogenase, therefore they can metabolise BCAA for these purposes (Brosnan et al., 2006). Indeed, it has been shown that immune cells can incorporate BCAA into proteins and their (immune cells) activation commits them to specific energy pathways with an upregulation of the TCA cycle to produce reducing equivalents that donate electrons to the electron transport chain to fuel oxidative phosphorylation (Calder, 2006; Pearce et

al., 2013). The catabolism of isoleucine and leucine can contribute to the TCA cycle by producing acetyl-CoA (from both isoleucine and isoleucine) and succinyl-CoA (from isoleucine) (**Figure 5.14**). Thus, the lower concentrations of isoleucine and leucine observed during MS relapses may reflect a consumptive deficiency as a result of these catabolic processes. Functionally, the importance of BCAA to the immune system has been shown across cell culture and animal studies (Calder, 2006). These demonstrate that: (1) lymphocyte proliferation is compromised by a reduction in BCAA availability, and (2) a decrease in BCAA in the diet of rodents affected the function of natural killer cells and cytotoxic T cells, thus demonstrating the potential role BCAA in immune activation.

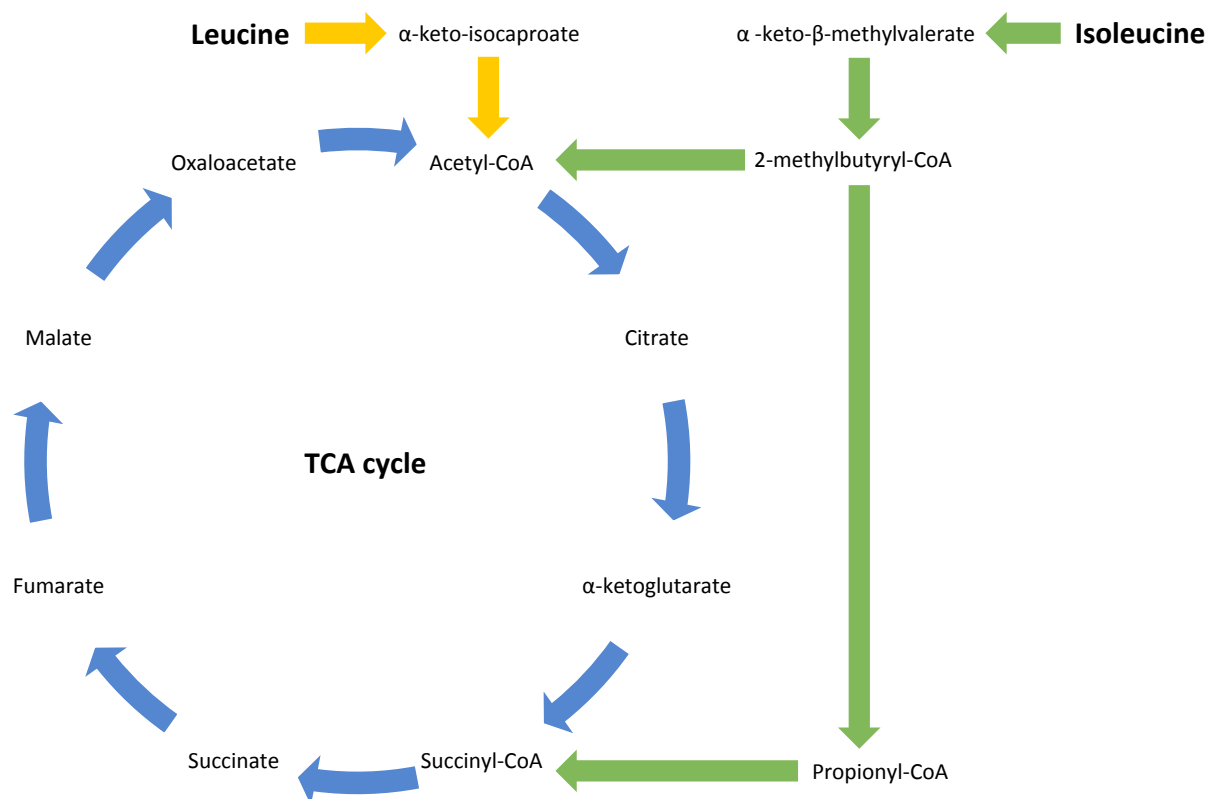


Figure 5.14: Substrates derived from the catabolism of isoleucine and leucine contribute to the TCA cycle during periods of stress and inflammation. Adapted from Zhang et al (2017) (available without permissions required via open access).

The concentration of the third BCAA, valine, was not significantly altered during relapses on ANOVA comparing across patient groups in this chapter, although it was ranked the fourth most

discriminatory metabolite (VIP score of 1.35) in targeted metabolomics when comparing In R vs. LR ≥ 2 years patients. As the three BCAA (isoleucine, leucine and valine) have common catabolic enzymes, the levels of the three BCAA should correlate (Brosnan et al., 2006). Further analysis (not reported in Results) showed that within paired relapse-remission samples, valine was lower in seven of the nine patients during relapse (the direction of change being consistent as that seen in isoleucine and leucine), but the magnitude of change was modest and thus not significant on paired t-test.

Lysine concentrations were observed to be elevated during relapses in this study. This is consistent with observations in the MBP-EAE model showing raised lysine in both CSF and intact CNS tissues using mass spectroscopy and ^1H NMR techniques (Battini et al., 2018; Blanchet et al., 2011). The putative role of lysine within the context of MS pathogenesis is less clear although it is considered to be involved in the entry of arginine into leucocytes and thus on nitric oxide metabolism (Blanchet et al., 2011). Lysine is also an essential amino acid and most its functions relate to proteinogenesis, epigenetic regulation by means of histone modification (via acetylation) and crosslinking of collagen polypeptides (Choudhary et al., 2014; Yamauchi et al., 2012). It is interesting to note that lysine is a component (together with alanine, tyrosine and glutamic acid) of the complex mixture of linear and random polypeptides that make up glatiramer acetate, originally synthesised to mimic MBP (Scott, 2013). The use of glatiramer acetate (and potential catabolism to its constituent amino acids) is unlikely to be a confounder in this study as: (1) the proportions of In R and LR ≥ 24 M patients on glatiramer acetate are the same, and (2) the concentrations of the alanine, tyrosine and glutamic acid are not correspondingly increased. Another noteworthy mention is the condition of hyperlysinemia, which is an autosomal recessive disorder caused by mutations in the AASS gene leading to α -aminoacidic semialdehyde synthase deficiency, impairing the degradation of lysine. This disorder usually manifests in childhood as progressive spastic paraparesis, epilepsy and intellectual impairment (Houten et al., 2013). Although the association of elevated lysine to neurological

deterioration is clear, the mechanisms by which this occur is less certain, as are the biological processes leading to the raised concentrations during MS relapses observed in this study.

In this study, asparagine levels were noted to be higher during relapses. It has been observed that CSF asparagine is increased in MBP-EAE model (compared to non-immunised Lewis rats) as well as in the plasma and CSF of MS patients (compared to HC) (Barkhatova et al., 1998; Noga et al., 2012; Poddighe et al., 2017). Asparagine is a non-essential amino acid as it can be synthesised from metabolic pathways and is involved in the production of proteins and glycoproteins. Asparagine has been shown to be involved in the priming of mTOR complex 1 for activation and within the context of Salmonella infection, the deprivation of asparagine in media abolishes T cell activation, T cell blastogenesis and IL-2 secretion (Dyachok et al., 2016; Torres et al., 2016). This illustrates that asparagine may have a role in proper immune functioning, although the exact mechanisms in MS pathogenesis are not as yet clear.

No confounders for the relationship between the identified metabolites and relapses were found in this study. Steroids (commonly prescribed as oral methylprednisolone for 3 to 5 days in the UK) are given to patients in relapse after clinical assessment and there is a potential for steroids to induce metabolic changes, particularly with regards to glucose and lipid metabolism (Bordag et al., 2015; Pranger et al., 2018). However, only five of the 38 In R patients received steroids prior to blood sampling and none were on steroids during sampling. Of interest, targeted metabolomics (which measures predominantly lipid parameters and also glucose) showed that there is no difference in the metabolome of LR 1 – 6 M vs. LR ≥ 24 M patients (the mean predictive accuracy of the OPLS-DA models was $51.7 \pm 8.0\%$ and was not statistically different from random chance, $p = 0.482$), demonstrating that metabolic effects of short course, albeit high dose, steroids are unlikely to persist in the longer term.

As mentioned, all four metabolites, individually, performed better than serum NfL in distinguishing In R from LR ≥ 24 M patients, and the use of a composite metabolite marker appears to have higher discriminatory ability. This is particularly applicable for within-individual analysis, given that the direction of the four metabolites at an individual level may not always be concordant. Indeed, using serum NfL together with the four metabolites in combination gave the highest AUC (at group level) and this could be an attractive approach for future research. Future work will look to recruit more paired relapse-remission patients, and indeed remission-relapse patients to assess the predictive ability of the metabolites, with more frequent sampling (e.g. monthly) to increase the power of the multivariate models, be it with ROC analysis or a vector-based approach. To further validate the vector-based approach, serial samples from stable patients without further relapses are required to show that the repeat samples from these patients do not move along the vector representing high to low inflammation. The current study was unable to address this as the MET cohort was recruited in a pragmatic manner based on patients' clinical visits, hence stable patients would not have had repeat samples within 6 months. A control group consisting of patients with infections (e.g. urinary and chest infections) should be recruited in the future. This would determine if the signatures seen in relapses are generic for inflammation per se or MS-specific, and could help explore metabolic markers of pseudo-relapses. Future collaboration with industry partners will also help to establish the absolute concentrations of lysine and asparagine.

The pragmatic, observational nature of the MET cohort also meant that patients underwent MRI only when clinically indicated, which would have reduced the detection of subclinical MRI-defined inflammation up till the point of relapse, especially for In R patients. This could account for the fact that there was no association between the four metabolites with the presence of new T₂ lesion/s, while only lysine and asparagine were associated with the presence of new GAD-enhancing lesion/s. Also, further analysis (not shown in Results) with the addition of MRI parameters which would theoretically increase the phenotypic distinction between In R vs. LR ≥ 24 years patients did not

result in higher accuracies, i.e. the mean predictive accuracy of OPLS-DA models of CPMG data from In R patients with [presence of new GAD-enhancing lesion/s or new T₂ lesion/s (referenced to baseline scan done ≤1 year apart) in the next six months after blood sampling] (n = 13) vs. LR ≥2 years patients with [no new T₂ lesion/s (referenced to baseline scan done ≤1 year apart) in the last one year] (n = 19) was only 58.6 ± 7.8%. Future work will involve the use of scheduled, regular MRI examinations to establish patient groups stratified by subclinical MRI inflammation (with and without clinical relapses) to explore metabolic signatures underlying these events.

5.4.1 Chapter summary

Taking the study observations in totality, it is demonstrated that metabolic perturbations present in MS patients during relapses can be detected by serum global and targeted metabolomics, and interrogating the top metabolites identified four metabolites that could be potential biomarkers of clinical relapses, either singly or in combination. It is also not surprising that serum metabolites identified during acute neuroinflammation within the rat DTH model of MS differ from relapses in MS patients as the DTH lesion represents one single neuroinflammatory lesion while in MS, the metabolic signature is contributed by the cumulative lesion load, both active and quiescent lesions as well as existing neurodegenerative processes. The next chapter will explore the blood metabolomics signature of MS neurodegeneration, specifically in patients with SPMS, as compared to RRMS patients. This could enable the development of a metabolomics-based blood test to identify SPMS, which would be invaluable for disease management and recruitment into SPMS clinical trials.

Chapter 6: A metabolomics blood test to define the secondary progressive stage of MS – validation and clinical performance

6.1 Introduction

The clinical transition from RRMS to SPMS represents a diagnostic challenge as progression is typically gradual and objective clinical signs often lag behind patient's symptoms (Katz Sand et al., 2014). Furthermore, secondary progression often occurs in a non-uniform manner (Plantone et al., 2016), and is difficult to distinguish from clinical inflammatory activity, particularly when there is no accompanying evidence of MRI activity. As such, the diagnosis of SPMS is always established retrospectively when an irreversible accrual of disability has already occurred (Lublin et al., 2014).

There are currently no validated biomarkers that can reliably separate these two stages of MS (Lublin et al., 2014). As mentioned in the Introduction of this thesis, conventional MRI offer little insight into pathogenesis, and interval measurements of brain atrophy are relatively small and are subject to biological and technical variability. The Oxford group has previously shown, using serum ^1H NMR metabolomics, that RRMS and SPMS can be differentiated with high accuracy (AUC = 0.94, predictive accuracy = 87%) (Dickens et al., 2014). This holds huge potential as a diagnostic test to objectively distinguish these two phases of MS.

However, it is well-known that metabolic alterations within blood samples can occur due to variations in pre-analytical sample-handling, in particular, delayed centrifugation of blood (to obtain serum or plasma) and repeated freeze-thaw cycles (Anton et al., 2015; Bernini et al., 2011; Bervoets et al., 2015; Fliniaux et al., 2011; Jobard et al., 2016; Malm et al., 2016; Pinto et al., 2014; Teahan et al., 2006; Yin et al., 2013). ^1H NMR spectroscopy and mass spectrometry metabolomics studies have consistently shown distinctive perturbations in the serum/plasma metabolome, occurring as early as two hours between blood draw and centrifugation. Metabolites most susceptible to change as a

result of delayed centrifugation are energy related metabolites, especially glucose (low with delay), lactate (high) and pyruvate (low). This is likely due to ongoing anaerobic glycolysis by erythrocytes in the hypoxic environment of the blood collection tube, which is exacerbated if a delay occurs at room temperature (Bernini et al., 2011; Bervoets et al., 2015). Increased choline likely due to the breakdown of phospholipids, higher HDL resulting from the conversion of VLDL/LDL, higher hypoxanthine and higher SIP have also been observed (Pinto et al., 2014; Yin et al., 2013). Also, repeated freeze-thaw cycles are not uncommon, especially in biobanks whereby limited sample aliquots are available from each participant. Interestingly, it has been noted that the metabolic alteration resulting from repeated freeze-thaw cycles is less of an issue for mass spectrometry metabolomics as compared to ^1H NMR spectroscopy metabolomics (Anton et al., 2015; Bervoets et al., 2015; Pinto et al., 2014; Yin et al., 2013).

It is clear that stringent processing and storage protocols are necessary in the initial phase of metabolomics blood test development (Hernandes et al., 2017), which can be difficult to follow in clinical practice. While metabolic perturbations introduced by freeze-thaw and delayed centrifugation have been previously described, these have been performed solely in healthy individuals. There is a paucity of studies addressing how these factors may affect the accuracy of a diagnostic metabolomics test in a clinical environment. Indeed, the extent to which sample-handling variation affects overall diagnostic accuracy is likely to be disease and application specific, and dependent on the stability of the main discriminatory metabolites identified. Therefore, to ensure that the metabolic distinction of RRMS vs. SPMS is applicable in a clinical setting, it is vital that high diagnostic accuracy is maintained even in instances when the sample-handling protocol may vary.

6.1.1 Aims of Chapter 6

The aims of this chapter are:

1. To validate the RRMS vs. SPMS metabolomics diagnostic test on an independent, prospective, well-characterised set of RRMS and SPMS samples by using the *optimal* sample-handling protocol detailed in the Material and Methods.
2. To investigate the impact of two of the most common sources of variation in sample-handling identified in the clinics – freeze-thaw and delayed blood centrifugation, on the diagnostic performance of the test.
3. To identify the main discriminatory metabolites separating RRMS and SPMS, and to determine how sub-optimal sample-handling protocols affect their discriminatory ability.

As mentioned in the Methods, Section 2.3, all patients involved in this study were recruited under a new prospective cohort (i.e. MET cohort) established by the DPhil candidate. The candidate's individual contributions to this work include: (1) design and conceptualisation of the study, (2) recruitment of patients and blood sample collection, (3) acquisition of metabolomics and clinical data, (4) analysis of all data as well as their interpretation. The findings of this chapter have been accepted for publication in a journal, with the candidate as first author.

6.2 Methods

6.2.1 Patients

In this cross-sectional study, 31 RRMS patients and 28 SPMS patients were prospectively recruited from the Oxford University Hospitals (i.e. MET cohort). All patients fulfilled the 2017 revisions to the McDonald criteria for MS (Thompson et al., 2018). SPMS status was established clinically by MS neurologists; all SPMS patients demonstrated progressive accrual of disability over at least one year independent of relapses (Kremenchutzky et al., 2006), and had EDSS ≥ 4.5 at the time of confirmed disability progression (Kurtzke, 1983; Lorscheider et al., 2016). Clinical and demographic data were obtained from medical notes and patient interviews. Current EDSS was assessed on the day of recruitment, prior to blood sampling. ARR was calculated as number of relapses in the previous two

years divided by two, and only if the disease duration is at least two years. Detailed information on smoking, alcohol intake, time from last meal and type of food for last meal was collected at the time of recruitment.

6.2.2 Blood collection, serum processing and NMR sample preparation

The *optimised* protocol, as described in Material and Methods, was used for pre-analytical sample handling. This protocol is widely used in metabolomics literature and represents the ideal sample-handling condition (Bervoets et al., 2015; Yin et al., 2015). To reiterate in brief, blood was collected in BD™ Vacutainer™ serum tubes (BD 367837). Blood was left to stand for 30 minutes at room temperature, then centrifuged at 1,300 *g* for 10 minutes at room temperature for erythrocyte separation to obtain serum. Serum was then immediately aliquoted and stored at –80 °C until NMR sample preparation. For NMR sample preparation, serum was thawed at room temperature followed by ultra-centrifugation at 100,000 *g* for 30 minutes at 4 °C. The supernatant was then diluted with sodium phosphate buffer and stored at –80 °C until NMR analysis. Immediately before NMR analysis, the buffered NMR sample was thawed at room temperature and then transferred to a 5 mm borosilicate glass tube.

6.2.3 Variations to the optimised protocol

Three variations to the *optimised* protocol were introduced to resemble practical considerations encountered in the clinic and laboratory (**Figure 6.1**). The *freeze-thaw* protocol was identical to the *optimised* protocol, but with an additional freeze-thaw to simulate a scenario whereby the NMR sample has already been prepared, but the NMR spectrometer was unavailable due to logistical or technical issues. The *120 minutes* and *240 minutes* protocols differed from the *optimised* protocol with 120 minutes and 240 minutes of standing time respectively before erythrocyte separation. These two protocol variations parallel a very common scenario whereby blood has been taken but

not centrifuged in a timely manner due to manpower demands in a busy clinic or laboratory. Apart from the protocol variations of interest, all other processes were kept strictly the same.

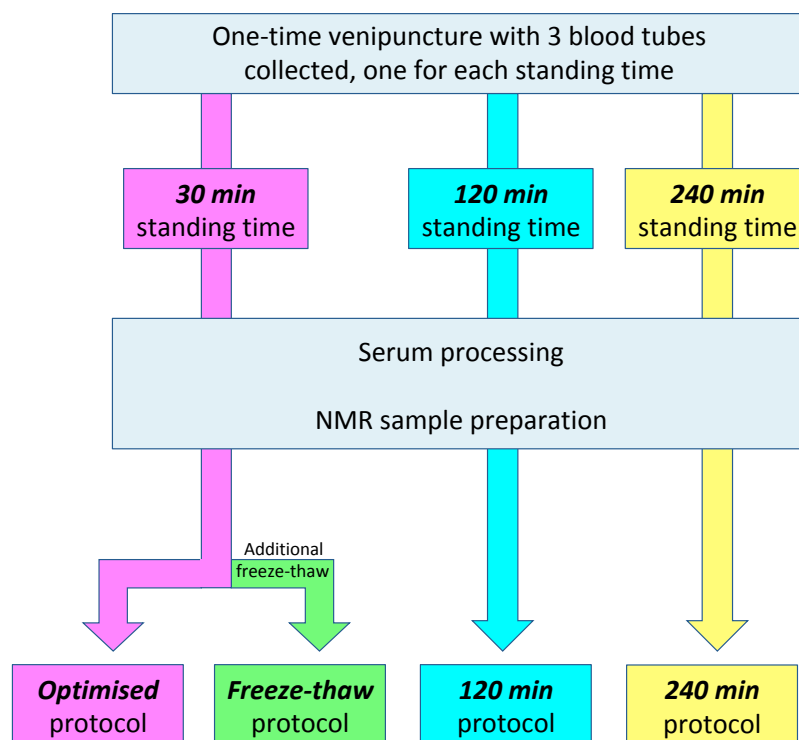


Figure 6.1: Flow diagram illustrating the sample-handling protocols investigated. The effect of freeze-thaw and increased standing time were investigated, and compared to the ideal, optimised (30 minutes) protocol.

6.2.4 ^1H NMR metabolomics CPMG spectra acquisition

All ^1H NMR experiments for global metabolomics were performed in-house at the Department of Chemistry, University of Oxford using a 700-MHz Bruker AVIII spectrometer, with the CPMG relaxation editing pulse sequence for spectra acquisition. Technical specifications of the NMR experiments and details of data handling are as described in the Material and Methods. To reduce the possibility of ‘batch effect’ bias, samples from the four protocols and the different classes (RRMS and SPMS), along with quality control samples (pooled HC serum) were mixed throughout the run. ‘Bins’ which contained no spectral resonances on visual inspection (i.e. noise) were excluded, as

were 'bins' with a coefficient of variation exceeding 15% (calculated across the quality control samples). In all, 185 metabolite 'bins' were available for supervised multivariate statistical analysis.

6.2.5 Univariate statistical analysis

All statistical analysis, apart from spectral analysis (see below), were performed with STATA software (release 14, Texas, USA) and GraphPad Prism (version 6, California, USA). Comparative analyses between RRMS and SPMS patients were performed using Mann-Whitney U test or two-sample t-test as appropriate for continuous variables, and with Chi-squared test for categorical variables. Pearson's or Spearman's correlation was used to explore correlations depending on data normality. Repeated-measures two-way ANOVA was used to explore potential interactions and Sidak's test was applied for multiple comparisons. Two-tailed p values of <0.05 were considered statistically significant and data was presented as mean $\pm$ SD unless stated otherwise.

6.2.6 Multivariate statistical analysis

OPLS-DA was used to interrogate the CPMG spectral metabolomics data set to identify and validate the metabolic differences between RRMS and SPMS patients. Details of the OPLS-DA approach are as described in the Materials and Methods. For this analysis, both the training sets and test sets (for external cross-validation) were derived from *optimised* samples.

To determine how well the above diagnostic model developed performs on samples that have experienced freeze-thaw or increased standing time, the same external ten-fold cross-validation strategy was employed with random subsets of the (1) *freeze-thaw*, (2) *120 minutes*, and (3) *240 minutes* samples selected as *test* sets, with samples from the *optimised* protocol being used as the training set for model building. This replicates a scenario in which a diagnostic model developed on samples collected in a research setting is applied to samples collected in a clinical setting. No sample

from the same patient existed in both the training and test sets simultaneously, i.e. the test set was always independent of the training set.

To explore whether samples collected with sub-optimal protocols can be used in a research setting to develop multivariate models and accurately identify metabolite biomarkers by using the OPLS-DA strategy described above, only samples from the *freeze-thaw*, *120 minutes*, and *240 minutes* protocols (i.e. in both training and test sets) were used. Finally, we investigated whether samples stored long-term (up to ten years) can be used to identify metabolite biomarkers by applying the same analytical strategy to a cohort of RRMS and SPMS patients from the Welsh Neuroscience Research Tissue Bank, Cardiff University (REC reference: 19/WA/0058). Direct comparison of the diagnostic accuracies and discriminatory metabolites identified by these models against the model developed using the *optimised* protocol provides valuable insight into the advantages and pitfalls of sample-handling variations for metabolomics analysis and biomarker identification.

6.3 Results

6.3.1 Clinical characteristics of study population

Thirty-one RRMS patients and 28 SPMS patients were recruited into the study. As expected, SPMS patients were older, had longer disease duration, lower ARR in the previous two years, higher EDSS, and none were on DMT (**Table 6.1**). No differences in gender were observed, with a preponderance of females in both groups.

Table 6.1: Clinical and demographic characteristics of the study cohort. *ARR calculated only if disease duration is at least two years.

	RRMS (n = 31)	SPMS (n = 28)	p value
Age (years,) mean $\pm$ SD	43.5 $\pm$ 9.7	58.1 $\pm$ 9.6	<0.001
Female, no. (%)	23 (74.2)	20 (71.4)	0.811
Disease duration (years), median (range)	9.2 (0.8 – 24.2)	26.6 (3.9 – 50.3)	<0.001
*ARR last two years, median	0.0 (0.0 – 1.5)	0.0 (0.0 – 0.0)	<0.001

(range)			
EDSS, median (range)	2.5 (1.0 – 6.5)	7.0 (4.5 – 8.5)	<0.001
DMT use, no. (%)	20 (64.5)	0 (0.0)	<0.001
Glatiramer acetate	8 (25.8)	-	-
Interferon beta	2 (6.5)	-	-
Dimethyl fumarate	7 (22.6)	-	-
Fingolimod	1 (3.2)	-	-
Natalizumab	1 (3.2)	-	-
Alemtuzumab	1 (3.2)	-	-
BMI, mean $\pm$ SD	25.9 $\pm$ 4.6	28.0 $\pm$ 5.9	0.132

6.3.2 OPLS-DA modelling using samples from the optimised protocol (training set: optimised protocol, test set: optimised protocol)

In order to validate the results from the previous study using a new independent cohort, samples collected using the *optimised* protocol were used to construct discriminatory OPLS-DA models to segment RRMS from SPMS (i.e. both training and test data from the *optimised* protocol). The representative OPLS-DA scores plot showed excellent separation between RRMS and SPMS patients (**Figure 6.2 A**). The mean predictive accuracy for the ensemble of the OPLS-DA models of RRMS vs. SPMS was significantly higher than the mean predictive accuracy of the ensemble created by random class assignments (mean $\pm$ SD, 91.0 $\pm$ 3.0% vs. 47.8 $\pm$ 9.5%; p <0.001) (**Figure 6.2 B**). Sensitivity and specificity indices can be found in **Table 6.2**.

In summary, using *optimised* samples resulted in well-validated OPLS-DA models with an excellent mean predictive accuracy in distinguishing RRMS and SPMS, validating the previous study by the Oxford group.

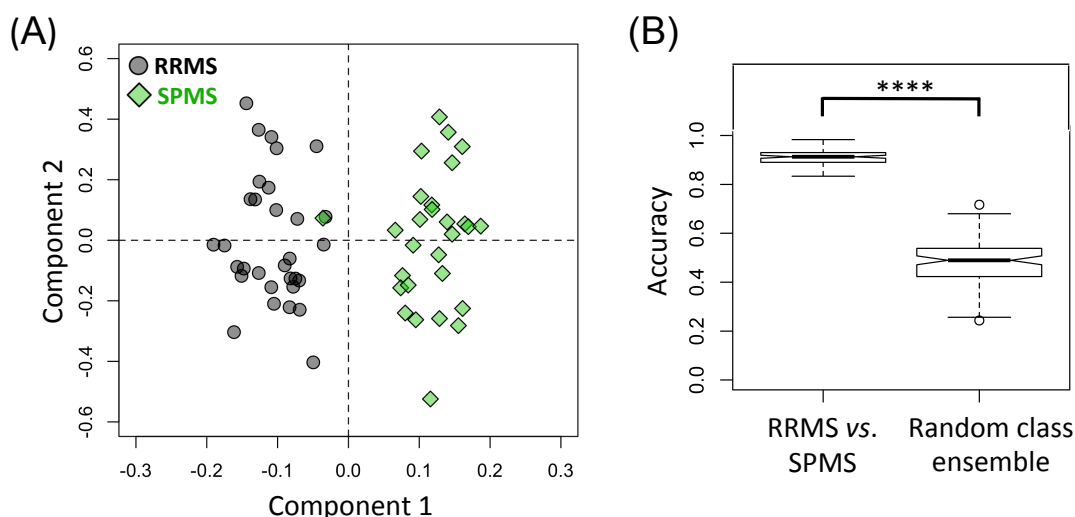


Figure 6.2: Performance of the OPLS-DA models generated using samples from the optimised sample-handling protocol. (A) The representative OPLS-DA scores plot of this well-validated model illustrates a distinct separation between RRMS and SPMS patients, (B) with a high predictive accuracy of $91.0 \pm 3.0\%$. Each point in the scores plot represents all metabolomics spectral data from one patient condensed into a single data point. Points (i.e. patients) located closer to one another on the scores plot are metabolically more similar than those further apart. RRMS = black circles, SPMS = green diamonds. **** indicates $p < 0.0001$ by Kolmogorov-Smirnov test.

Table 6.2: Summary indices of OPLS-DA models using samples collected within and across the different sample-handling protocols.

	<u>Train: Optimised protocol</u> <u>Test: Optimised protocol</u>	<u>Train: Freeze-thaw protocol</u> <u>Test: Freeze-thaw protocol</u>	<u>Train: 120 minutes protocol</u> <u>Test: 120 minutes protocol</u>	<u>Train: 240 minutes protocol</u> <u>Test: 240 minutes protocol</u>
Accuracy $\pm$ SD (%)	91.0 ± 3.0	89.6 ± 3.3	81.7 ± 3.5	84.9 ± 3.2
Sensitivity $\pm$ SD (%)	92.3 ± 4.4	87.2 ± 4.5	83.8 ± 5.4	88.5 ± 4.6
Specificity $\pm$ SD (%)	91.6 ± 3.7	94.1 ± 4.0	83.4 ± 4.0	84.1 ± 4.5
	<u>Train: Optimised protocol</u> <u>Test: Freeze-thaw protocol</u>	<u>Train: Optimised protocol</u> <u>Test: 120 minutes protocol</u>	<u>Train: Optimised protocol</u> <u>Test: 240 minutes protocol</u>	–
Accuracy $\pm$ SD (%)	85.5 ± 3.8	85.9 ± 3.1	88.0 ± 3.0	–
Sensitivity $\pm$ SD (%)	91.1 ± 4.1	78.5 ± 5.9	83.4 ± 5.4	–
Specificity $\pm$ SD (%)	80.6 ± 6.6	94.0 ± 4.1	93.0 ± 5.3	–
	<u>Combination 1</u> <u>Train: Optimised + Freeze-thaw protocol</u> <u>Test: Optimised + Freeze-thaw protocol</u>	<u>Combination 2</u> <u>Train: Optimised + 120 minutes protocol</u> <u>Test: Optimised + 120 minutes protocol</u>	<u>Combination 3</u> <u>Train: Optimised + 240 minutes protocol</u> <u>Test: Optimised + 240 minutes protocol</u>	–
Accuracy $\pm$ SD (%)	87.7 ± 3.9	86.2 ± 4.1	87.1 ± 5.0	–
Sensitivity $\pm$ SD (%)	87.9 ± 5.9	86.0 ± 5.1	86.3 ± 6.1	–
Specificity $\pm$ SD (%)	88.3 ± 6.0	86.7 ± 6.9	88.6 ± 7.4	–

6.3.2.1 Identifying discriminatory metabolites from the well-validated OPLS-DA model

To identify the most discriminatory metabolites driving the distinction between RRMS and SPMS, VIP scores were generated. Seven metabolites were identified as highly discriminatory; the VIP cut-off of

1.48 was obtained by identifying the inflexion point in the VIP ranking plot (**Figure 6.3**). Details of these seven discriminatory metabolites are shown in **Table 6.3**. In summary, lipoproteins, choline and β -hydroxybutyrate concentrations are lower in SPMS compared to RRMS, while glucose and N-acetylated glycoproteins/glycolipids are higher. Of note, these discriminatory metabolites were consistent with those found in the previous cohort (Dickens et al., 2014). Indeed, the direction of change of the metabolites in SPMS with respect to RRMS were also similar, with the exception of the mobile $-N(CH_3)_3$ /free choline signal. This peak was previously reported as phosphocholine (chemical shift 3.23 ppm). Overall, these observations provide strong evidence that the identified metabolites are biomarkers of MS disease stage.

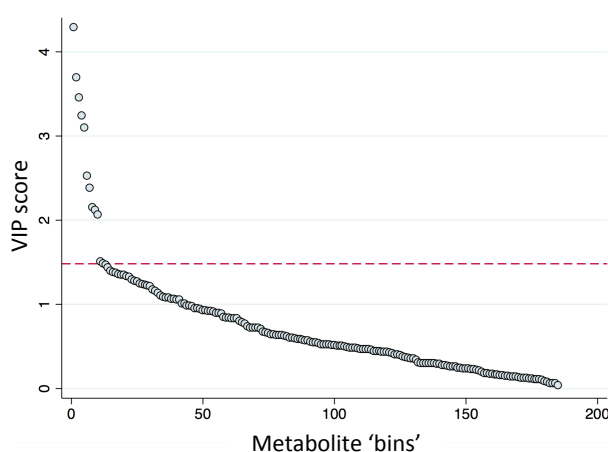


Figure 6.3: VIP ranking plot identifies the top discriminatory metabolites from the optimised protocol. The dashed line indicates the VIP score cut-off at 1.48.

Table 6.3: The seven identified discriminatory metabolites from the well-validated OPLS-DA model.

Discriminatory metabolites	Chemical shift of contributing spectral 'bins' (VIP score, VIP rank)	Fold change in SPMS relative to RRMS
Mobile $-CH_3$ HDL/LDL	0.80....0.82 ppm (2.15, 8) 0.82....0.84 ppm (3.10, 5) 0.84....0.86 ppm (4.29, 1) 0.86....0.88 ppm (2.52, 6)	0.83
β -hydroxybutyrate	1.20....1.22 ppm (2.07, 10)	0.84
Mobile $(-CH_2-)_n$ LDL	1.22....1.24 ppm (3.24, 4)	0.84

	1.24....1.26 ppm (3.46, 3)	
Mobile (-CH ₂ -) _n Chylomicron/VLDL	1.26....1.28 ppm (2.12, 9)	0.92
NAC1/=CH-CH ₂ -CH ₂ -	2.04....2.06 ppm (1.48, 12)	1.07
Mobile -N(CH ₃) ₃ /free choline	3.20....3.22 ppm (2.38, 7) 3.22....3.24 ppm (3.69, 2)	0.86
Glucose	3.88....3.90 ppm (1.50, 11)	1.07

6.3.2.2 Addressing potential confounders for the identified metabolite biomarkers

As illustrated in **Table 6.1**, several baseline characteristics were different between RRMS and SPMS patients. Two approaches were used to explore if these differences could have accounted for their metabolic separation. Firstly, there were no strong correlations (i.e. all $R^2 < 0.3$) of any of the discriminatory metabolites with age, disease duration, number of relapses in the last two years, BMI, time from last meal, or number of units of alcohol consumed per week, and there were no significant associations between any of the metabolites with comorbidity status. In particular, none of the top seven metabolites had significant correlations with EDSS, indeed, the highest correlation coefficient was 0.34 ($p = 0.066$) for mobile (-CH₂-)_n Chylomicron/VLDL (bin 1.26....1.28 ppm) with EDSS. Within RRMS patients, there were no differences in any of the metabolite levels stratified by DMT status. Secondly, investigation of the OPLS-DA scores plot revealed no clustering of the potential confounders (expressed as binary variables) confirming that there was no confounding effect (**Figure 6.4**). Importantly, RRMS patients who were ≥ 50 years old or had EDSS ≥ 6.0 did not cluster near to the SPMS patients, and RRMS patients on DMT were spread throughout the RRMS cluster including those nearest to the SPMS cluster, suggesting that these factors were not confounding the separation of RRMS and SPMS patients. Patients with BMI ≥ 30 , consumed ≥ 1 units of alcohol per week, and were fed or fasted were also evenly distributed across both groups. Smoking was not explored as a confounder as there were only three current smokers in the entire cohort.

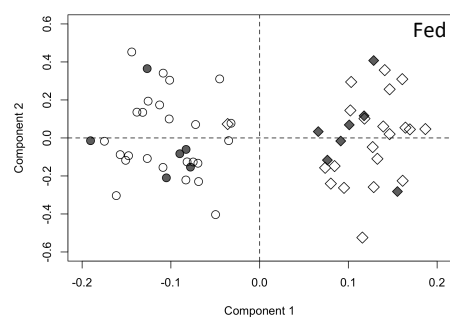
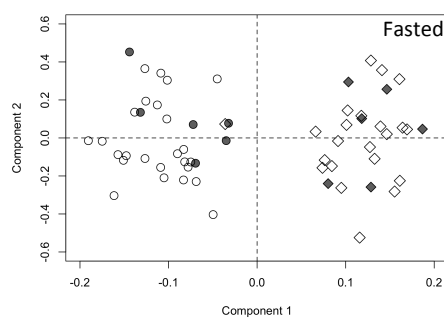
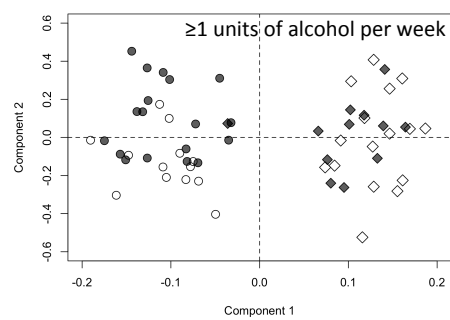
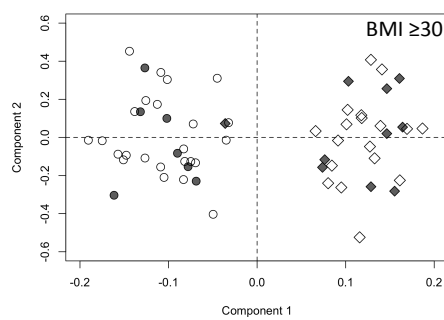
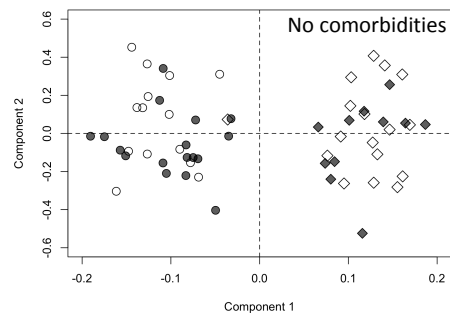
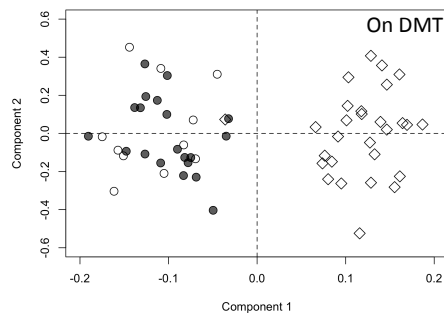
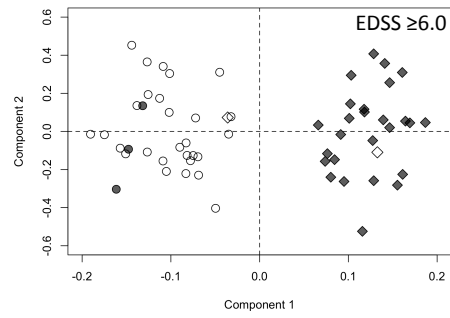
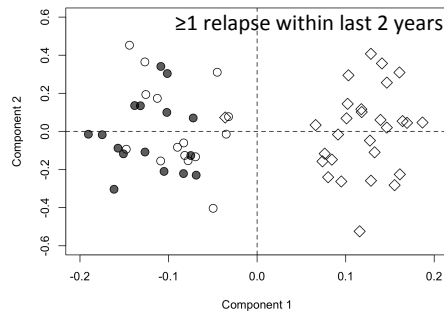
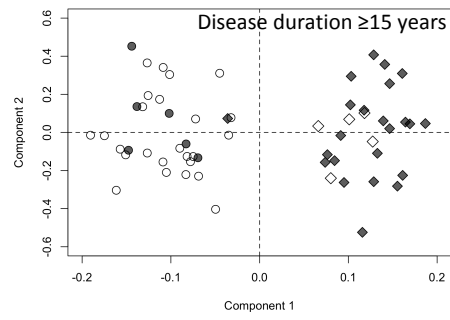
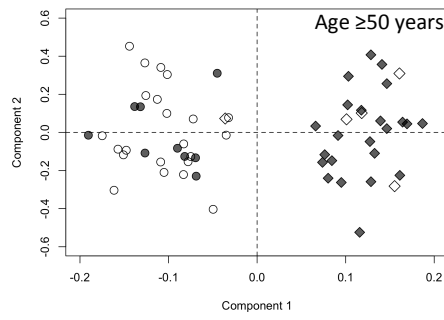


Figure 6.4: Scores plots of the well-validated RRMS (circle) and SPMS (diamond) OPLS-DA model shaded according to potential confounding factors. In each instance, the potential confounder of interest (age, disease duration, presence of relapses, disability status, DMT use, comorbidity status, BMI, alcohol intake, and prandial status) is evenly distributed throughout each disease classification. RRMS = circles, SPMS = diamonds. Fasted is defined as ≥ 6 hours since last meal, while fed is defined as < 2 hours since last meal.

6.3.3 OPLS-DA modelling to evaluate the diagnostic performance of the well-validated OPLS-DA model on sub-optimal samples (training set: *optimised* protocol, test set: *freeze-thaw/120 minutes/240 minutes* protocols)

Next, to explore if the high predictive accuracy of the well-validated model (**Figure 6.2**) is maintained when the handling of samples is sub-optimal, i.e. increasing standing time, or an additional freeze-thaw, samples from the *optimal* protocol was used as the training set for model building while samples from sub-optimal protocols were utilised as the test set to obtain the predictive accuracy. This is akin to developing a metabolomics blood test using stringent/optimal samples (in a research setting) and then applying the test in a clinical setting. The well-validated OPLS-DA models were able to predict the diagnosis of samples from the *freeze-thaw*, *120 minutes*, and *240 minutes* protocols with mean predictive accuracies of $85.5 \pm 3.8\%$, $85.9 \pm 3.1\%$, and $88.0 \pm 3.0\%$ respectively (**Figure 6.5**). This translates to a relative accuracy reduction of 6.0%, 5.6% and 3.3% respectively, when compared to using *optimised* protocol samples as the *test* set. Sensitivity and specificity indices can be found in **Table 6.2**. Of note, samples left standing for *120 minutes* and *240 minutes* after venepuncture maintained very high specificities for SPMS (94.0% and 93.0% respectively), allowing SPMS to be 'ruled in'.

In summary, the well-validated OPLS-DA model is resistant to variations in sample-handling and is still able to stage MS accurately.

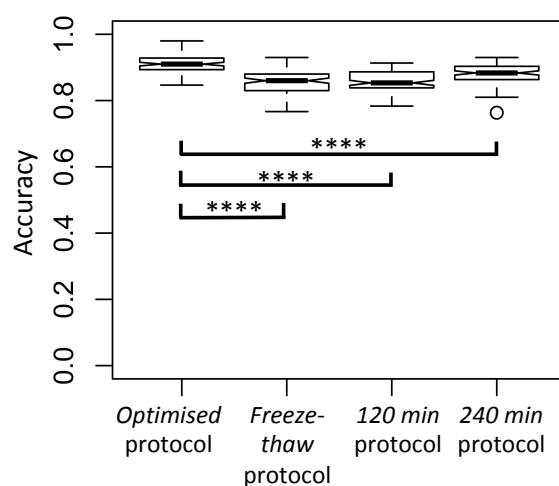


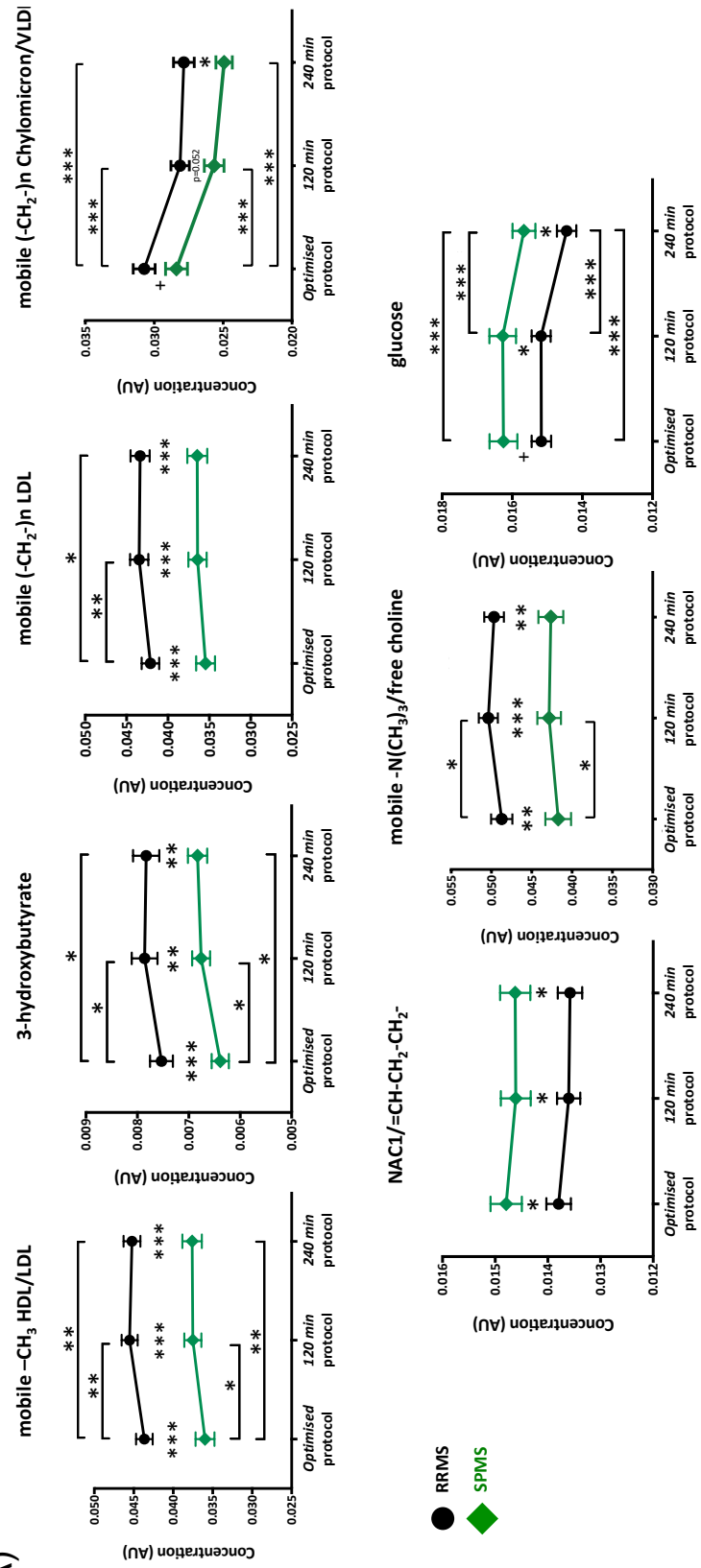
Figure 6.5: Box plots illustrating the diagnostic accuracy of the well-validated discriminatory RRMS vs. SPMS OPLS-DA model on sub-optimal samples, which have undergone freeze-thaw or increased standing time of 120 minutes and 240 minutes. **** indicates $p < 0.0001$ by Tukey's post-hoc test, after one-way ANOVA.

6.3.4 Exploring the effects of freeze-thaw and delayed centrifugation on the seven discriminatory metabolites

Next, to explore if the seven discriminatory metabolites (**Table 6.3**) identified from the well-validated model remained discriminatory with sub-optimal protocols, repeated-measures two-way ANOVA (the two factors being MS subtype and protocol variation) was performed. All metabolites remained discriminatory with variations in standing time (**Figure 6.6 A**), while most were still able to distinguish RRMS vs. SPMS despite an additional freeze-thaw (**Figure 6.6 B**). There was no interaction between MS subtype with any of the protocol variations within all the metabolite biomarkers indicating that the differences in metabolite levels between RRMS and SPMS are similar despite sample-handling variations, and analogous metabolic perturbations occur in RRMS and SPMS serum.

In summary, while serum metabolite concentrations vary as a result of sampling-handling, the identified metabolite biomarkers remain discriminatory for RRMS and SPMS.

(A)



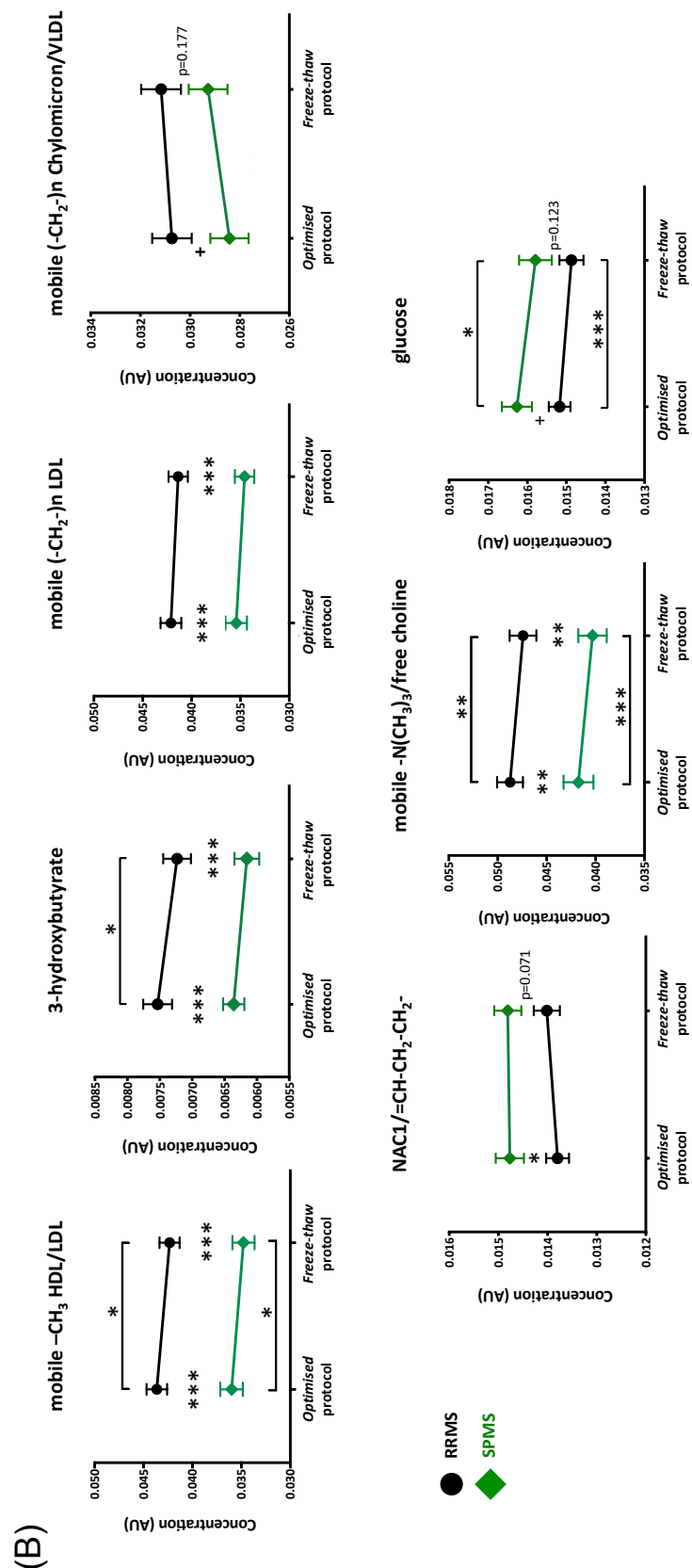


Figure 6.6: The effect of (A) increasing standing time, and (B) freeze-thaw on the identified serum metabolite biomarkers distinguishing RRMS (black circle) and SPMS (green diamond). *indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ on Sidak's post-hoc test, after repeated measures two-way ANOVA. *Statistically significant from multivariate OPLS-DA. Error bars represent SEM.

6.3.5 OPLS-DA modelling using samples from the sub-optimal protocols (training set: *freeze-thaw/120 minutes/240 minutes* protocols, test set: *freeze-thaw/120 minutes/240 minutes* protocols)

Next, to explore if samples from the *optimised* protocol are strictly required for metabolomics test development, or if samples from sub-optimal protocols could be utilised for this purpose, OPLS-DA models were developed using samples from sub-optimal protocols (i.e. *freeze-thaw/120 minutes/240 minutes*) as both the training and test sets. OPLS-DA modelling using samples within the *freeze-thaw*, *120 minutes* and *240 minutes* protocols resulted in mean predictive accuracies of $89.6 \pm 3.3\%$, $81.7 \pm 3.5\%$ and $84.9 \pm 3.2\%$ respectively (**Figure 6.7 A**). There was no advantage to using a combination of samples from the *optimised* protocol and sub-optimal protocols (*optimised/freeze-thaw*, *optimised/120 minutes*, *optimised/240 minutes*) for test development (**Figure 6.7 B**). Sensitivity and specificity indices can be found in **Table 6.2**.

In summary, using samples collected under sub-optimal protocols for model development reduces predictive accuracies.

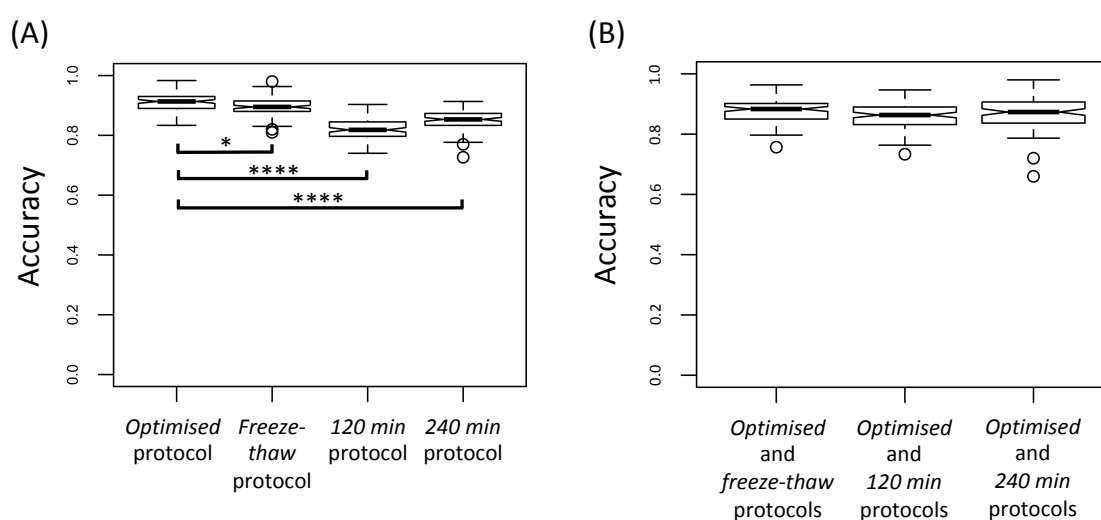


Figure 6.7: (A) Box plots of OPLS-DA model predictive accuracies using samples within each protocol as both the training and test sets. (B) Box plots of OPLS-DA model predictive accuracies using combinations of samples from the optimised protocol and from sub-optimal protocols as both the training and test sets. *indicates $p < 0.05$, **** $p < 0.0001$ on Tukey's post-hoc test, after one-way ANOVA.

As previous studies have reported significant alterations in serum metabolite concentrations as a result of long-term (>5 years) storage at -80 °C (Haid et al., 2018; Yang et al., 2013), it was investigated if biobanked samples could be used for model development. OPLS-DA models built using samples from the Welsh Neuroscience Research Tissue Bank stored for between one and ten years resulted in a predictive accuracy of $58 \pm 5.6\%$, which is significantly better than random chance, confirming that biobanked samples could be useful in a research setting. However, it should be noted that the predictive accuracy of the models developed using the biobanked samples is significantly lower than that obtained using samples collected under optimal conditions. This is a result of significant alterations in metabolite concentrations in samples stored for >5 years (**Figure 6.8**), highlighting the importance of using strict protocols for clinical test development and validation. In contrast, the *well-validated* OPLS-DA model was still able to diagnose (RRMS vs. SPMS) biobanked samples up to five years old with an accuracy of 66%.

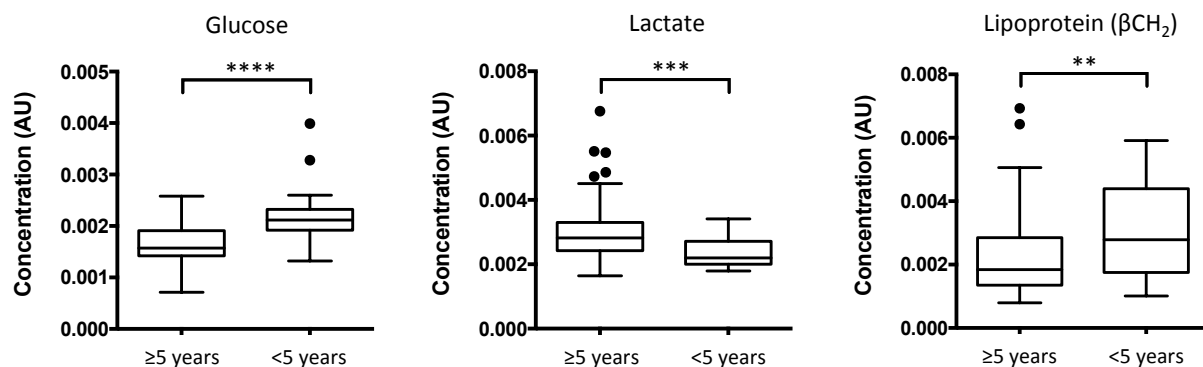


Figure 6.8: Box plots showing that long-term (>5 years) storage at -80 °C results in significant changes to glucose, lactate and lipoprotein concentrations. ****indicates $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ on two sample t-test.

6.3.6 Exploring the discriminatory ability of serum NfL in distinguishing RRMS and SPMS

As mentioned in the Introduction of this thesis, there is emerging evidence for NfL to distinguish between RRMS and progressive MS, thus it was explored if serum NfL could be useful in this cohort. Serum NfL levels were statistically higher in SPMS patients than in RRMS patients (mean $\pm$ SD, $16.8 \pm$

7.8 pg/mL vs. 10.5 ± 8.5 pg/mL; $p = 0.005$) (**Figure 6.9 A**), consistent with the current literature (Disanto et al., 2017). On ROC analysis, the AUC was 0.802 (95% CI 0.685 – 0.920; $p < 0.0001$) (**Figure 6.9 B**). Using the Liu method which defines the cut off point by maximising the product of sensitivity and specificity (Liu, 2012), the optimal cut off level was calculated to be at 10.8 pg/mL, resulting in an accuracy of 78.0%. There was only one RRMS patient who presented within one month of a relapse (i.e. 28 days after symptom onset) and this sample corresponded to the highest serum NfL level (44.1 pg/mL) in the entire cohort.

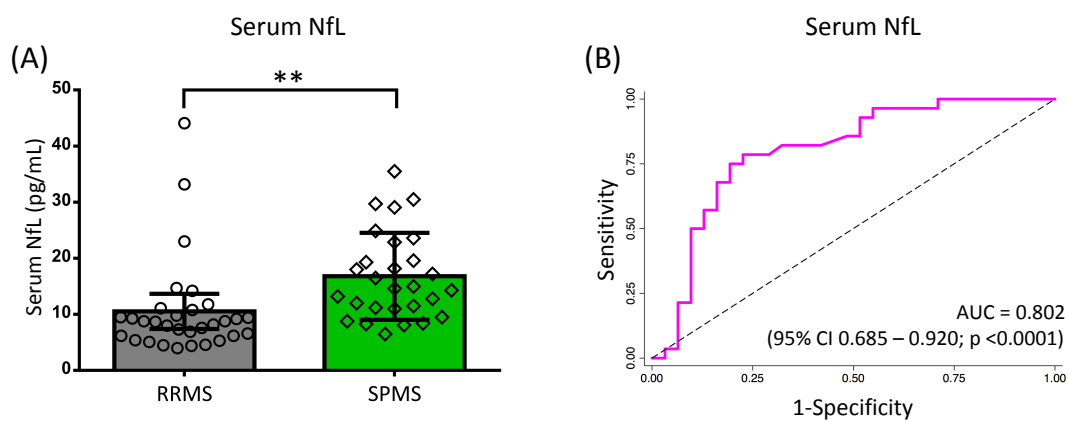


Figure 6.9: Serum NfL concentration in the study cohort. (A) SPMS patients had higher serum NfL levels compared to RRMS patients. Data presented as mean and error bars represent SD. **indicates $p < 0.01$ on two sample t-test. (B) ROC analysis shows an AUC of 0.802 in discriminating RRMS and SPMS.

6.4 Discussion

In this chapter, using a prospective cohort of RRMS and SPMS patients, OPLS-DA modelling of serum metabolomics data was able to stage MS with 91% accuracy and the metabolite biomarkers identified agree with those in the previous work by the Oxford group. Commonly encountered sample-handling variations was also introduced to ‘stress-test’ the current metabolomics algorithm and observed only a modest decrease in accuracy. Therefore, this study supports the clinical applicability of the *well-validated* metabolomics test, by (1) further validating the results on an independent cohort, (2) confirming that the identified metabolite biomarkers remain discriminatory

despite variations in sample-handling, and, thus, demonstrating that (3) samples collected prospectively from a 'real-world' setting are accurately diagnosed by the mathematical models.

The transition from RRMS to SPMS is phenotypically gradual and clinicians often diagnose SPMS much later (the mean period of diagnostic uncertainty was 2.9 years in one study) when there is clear sustained accumulation of disability (Katz Sand et al., 2014). Therefore, the diagnosis of SPMS is retrospective and further aggravated by a lack of consensus on clinical definitions (Lorscheider et al., 2016). As the paradigm of MS treatment shifts towards halting progression instead of merely reducing relapses, there is an urgent need for molecular biomarkers to objectively confirm SPMS and to monitor disease progression. The current metabolomics approach has the potential to identify the onset of progression for clinical management purposes, as well as provide robust inclusion criteria for entry into SPMS clinical trials. Moreover, the use of serum allows serial samples to be taken for prospective monitoring of both the onset and rate of progression. Although it is recommended that blood samples are processed within 30 minutes of collection, immediately aliquoted, and stored at -80 °C until analysis for model development and validation, accurate results are still obtained using samples collected in a clinical setting. Furthermore, samples stored for >5 years can still provide significant and useful results for biomarker discovery in a research setting, although care should be taken as serum metabolite concentrations may change.

While the principal advantage of the OPLS-DA multivariate analysis is to identify disease-specific metabolic signatures (i.e. combination of all metabolites), such modelling also allows specific discriminatory metabolites distinguishing RRMS and SPMS to be discerned; higher lipoproteins, choline, β -hydroxybutyrate in RRMS, and higher glucose and N-acetylated glycoproteins/glycolipids in SPMS. These are remarkably consistent (in terms of their importance and direction) across the current and previous cohorts, and may provide further clues to disentangle the neuroinflammatory and neurodegenerative processes that are present in the two MS disease phases, though to varying degrees. Indeed, recent ^1H NMR lipidomics studies have reported on the differences in lipoprotein

subclasses between RRMS, SPMS and age-gender matched healthy controls, and also demonstrated reduction in anti-inflammatory capability of HDL in RRMS patients (Gafson et al., 2018; Jorissen et al., 2017). Our findings of higher β -hydroxybutyrate and lower glucose in RRMS patients also parallel the observations of another ^1H NMR metabolomics study comparing MS patients (84% of whom were RRMS) and healthy controls (Cocco et al., 2016). The higher levels of β -hydroxybutyrate observed in RRMS relative to SPMS could be due to greater energy deficiency (from increased energy demands) in RRMS patients as a result of more intense neuroinflammation. The lower glucose level seen in RRMS patients is consistent with this hypothesis and it is now recognised that astrocytes can perform gluconeogenesis when glucose levels are low. Therefore, these observations suggest an energy deficient state in which fatty acids are converted to ketone bodies via β -oxidation by astrocytes (Barber et al., 2019), and the ketone bodies are transported to neurons to be used as energy substrates. This hypothesis is supported by clinical studies in which higher β -hydroxybutyrate concentration was observed in MS patients with GAD-enhancing lesions compared to those without (Lutz et al., 2007).

Taking these findings in totality, these metabolites could provide inference to the pro-inflammatory state within RRMS. While there have been no studies reporting differences in blood choline levels between RRMS and progressive MS patients, ^1H NMR studies have found increased choline (in both CSF and blood) in RRMS with respect to HC as well as to non-MS controls (Cocco et al., 2016; Reinke et al., 2014). The finding of higher serum choline in RRMS in this study may suggest higher myelin turnover (increased myelin breakdown and/or failure to synthesise myelin), as demonstrated by brain MRS studies showing higher choline levels in RRMS patients compared to controls (Inglese et al., 2003; Kirov et al., 2009; Tartaglia et al., 2002). It is likely that the identified metabolites (and indeed the metabolomics profile) change gradually from RRMS to SPMS, rather than an abrupt increase or decrease in concentrations. This is because the metabolites identified are likely to reflect the underlying pathogenic/pathological substrate, which evolves gradually from focal intense neuroinflammation as seen in RRMS, to more diffuse and cortical/leptomeningeal inflammation

observed in SPMS. Serial samples from patients transitioning from RRMS to SPMS coupled with detailed clinical follow-up will be crucial to answer this question – to determine their metabolites levels and where their metabolomic profile lie in the OPLS-DA scores plot.

While the remarkable accuracy achieved in distinguishing RRMS from SPMS by metabolomics in this study could be, in part, due to recruiting patient groups that are phenotypically more distinct along the continuum of MS disease course, it is in this instance necessary as patients with unequivocal SPMS status (and indeed RRMS) were required to allow for the construction of discriminatory models, and to use these to explore the accuracy changes arising from protocol variations. The ‘ground truth’ used to diagnose RRMS and SPMS was clinical judgement by MS neurologists, guided by established clinical recommendations, i.e. all SPMS patients demonstrated progressive accrual of disability over at least one year independent of relapses, and had EDSS ≥ 4.5 at the time of confirmed disability progression (refer to Section 6.2.1). There were indeed RRMS patients with EDSS ≥ 4.5 (it was important that these patients were included to explore the confounding effect of EDSS), but these were relapse-associated disability worsening rather than slow insidious progression as seen in SPMS. None of the SPMS patients had a relapse in the last two years prior to recruitment or on DMT. The higher disease duration, older age, and higher EDSS in SPMS patients in the study add further support that this clinically based decision-making process for patient recruitment was appropriate. Due to inherent baseline differences in the two groups of patients, as would be expected as a natural corollary of the MS disease course, it was explored if these factors were potential confounders. There were no confounders accounting for the metabolic differences between RRMS and SPMS patients despite extensive analyses, demonstrating that the metabolite biomarkers identified are MS stage specific. Of note, the diagnostic accuracy of serum metabolomics outperform that of serum NfL in this well-characterised cohort of patients, highlighting that a biomarker signature could be more advantageous compared to a single candidate biomarker, although this needs to be validated in a larger cohort of patients.

6.4.1 Chapter summary

There is an unmet need for biofluid markers to differentiate these two phases of MS. The current study showed that RRMS and SPMS can be distinguished with high accuracy using serum metabolomics, and the accuracy of the RRMS vs. SPMS test remained robust to variations in sample-handling encountered in the clinics, therefore demonstrating its clinical applicability. Future work will determine how the *well-validated* model performs on a large prospective cohort of patients that includes patients suspected to be transitioning to SPMS. Serial samples from these ‘transitional’ patients coupled with detailed clinical follow-up will determine the diagnostic accuracy of the test in a clinical setting, and these samples could also be used to model progression to allow predictive models to be constructed.

Chapter 7: General discussion

7.1 Principal aims and findings of this thesis

1. This thesis sought to discover whether metabolomics could be used to differentiate MS patients from those with antibody-positive NMOSD, and explored if the identified metabolite biomarkers could aid in classifying antibody-negative individuals with overlapping MS and NMOSD phenotypes. It was possible to distinguish MS from antibody-positive NMOSD with high accuracy and the discriminatory metabolites, myoinositol and formate, were able to help validate the phenotypic classifications within antibody-negative patients with pathogenic relevance.
2. This thesis explored the use of CSF and serum metabolomics in the focal DTH model of MS to determine the extent of metabolic perturbation at different experimental time points representing the active inflammatory, chronic active and chronic inactive phases. It was possible to distinguish diseased and controls at each time point, in both serum and CSF, with the greatest metabolic distinction at the earliest time point when neuroinflammation was most intense, rather than when axonal loss or demyelination were most pronounced. The principal discriminatory serum and CSF metabolites were specific for the presence of the DTH lesion and correlated with histological markers of neuroinflammation, in particular, serum allantoin and CSF glutamine with reactive astrogliosis.
3. Drawing from the observations in rodents, this thesis aimed to investigate if serum metabolomics could be used to detect clinically-defined neuroinflammation, i.e. in patients who are in relapse, and if so, to identify metabolite biomarkers that could be clinically useful. It was possible to metabolically differentiate patients in relapses from 'inactive' patients, and this metabolic perturbation appeared to last up to six months. The candidate metabolite biomarkers (lysine, asparagine, isoleucine, leucine) showed a clear trend at group

level, either increasing or decreasing, with time away from a relapse, and it was demonstrated that these biomarkers were applicable in an individualised and responsive manner.

4. In addition to the application of metabolomics in MS diagnosis and disease activity, this thesis also sought to validate the metabolic separation between RRMS and SPMS, and to determine if this separation is maintained in a clinical environment under which variations in sample-handling may be introduced. It was possible to distinguish RRMS and SPMS with high predictive accuracy on an independent, prospective patient cohort, validating the previous work by the Oxford group. In addition, the introduction of freeze-thaw and delayed blood centrifugation resulted in only a small reduction in accuracies when these sub-optimal samples were tested upon the validated models, as most of the key metabolites remained discriminatory in spite of sample-handling variations.

7.2 Interpretation of findings within the context of current knowledge

This thesis demonstrates that comprehensive metabolic profiling in combination with pattern recognition multivariate statistical analysis is a biologically relevant and practicable approach to identify disease and disease-state specific metabolic signatures and metabolite biomarkers. The findings arising from this thesis and their potential utility will now be discussed in the context of current MS literature.

7.2.1 Diagnosis

The McDonald criteria is as much as prognostic criteria as it is a diagnostic criteria. It was developed to identify individuals with a first MS-like clinical attack who are at increased risk of developing MS. Subsequent modifications to the criteria have served to improve its sensitivity, but not specificity (reference to the development of CDMS) when applied to real-world CIS cohorts (Hyun et al., 2019; van der Vuurst de Vries et al., 2018). Indeed, using the MRI criteria from the 2010 McDonald criteria

in CIS patients followed up for five years, the sensitivity, specificity and AUC was 72%, 52% and 62% respectively (Filippi et al., 2018). It is important to note that the components that make up the criteria have not changed in the last two decades, highlighting the lack of (and indeed slow pace in developing) well-validated diagnostic biomarkers that can infer on MS pathophysiology. Indeed, CSF OCB, first used in the Poser criteria in 1983, was reintroduced into the 2017 McDonald criteria as a proxy for DIT within the context of the criteria (Thompson et al., 2018).

It should be emphasised that the McDonald criteria must be applied in the correct context, with the caveat that there must be 'no better explanation', apart from an assumed MS pathology, for the clinical presentation. This is likely to be applied corrected in tertiary neurological centres with MS expertise. However, it is also likely that other centres with less experience in MS may be applying this criteria to patients who are not likely to have MS in the first instance, e.g. in NMOSD or in monophasic post-infectious CNS inflammation. Such misdiagnosis has significant implications with regards to treatment as well as prognosis, not to mention the social and psychological burden imposed upon patients labelled with an 'MS' diagnosis. Therefore, it can be argued that an MS diagnostic criteria must have the capability to exclude other competing conditions that can look like MS, but clearly do not have MS pathology (Solomon, 2019). As such, biofluid markers as well as advanced MRI techniques that can infer on MS pathophysiology should be incorporated into these criteria, should they be standardised and validated in independent cohorts.

The majority of metabolomics studies in MS have thus far explored the metabolic differences between MS and HC/non-MS controls. As mentioned in the Introduction of this thesis, many of these controls are not disease relevant and would not have been considered in the diagnostic workup of patients suspected to have MS. The results from Chapter 3 show that distinctive metabolic profiles of MS, as well as Ab-NMOSD, with respect to each other, can be established. The discriminatory metabolites show utility within difficult-to-diagnose antibody-negative patients, allowing for the identification of individuals with likely MS pathology and those with antibody-mediated pathology.

This has important implications with regards to treatment. This approach demonstrates the potential of metabolomics to detect a MS-specific metabolic signature, with respect to other competing diseases, indeed with NMOSD being the most relevant. The findings will need to be validated in a larger cohort of patients and more diagnostic metabolic models need to be established by comparing early MS with other relevant diseases, e.g. MS vs. post-infectious neuroinflammation, MS vs. CNS sarcoidosis, MS vs. migraine with white matter lesions. This will allow for a comprehensive modelling database in which the metabolic profile of future patients suspected to have MS can be inserted to establish the diagnosis of MS, in conjunction with MRI findings and other ancillary investigations.

7.2.2 Disease activity

The role of clinical relapses and MRI activity (presence of GAD-enhancing and/or new T₂ lesions on conventional MRI) is well-established in determining disease activity within MS patients, whereby disease activity refers to the pathological substrates of immune/inflammatory cell infiltration, and neuroinflammation driven demyelination and neuroaxonal loss. The use of these measures is important as these provide topological and functional information on MS disease activity. However, it must be pointed out that these are likely to represent the ‘tip-of-the-iceberg’ with regards to measuring disease activity in its entirety, the reasons being that relapses are dependent on the involvement of clinically eloquent regions, and that conventional MRI is unable to accurately determine the pathogenic changes occurring in NAWM, grey matter, or in the spinal cord. Therefore, a biofluid marker that can provide a measure of this ‘occult’ neuroinflammation would be invaluable, especially when used with clinico-MRI measures in a complementary fashion.

As mentioned in the Introduction, NfL concentration has shown promising results in reflecting disease activity, being associated with relapses as well as MRI indices of disease activity, as it is likely to reflect inflammation-driven neuroaxonal loss. However, its application at an individual level

remains to be established. The results from Chapter 4 show that serum and CSF metabolomics are responsive and accurate markers of neuroinflammation, in that it can detect the DTH lesion early (indeed earlier than serum and CSF NfL) when neuroinflammation was at its most intense, with the magnitude of metabolic perturbation decreasing with time. Importantly, these results provide proof that a clinically asymptomatic brain lesion is able to produce specific metabolic signatures, and indeed metabolites, which are detectable in serum, supporting the metabolomics approach in detecting disease activity in human MS. The results from Chapter 5 agree with those observed in rodents; patients in relapse are metabolically perturbed in comparison to 'inactive' patients, again with the magnitude of the metabolic disturbance reducing with time. Notably, the identified metabolites are (1) applicable in an individualised manner, albeit in a small subset of the study population, and (2) offer higher discriminatory value, either singly or in combination, compared to serum NfL at both group and individual level. This has potential to be developed into a biomarker test for clinical relapses, but not before validation on a larger cohort of patients with prospective/predictive samples (i.e. remission then relapse). This is best suited to be performed in the context of a clinical trial, which permits regular blood sampling as well as scheduled MRI to determine if these metabolites also measure radiological disease activity. Metabolites proposed by other studies that are associated with MS disease activity, such as certain lysoPC species, PC, lactate, β -hydroxybutyrate can be assessed, and if validated, incorporated into a metabolic composite biomarker together with those shown in Chapter 5.

7.2.3 Progression

Perhaps the most pressing biomarker need in MS currently is defining progressive MS and quantifying the neurodegenerative burden within MS patients. The newer DMT have been highly successful in ablating clinico-MRI disease activity, but their role in staving off future progression is debatable. Indeed, this has been a focus of recent clinical research with RCT exploring the efficacy of DMT in progressive patients. It is apparent that the success of some DMT in progressive MS is driven

by individuals who still have a strong neuroinflammatory component and calls into question the efficacy of these drugs in patients with more advanced progression (Kappos et al., 2018; Montalban et al., 2017). This highlights the urgent need to objectively define progressive MS, independent of any inflammatory component, and perform RCT on these patients with candidate therapies to halt progression.

As mentioned in the Introduction of this thesis, brain atrophy is a promising marker to reflect the degenerative component, however interval measurements have been observed to be small, and its widespread use is prohibited by technical and biological variability (Miller et al., 2018). While CSF and serum NfL has been reported to distinguish RRMS and progressive patients, the discordance in CSF and serum findings deserves to be further investigated, as well as its diagnostic value in individual patients.

The results from Chapter 6 validates previous work, showing that serum metabolomics has excellent discriminatory value in separating RRMS and SPMS, and this was not affected by sample-handling variations. The findings also demonstrate that the discriminatory value of serum metabolomics is higher than that of serum NfL. This represents the first metabolomics study in progressive MS to validate previous findings and to demonstrate that it can be practicably implemented in the clinics. Expectedly, this has substantial potential for both clinical management and for use as an inclusion criterion in progressive MS RCT. A commercial NMR company is currently working with the Oxford group to develop this as a clinical test (numares, 2020b). At this juncture, it has to be reiterated that test development and implementation with NMR metabolomics is highly practicable given its reproducibility, quantifiable nature, simple sample preparation, and low sample processing cost.

7.3 Future research

Metabolomics is a relatively new field in biomedical research, much less in CNS IDD and MS. The existing literature and the findings from this thesis provide encouraging results that metabolomics,

either singly or as an adjunct to existing tools, can contribute to precision medicine in CNS IDD. There is still much work to be done in terms of validating previous results and exploring new uses in larger prospective patient cohorts for biomarker discovery. Beyond this, metabolomics has much to offer in terms of finding perturbed metabolic pathways and targeted studies can be used to identify potential druggable targets. These promising targets can be then evaluated using in vitro and in vivo platforms to establish mechanistic relevance and therapeutic effect. The success of the SIP modulator, fingolimod, highlights that metabolites and lipids play important bioactive roles relevant to MS pathogenesis, and intervening at appropriate metabolic checkpoints represents a viable treatment option. Future studies will also need to incorporate upstream 'omics' platforms, e.g. proteomics, transcriptomics, genomics, to find relevant associations with the downstream metabolites, within the context of MS pathogenesis. Adopting this approach will allow summative markers to be developed and increase our understanding of MS from a holistic systems biology perspective.

Bibliography

- Adelman, G., Rane, S. G., & Villa, K. F. (2013). The cost burden of multiple sclerosis in the United States: a systematic review of the literature. *J Med Econ*, 16(5), 639-647. doi:10.3111/13696998.2013.778268
- Akarachantachote N., C. S., Saithanu K. (2014). Cutoff Threshold of Variable Importance in Projection for Variable Selection. *IJPAM*, 94(3), 307-322.
- Akgun, K., Kretschmann, N., Haase, R., Proschmann, U., Kitzler, H. H., Reichmann, H., & Ziemssen, T. (2019). Profiling individual clinical responses by high-frequency serum neurofilament assessment in MS. *Neurol Neuroimmunol Neuroinflamm*, 6(3), e555. doi:10.1212/NXI.0000000000000555
- Albargothy, N. J., Johnston, D. A., MacGregor-Sharp, M., Weller, R. O., Verma, A., Hawkes, C. A., & Carare, R. O. (2018). Convective influx/glymphatic system: tracers injected into the CSF enter and leave the brain along separate periaxonal basement membrane pathways. *Acta Neuropathologica*, 136(1), 139-152. doi:10.1007/s00401-018-1862-7
- Alum, M. F., Shaw, P. A., Sweatman, B. C., Ubhi, B. K., Haselden, J. N., & Connor, S. C. (2008). 4,4-Dimethyl-4-silapentane-1-ammonium trifluoroacetate (DSA), a promising universal internal standard for NMR-based metabolic profiling studies of biofluids, including blood plasma and serum. *Metabolomics*, 4(2), 122-127. doi:10.1007/s11306-008-0103-9
- Alvarez, J. I., Cayrol, R., & Prat, A. (2011). Disruption of central nervous system barriers in multiple sclerosis. *Biochim Biophys Acta*, 1812(2), 252-264. doi:10.1016/j.bbdis.2010.06.017
- Ananieva, E. A., Powell, J. D., & Hutson, S. M. (2016). Leucine Metabolism in T Cell Activation: mTOR Signaling and Beyond. *Adv Nutr*, 7(4), 798S-805S. doi:10.3945/an.115.011221
- Andersen, S. L., Briggs, F. B. S., Winnike, J. H., Natanzon, Y., Maichle, S., Knagge, K. J., . . . Gregory, S. G. (2019). Metabolome-based signature of disease pathology in MS. *Mult Scler Relat Disord*, 31, 12-21. doi:10.1016/j.msard.2019.03.006
- Anthony, D. C., & Couch, Y. (2014). The systemic response to CNS injury. *Exp Neurol*, 258, 105-111. doi:10.1016/j.expneurol.2014.03.013
- Anthony, D. C., Couch, Y., Losey, P., & Evans, M. C. (2012). The systemic response to brain injury and disease. *Brain Behav Immun*, 26(4), 534-540. doi:10.1016/j.bbi.2011.10.011
- Anthony, D. C., Sibson, N. R., Losey, P., Meier, D. P., & Leppert, D. (2014). Investigation of immune and CNS-mediated effects of fingolimod in the focal delayed-type hypersensitivity multiple sclerosis model. *Neuropharmacology*, 79, 534-541. doi:10.1016/j.neuropharm.2013.12.022
- Anton, G., Wilson, R., Yu, Z. H., Prehn, C., Zukunft, S., Adamski, J., . . . Waldenberger, M. (2015). Pre-analytical sample quality: metabolite ratios as an intrinsic marker for prolonged room temperature exposure of serum samples. *PLoS One*, 10(3), e0121495. doi:10.1371/journal.pone.0121495
- Arrambide, G., Tintore, M., Espejo, C., Auger, C., Castillo, M., Rio, J., . . . Montalban, X. (2018). The value of oligoclonal bands in the multiple sclerosis diagnostic criteria. *Brain*, 141(4), 1075-1084. doi:10.1093/brain/awy006
- Asgari, N., Lillevang, S. T., Skejoe, H. P., Falah, M., Stenager, E., & Kyvik, K. O. (2011). A population-based study of neuromyelitis optica in Caucasians. *Neurology*, 76(18), 1589-1595. doi:10.1212/WNL.0b013e3182190f74
- Ayzenberg, I., Schollhammer, J., Hoepner, R., Hellwig, K., Ringelstein, M., Aktas, O., . . . Neuromyelitis Optica Study, G. (2016). Efficacy of glatiramer acetate in neuromyelitis optica spectrum disorder: a multicenter retrospective study. *J Neurol*, 263(3), 575-582. doi:10.1007/s00415-015-7991-1

- Azzopardi, L., Cox, A. L., McCarthy, C. L., Jones, J. L., & Coles, A. J. (2016). Alemtuzumab use in neuromyelitis optica spectrum disorders: a brief case series. *J Neurol*, 263(1), 25-29. doi:10.1007/s00415-015-7925-y
- Bacioglu, M., Maia, L. F., Preische, O., Schelle, J., Apel, A., Kaeser, S. A., . . . Jucker, M. (2016). Neurofilament Light Chain in Blood and CSF as Marker of Disease Progression in Mouse Models and in Neurodegenerative Diseases. *Neuron*, 91(1), 56-66. doi:10.1016/j.neuron.2016.05.018
- Barber, C. N., & Raben, D. M. (2019). Lipid Metabolism Crosstalk in the Brain: Glia and Neurons. *Front Cell Neurosci*, 13, 212. doi:10.3389/fncel.2019.00212
- Barkhatova, V. P., Zavalishin, I. A., Askarova, L., Shavratskii, V., & Demina, E. G. (1998). Changes in neurotransmitters in multiple sclerosis. *Neurosci Behav Physiol*, 28(4), 341-344. doi:10.1007/bf02464784
- Battini, S., Bund, C., Moussallieh, F. M., Cicek, A. E., De Seze, J., & Namer, I. J. (2018). Metabolomics approaches in experimental allergic encephalomyelitis. *J Neuroimmunol*, 314, 94-100. doi:10.1016/j.jneuroim.2017.11.018
- Bauer, A. T., Burgers, H. F., Rabie, T., & Marti, H. H. (2010). Matrix metalloproteinase-9 mediates hypoxia-induced vascular leakage in the brain via tight junction rearrangement. *J Cereb Blood Flow Metab*, 30(4), 837-848. doi:10.1038/jcbfm.2009.248
- Bechmann, I., & Woodroffe, N. (2014). Immune Privilege of the Brain. *Neuroinflammation and Cns Disorders*, 1-8. doi:Book_Doi 10.1002/9781118406557
- Bernini, P., Bertini, I., Luchinat, C., Nincheri, P., Staderini, S., & Turano, P. (2011). Standard operating procedures for pre-analytical handling of blood and urine for metabolomic studies and biobanks. *J Biomol NMR*, 49(3-4), 231-243. doi:10.1007/s10858-011-9489-1
- Bervoets, L., Louis, E., Reekmans, G., Mesotten, L., Thomeer, M., Adriaenssens, P., & Linsen, L. (2015). Influence of preanalytical sampling conditions on the ¹H NMR metabolic profile of human blood plasma and introduction of the Standard PREanalytical Code used in biobanking. *Metabolomics*, 11(5), 1197-1207.
- Bhargava, P., Fitzgerald, K. C., Calabresi, P. A., & Mowry, E. M. (2017). Metabolic alterations in multiple sclerosis and the impact of vitamin D supplementation. *JCI Insight*, 2(19). doi:10.1172/jci.insight.95302
- Bhargava, P., Fitzgerald, K. C., Venkata, S. L. V., Smith, M. D., Kornberg, M. D., Mowry, E. M., . . . Calabresi, P. A. (2019). Dimethyl fumarate treatment induces lipid metabolism alterations that are linked to immunological changes. *Ann Clin Transl Neurol*, 6(1), 33-45. doi:10.1002/acn3.676
- Bhinderwala, F., Wase, N., DiRusso, C., & Powers, R. (2018). Combining Mass Spectrometry and NMR Improves Metabolite Detection and Annotation. *J Proteome Res*, 17(11), 4017-4022. doi:10.1021/acs.jproteome.8b00567
- Bingol, K., Bruschweiler-Li, L., Yu, C., Somogyi, A., Zhang, F., & Bruschweiler, R. (2015). Metabolomics beyond spectroscopic databases: a combined MS/NMR strategy for the rapid identification of new metabolites in complex mixtures. *Anal Chem*, 87(7), 3864-3870. doi:10.1021/ac504633z
- Bingol, K., & Bruschweiler, R. (2015). NMR/MS Translator for the Enhanced Simultaneous Analysis of Metabolomics Mixtures by NMR Spectroscopy and Mass Spectrometry: Application to Human Urine. *J Proteome Res*, 14(6), 2642-2648. doi:10.1021/acs.jproteome.5b00184
- Biomarkers Definitions Working, G. (2001). Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther*, 69(3), 89-95. doi:10.1067/mcp.2001.113989
- Bittner, S., Afzali, A. M., Wiendl, H., & Meuth, S. G. (2014). Myelin oligodendrocyte glycoprotein (MOG35-55) induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice. *J Vis Exp*(86). doi:10.3791/51275

- Blanchet, L., Smolinska, A., Attali, A., Stoop, M. P., Ampt, K. A., van Aken, H., . . . Buydens, L. M. (2011). Fusion of metabolomics and proteomics data for biomarkers discovery: case study on the experimental autoimmune encephalomyelitis. *BMC Bioinformatics*, 12, 254. doi:10.1186/1471-2105-12-254
- Bolton, S. J., Anthony, D. C., & Perry, V. H. (1998). Loss of the tight junction proteins occludin and zonula occludens-1 from cerebral vascular endothelium during neutrophil-induced blood-brain barrier breakdown in vivo. *Neuroscience*, 86(4), 1245-1257. doi:10.1016/s0306-4522(98)00058-x
- Bordag, N., Klie, S., Jurchott, K., Vierheller, J., Schiewe, H., Albrecht, V., . . . Selbig, J. (2015). Glucocorticoid (dexamethasone)-induced metabolome changes in healthy males suggest prediction of response and side effects. *Sci Rep*, 5, 15954. doi:10.1038/srep15954
- Bradl, M., Misu, T., Takahashi, T., Watanabe, M., Mader, S., Reindl, M., . . . Lassmann, H. (2009). Neuromyelitis optica: pathogenicity of patient immunoglobulin in vivo. *Ann Neurol*, 66(5), 630-643. doi:10.1002/ana.21837
- Bramow, S., Frischer, J. M., Lassmann, H., Koch-Henriksen, N., Lucchinetti, C. F., Sorensen, P. S., & Laursen, H. (2010). Demyelination versus remyelination in progressive multiple sclerosis. *Brain*, 133(10), 2983-2998. doi:10.1093/brain/awq250
- Brereton, R. G., & Lloyd, G. R. (2014). Partial least squares discriminant analysis: taking the magic away. *Journal of Chemometrics*, 28(4), 213-225. doi:10.1002/cem.2609
- Bridel, C., van Wieringen, W. N., Zetterberg, H., Tijms, B. M., Teunissen, C. E., and the, N. F. L. G., . . . Wild, E. J. (2019). Diagnostic Value of Cerebrospinal Fluid Neurofilament Light Protein in Neurology: A Systematic Review and Meta-analysis. *JAMA Neurol*. doi:10.1001/jamaneurol.2019.1534
- Briggs, J. P., Levitt, M. F., & Abramson, R. G. (1977). Renal Excretion of Allantoin in Rats - Micropuncture and Clearance Study. *American Journal of Physiology*, 233(5), F373-F381. Retrieved from <Go to ISI>://WOS:A1977EC66300053
- Brites, P., Mooyer, P. A., El Mrabet, L., Waterham, H. R., & Wanders, R. J. (2009). Plasmalogens participate in very-long-chain fatty acid-induced pathology. *Brain*, 132(Pt 2), 482-492. doi:10.1093/brain/awn295
- Broadhurst, D. I., & Kell, D. B. (2006). Statistical strategies for avoiding false discoveries in metabolomics and related experiments. *Metabolomics*, 2(4), 171-196. doi:10.1007/s11306-006-0037-z
- Broom, K. A., Anthony, D. C., Blamire, A. M., Waters, S., Styles, P., Perry, V. H., & Sibson, N. R. (2005). MRI reveals that early changes in cerebral blood volume precede blood-brain barrier breakdown and overt pathology in MS-like lesions in rat brain. *J Cereb Blood Flow Metab*, 25(2), 204-216. doi:10.1038/sj.jcbfm.9600020
- Brosnan, J. T., & Brosnan, M. E. (2006). Branched-chain amino acids: enzyme and substrate regulation. *J Nutr*, 136(1 Suppl), 207S-211S. doi:10.1093/jn/136.1.207S
- Calabrese, M., Agosta, F., Rinaldi, F., Mattisi, I., Grossi, P., Favaretto, A., . . . Filippi, M. (2009). Cortical lesions and atrophy associated with cognitive impairment in relapsing-remitting multiple sclerosis. *Arch Neurol*, 66(9), 1144-1150. doi:10.1001/archneurol.2009.174
- Calder, P. C. (2006). Branched-chain amino acids and immunity. *J Nutr*, 136(1 Suppl), 288S-293S. doi:10.1093/jn/136.1.288S
- Campbell, S. J., Perry, V. H., Pitossi, F. J., Butchart, A. G., Chertoff, M., Waters, S., . . . Anthony, D. C. (2005). Central nervous system injury triggers hepatic CC and CXC chemokine expression that is associated with leukocyte mobilization and recruitment to both the central nervous system and the liver. *Am J Pathol*, 166(5), 1487-1497. doi:10.1016/S0002-9440(10)62365-6
- Campbell, S. J., Zahid, I., Losey, P., Law, S., Jiang, Y., Bilgen, M., . . . Anthony, D. C. (2008). Liver Kupffer cells control the magnitude of the inflammatory response in the injured brain and spinal cord. *Neuropharmacology*, 55(5), 780-787. doi:10.1016/j.neuropharm.2008.06.074

- Camu, W., Leherter, P., Pierrot-Deseilligny, C., Hautecoeur, P., Besserve, A., Jean Deleglise, A. S., . . . Souberbielle, J. C. (2019). Cholecalciferol in relapsing-remitting MS: A randomized clinical trial (CHOLINE). *Neurol Neuroimmunol Neuroinflamm*, 6(5). doi:10.1212/NXI.0000000000000597
- Cansev, M. (2006). Uridine and cytidine in the brain: their transport and utilization. *Brain Res Rev*, 52(2), 389-397. doi:10.1016/j.brainresrev.2006.05.001
- Canto, E., Tintore, M., Villar, L. M., Costa, C., Nurdinov, R., Alvarez-Cermeno, J. C., . . . Comabella, M. (2015). Chitinase 3-like 1: prognostic biomarker in clinically isolated syndromes. *Brain*, 138(Pt 4), 918-931. doi:10.1093/brain/awv017
- Carlsson, H., Abujrais, S., Herman, S., Khoonsari, P. E., Akerfeldt, T., Svenningsson, A., . . . Kulima, K. (2020). Targeted metabolomics of CSF in healthy individuals and patients with secondary progressive multiple sclerosis using high-resolution mass spectrometry. *Metabolomics*, 16(2), 26. doi:10.1007/s11306-020-1648-5
- Chamorro, A., Amaro, S., Castellanos, M., Segura, T., Arenillas, J., Marti-Fabregas, J., . . . Investigators, U.-I. (2014). Safety and efficacy of uric acid in patients with acute stroke (URICO-ICTUS): a randomised, double-blind phase 2b/3 trial. *Lancet Neurol*, 13(5), 453-460. doi:10.1016/S1474-4422(14)70054-7
- Chan, K. H., Tse, C. T., Chung, C. P., Lee, R. L., Kwan, J. S., Ho, P. W., & Ho, J. W. (2011). Brain involvement in neuromyelitis optica spectrum disorders. *Arch Neurol*, 68(11), 1432-1439. doi:10.1001/archneurol.2011.249
- Chard, D., & Trip, S. A. (2017). Resolving the clinico-radiological paradox in multiple sclerosis. *F1000Res*, 6, 1828. doi:10.12688/f1000research.11932.1
- Chard, D. T., Griffin, C. M., McLean, M. A., Kapeller, P., Kapoor, R., Thompson, A. J., & Miller, D. H. (2002). Brain metabolite changes in cortical grey and normal-appearing white matter in clinically early relapsing-remitting multiple sclerosis. *Brain*, 125(Pt 10), 2342-2352. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12244090>
- Cho, H. W., Kim, S. B., Jeong, M. K., Park, Y., Miller, N. G., Ziegler, T. R., & Jones, D. P. (2008). Discovery of metabolite features for the modelling and analysis of high-resolution NMR spectra. *Int J Data Min Bioinform*, 2(2), 176-192. doi:10.1504/ijdbm.2008.019097
- Choi, S. R., Howell, O. W., Carassiti, D., Magliozzi, R., Gveric, D., Muraro, P. A., . . . Reynolds, R. (2012). Meningeal inflammation plays a role in the pathology of primary progressive multiple sclerosis. *Brain*, 135(Pt 10), 2925-2937. doi:10.1093/brain/aws189
- Choi, W. T., Tosun, M., Jeong, H. H., Karakas, C., Semerci, F., Liu, Z., & Maletic-Savatic, M. (2018). Metabolomics of mammalian brain reveals regional differences. *BMC Syst Biol*, 12(Suppl 8), 127. doi:10.1186/s12918-018-0644-0
- Choudhary, C., Weinert, B. T., Nishida, Y., Verdin, E., & Mann, M. (2014). The growing landscape of lysine acetylation links metabolism and cell signalling. *Nat Rev Mol Cell Biol*, 15(8), 536-550. doi:10.1038/nrm3841
- Chun, J., & Hartung, H. P. (2010). Mechanism of action of oral fingolimod (FTY720) in multiple sclerosis. *Clin Neuropharmacol*, 33(2), 91-101. doi:10.1097/WNF.0b013e3181cbf825
- Cicalini, I., Rossi, C., Pieragostino, D., Agnifili, L., Mastropasqua, L., di Iorio, M., . . . Del Boccio, P. (2019). Integrated Lipidomics and Metabolomics Analysis of Tears in Multiple Sclerosis: An Insight into Diagnostic Potential of Lacrimal Fluid. *Int J Mol Sci*, 20(6). doi:10.3390/ijms20061265
- Ciccarelli, O., Thomas, D. L., De Vita, E., Wheeler-Kingshott, C. A., Kachramanoglou, C., Kapoor, R., . . . Thompson, A. J. (2013). Low myo-inositol indicating astrocytic damage in a case series of neuromyelitis optica. *Ann Neurol*, 74(2), 301-305. doi:10.1002/ana.23909
- Cocco, E., Murgia, F., Lorefice, L., Barberini, L., Poddighe, S., Frau, J., . . . Marrosu, M. G. (2016). (1)H-NMR analysis provides a metabolomic profile of patients with multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm*, 3(1), e185. doi:10.1212/NXI.0000000000000185

- Coles, A. J., Twyman, C. L., Arnold, D. L., Cohen, J. A., Confavreux, C., Fox, E. J., . . . investigators, C.-M. I. (2012). Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial. *Lancet*, 380(9856), 1829-1839. doi:10.1016/S0140-6736(12)61768-1
- Comabella, M., Fernandez, M., Martin, R., Rivera-Vallve, S., Borrás, E., Chiva, C., . . . Montalban, X. (2010). Cerebrospinal fluid chitinase 3-like 1 levels are associated with conversion to multiple sclerosis. *Brain*, 133(Pt 4), 1082-1093. doi:10.1093/brain/awq035
- Compston, A., & Coles, A. (2008). Multiple sclerosis. *Lancet*, 372(9648), 1502-1517. doi:10.1016/S0140-6736(08)61620-7
- Confavreux, C., & Vukusic, S. (2006). [The natural history of multiple sclerosis]. *Rev Prat*, 56(12), 1313-1320. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/16948219>
- Constantinescu, C. S., Farooqi, N., O'Brien, K., & Gran, B. (2011). Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *British Journal of Pharmacology*, 164(4), 1079-1106. doi:10.1111/j.1476-5381.2011.01302.x
- Couch, Y., Akbar, N., Roodseelaar, J., Evans, M. C., Gardiner, C., Sargent, I., . . . Anthony, D. C. (2017). Circulating endothelial cell-derived extracellular vesicles mediate the acute phase response and sickness behaviour associated with CNS inflammation. *Sci Rep*, 7(1), 9574. doi:10.1038/s41598-017-09710-3
- Cree, B. A. C., Hollenbach, J. A., Bove, R., Kirkish, G., Sacco, S., Caverzasi, E., . . . Hauser, S. L. (2019). Silent progression in disease activity-free relapsing multiple sclerosis. *Ann Neurol*, 85(5), 653-666. doi:10.1002/ana.25463
- Criste, G. A., & Trapp, B. D. (2006). N-acetyl-L-aspartate in multiple sclerosis. *Adv Exp Med Biol*, 576, 199-214; discussion 361-193. doi:10.1007/0-387-30172-0_14
- Cross, A. H., Manning, P. T., Keeling, R. M., Schmidt, R. E., & Misko, T. P. (1998). Peroxynitrite formation within the central nervous system in active multiple sclerosis. *J Neuroimmunol*, 88(1-2), 45-56. doi:10.1016/s0165-5728(98)00078-2
- Curti, E., Graziuso, S., Tsantes, E., Crisi, G., & Granella, F. (2018). Correlation between cortical lesions and cognitive impairment in multiple sclerosis. *Brain Behav*, 8(6), e00955. doi:10.1002/brb3.955
- Dale, R. C., Tantsis, E. M., Merheb, V., Kumaran, R. Y., Sinmaz, N., Pathmanandavel, K., . . . Brilot, F. (2014). Antibodies to MOG have a demyelination phenotype and affect oligodendrocyte cytoskeleton. *Neurol Neuroimmunol Neuroinflamm*, 1(1), e12. doi:10.1212/NXI.0000000000000012
- de Oliveira, E. M. L., Montani, D. A., Oliveira-Silva, D., Rodrigues-Oliveira, A. F., Matas, S. L. D., Fernandes, G. B. P., . . . Lo Turco, E. G. (2019). Multiple sclerosis has a distinct lipid signature in plasma and cerebrospinal fluid. *Arquivos De Neuro-Psiquiatria*, 77(10), 696-704. doi:10.1590/0004-282x20190122
- De Santis, S., Granberg, T., Ouellette, R., Treaba, C. A., Herranz, E., Fan, Q., . . . Toschi, N. (2019). Evidence of early microstructural white matter abnormalities in multiple sclerosis from multi-shell diffusion MRI. *Neuroimage Clin*, 22, 101699. doi:10.1016/j.nicl.2019.101699
- Del Boccio, P., Pieragostino, D., Di Ioia, M., Petrucci, F., Lugaresi, A., De Luca, G., . . . Urbani, A. (2011). Lipidomic investigations for the characterization of circulating serum lipids in multiple sclerosis. *J Proteomics*, 74(12), 2826-2836. doi:10.1016/j.jprot.2011.06.023
- Dendrou, C. A., Fugger, L., & Friese, M. A. (2015). Immunopathology of multiple sclerosis. *Nat Rev Immunol*, 15(9), 545-558. doi:10.1038/nri3871
- Deprez, S., Sweatman, B. C., Connor, S. C., Haselden, J. N., & Waterfield, C. J. (2002). Optimisation of collection, storage and preparation of rat plasma for ¹H NMR spectroscopic analysis in toxicology studies to determine inherent variation in biochemical profiles. *J Pharm Biomed Anal*, 30(4), 1297-1310. doi:10.1016/s0731-7085(02)00455-7
- Dickens, A. M., Larkin, J. R., Davis, B. G., Griffin, J. L., Claridge, T. D., Sibson, N. R., & Anthony, D. C. (2015). NMR-Based Metabolomics Separates the Distinct Stages of Disease in a Chronic

- Relapsing Model of Multiple Sclerosis. *J Neuroimmune Pharmacol*, 10(3), 435-444. doi:10.1007/s11481-015-9622-0
- Dickens, A. M., Larkin, J. R., Griffin, J. L., Cavey, A., Matthews, L., Turner, M. R., . . . Sibson, N. R. (2014). A type 2 biomarker separates relapsing-remitting from secondary progressive multiple sclerosis. *Neurology*, 83(17), 1492-1499. doi:10.1212/WNL.0000000000000905
- Disanto, G., Barro, C., Benkert, P., Naegelin, Y., Schadelin, S., Giardiello, A., . . . Swiss Multiple Sclerosis Cohort Study, G. (2017). Serum Neurofilament light: A biomarker of neuronal damage in multiple sclerosis. *Ann Neurol*, 81(6), 857-870. doi:10.1002/ana.24954
- Dobson, R., Ramagopalan, S., Davis, A., & Giovannoni, G. (2013). Cerebrospinal fluid oligoclonal bands in multiple sclerosis and clinically isolated syndromes: a meta-analysis of prevalence, prognosis and effect of latitude. *J Neurol Neurosurg Psychiatry*, 84(8), 909-914. doi:10.1136/jnnp-2012-304695
- Dunn, W. B., Broadhurst, D. I., Atherton, H. J., Goodacre, R., & Griffin, J. L. (2011). Systems level studies of mammalian metabolomes: the roles of mass spectrometry and nuclear magnetic resonance spectroscopy. *Chem Soc Rev*, 40(1), 387-426. doi:10.1039/b906712b
- Dyachok, J., Earnest, S., Iturran, E. N., Cobb, M. H., & Ross, E. M. (2016). Amino Acids Regulate mTORC1 by an Obligate Two-step Mechanism. *J Biol Chem*, 291(43), 22414-22426. doi:10.1074/jbc.M116.732511
- Eijlers, A. J. C., van Geest, Q., Dekker, I., Steenwijk, M. D., Meijer, K. A., Hulst, H. E., . . . Geurts, J. J. G. (2018). Predicting cognitive decline in multiple sclerosis: a 5-year follow-up study. *Brain*, 141(9), 2605-2618. doi:10.1093/brain/awy202
- Emwas, A. H. (2015). The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. *Methods Mol Biol*, 1277, 161-193. doi:10.1007/978-1-4939-2377-9_13
- Engelhardt, B., & Sorokin, L. (2009). The blood-brain and the blood-cerebrospinal fluid barriers: function and dysfunction. *Semin Immunopathol*, 31(4), 497-511. doi:10.1007/s00281-009-0177-0
- Eriksson, L. J., E.; Kettaneh-Wold, N.; Wold, S. (2001). Multi- and Megavariate Data Analysis. Principles and Applications. *Umetrics Academy*.
- Fernando, K. T., McLean, M. A., Chard, D. T., MacManus, D. G., Dalton, C. M., Miszkiel, K. A., . . . Miller, D. H. (2004). Elevated white matter myo-inositol in clinically isolated syndromes suggestive of multiple sclerosis. *Brain*, 127(Pt 6), 1361-1369. doi:10.1093/brain/awh153
- Ferretti, G., & Bacchetti, T. (2011). Peroxidation of lipoproteins in multiple sclerosis. *J Neurol Sci*, 311(1-2), 92-97. doi:10.1016/j.jns.2011.09.004
- Filippi, M., Preziosa, P., Meani, A., Ciccarelli, O., Mesaros, S., Rovira, A., . . . Rocca, M. A. (2018). Prediction of a multiple sclerosis diagnosis in patients with clinically isolated syndrome using the 2016 MAGNIMS and 2010 McDonald criteria: a retrospective study. *Lancet Neurol*, 17(2), 133-142. doi:10.1016/S1474-4422(17)30469-6
- Filippi, M., Rocca, M. A., Barkhof, F., Bruck, W., Chen, J. T., Comi, G., . . . Attendees of the Correlation between Pathological, M. R. I. f. i. M. S. w. (2012). Association between pathological and MRI findings in multiple sclerosis. *Lancet Neurol*, 11(4), 349-360. doi:10.1016/S1474-4422(12)70003-0
- Fisniku, L. K., Brex, P. A., Altmann, D. R., Miszkiel, K. A., Benton, C. E., Lanyon, R., . . . Miller, D. H. (2008). Disability and T2 MRI lesions: a 20-year follow-up of patients with relapse onset of multiple sclerosis. *Brain*, 131(Pt 3), 808-817. doi:10.1093/brain/awm329
- Flanagan, E. P., Kaufmann, T. J., Krecke, K. N., Aksamit, A. J., Pittock, S. J., Keegan, B. M., . . . Weinshenker, B. G. (2016). Discriminating long myelitis of neuromyelitis optica from sarcoidosis. *Ann Neurol*, 79(3), 437-447. doi:10.1002/ana.24582
- Fliniaux, O., Gaillard, G., Lion, A., Cailleu, D., Mesnard, F., & Betsou, F. (2011). Influence of common preanalytical variations on the metabolic profile of serum samples in biobanks. *J Biomol NMR*, 51(4), 457-465. doi:10.1007/s10858-011-9574-5

- Frey, B. B. (2018). *The Sage encyclopedia of educational research, measurement, and evaluation*. Los Angeles: SAGE Reference.
- Gafson, A. R., Thorne, T., McKechnie, C. I. J., Jimenez, B., Nicholas, R., & Matthews, P. M. (2018). Lipoprotein markers associated with disability from multiple sclerosis. *Sci Rep*, 8(1), 17026. doi:10.1038/s41598-018-35232-7
- GBD 2016 Multiple Sclerosis Collaborators. (2019). Global, regional, and national burden of multiple sclerosis 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol*, 18(3), 269-285. doi:10.1016/S1474-4422(18)30443-5
- Gebregiworgis, T., Massilamany, C., Gangaplara, A., Thulasingam, S., Kolli, V., Werth, M. T., . . . Powers, R. (2013). Potential of urinary metabolites for diagnosing multiple sclerosis. *ACS Chem Biol*, 8(4), 684-690. doi:10.1021/cb300673e
- Gebregiworgis, T., Nielsen, H. H., Massilamany, C., Gangaplara, A., Reddy, J., Illes, Z., & Powers, R. (2016). A Urinary Metabolic Signature for Multiple Sclerosis and Neuromyelitis Optica. *J Proteome Res*, 15(2), 659-666. doi:10.1021/acs.jproteome.5b01111
- Geraldes, R., Ciccarelli, O., Barkhof, F., De Stefano, N., Enzinger, C., Filippi, M., . . . group, M. s. (2018). The current role of MRI in differentiating multiple sclerosis from its imaging mimics. *Nat Rev Neurol*, 14(4), 199-213. doi:10.1038/nrneurol.2018.14
- Ghione, E., Bergsland, N., Dwyer, M. G., Hagemeier, J., Jakimovski, D., Paunkoski, I., . . . Zivadinov, R. (2018). Brain Atrophy Is Associated with Disability Progression in Patients with MS followed in a Clinical Routine. *AJNR Am J Neuroradiol*, 39(12), 2237-2242. doi:10.3174/ajnr.A5876
- Gil-Perotin, S., Castillo-Villalba, J., Cubas-Nunez, L., Gasque, R., Hervas, D., Gomez-Mateu, J., . . . Casanova, B. (2019). Combined Cerebrospinal Fluid Neurofilament Light Chain Protein and Chitinase-3 Like-1 Levels in Defining Disease Course and Prognosis in Multiple Sclerosis. *Front Neurol*, 10, 1008. doi:10.3389/fneur.2019.01008
- Gimeno-Bayon, J., Lopez-Lopez, A., Rodriguez, M. J., & Mahy, N. (2014). Glucose pathways adaptation supports acquisition of activated microglia phenotype. *J Neurosci Res*, 92(6), 723-731. doi:10.1002/jnr.23356
- Giovannoni, G., Comi, G., Cook, S., Rammohan, K., Rieckmann, P., Soelberg Sorensen, P., . . . Group, C. S. (2010). A placebo-controlled trial of oral cladribine for relapsing multiple sclerosis. *N Engl J Med*, 362(5), 416-426. doi:10.1056/NEJMoa0902533
- Gisslen, M., Price, R. W., Andreasson, U., Norgren, N., Nilsson, S., Hagberg, L., . . . Zetterberg, H. (2016). Plasma Concentration of the Neurofilament Light Protein (NFL) is a Biomarker of CNS Injury in HIV Infection: A Cross-Sectional Study. *EBioMedicine*, 3, 135-140. doi:10.1016/j.ebiom.2015.11.036
- Gonzalo, H., Brieva, L., Tatzber, F., Jove, M., Cacabelos, D., Cassanye, A., . . . Portero-Otin, M. (2012). Lipidome analysis in multiple sclerosis reveals protein lipoxidative damage as a potential pathogenic mechanism. *J Neurochem*, 123(4), 622-634. doi:10.1111/j.1471-4159.2012.07934.x
- Goodin, D. S., Reder, A. T., Bermel, R. A., Cutter, G. R., Fox, R. J., John, G. R., . . . Waubant, E. (2016). Relapses in multiple sclerosis: Relationship to disability. *Mult Scler Relat Disord*, 6, 10-20. doi:10.1016/j.msard.2015.09.002
- Griffin, J. L., Anthony, D. C., Campbell, S. J., Gauldie, J., Pitossi, F., Styles, P., & Sibson, N. R. (2004). Study of cytokine induced neuropathology by high resolution proton NMR spectroscopy of rat urine. *FEBS Lett*, 568(1-3), 49-54. doi:10.1016/j.febslet.2004.04.096
- Haghikia, A., Kayacelebi, A. A., Beckmann, B., Hanff, E., Gold, R., Haghikia, A., & Tsikas, D. (2015). Serum and cerebrospinal fluid concentrations of homoarginine, arginine, asymmetric and symmetric dimethylarginine, nitrite and nitrate in patients with multiple sclerosis and neuromyelitis optica. *Amino Acids*, 47(9), 1837-1845. doi:10.1007/s00726-015-2015-0
- Haid, M., Muschet, C., Wahl, S., Romisch-Margl, W., Prehn, C., Moller, G., & Adamski, J. (2018). Long-Term Stability of Human Plasma Metabolites during Storage at -80 degrees C. *J Proteome Res*, 17(1), 203-211. doi:10.1021/acs.jproteome.7b00518

- Hametner, S., Dal Bianco, A., Tractnig, S., & Lassmann, H. (2018). Iron related changes in MS lesions and their validity to characterize MS lesion types and dynamics with Ultra-high field magnetic resonance imaging. *Brain Pathol*, 28(5), 743-749. doi:10.1111/bpa.12643
- Harris, J. L., Choi, I. Y., & Brooks, W. M. (2015). Probing astrocyte metabolism in vivo: proton magnetic resonance spectroscopy in the injured and aging brain. *Front Aging Neurosci*, 7, 202. doi:10.3389/fnagi.2015.00202
- Hauser, S. L., Bar-Or, A., Comi, G., Giovannoni, G., Hartung, H. P., Hemmer, B., . . . Investigators, O. I. C. (2017). Ocrelizumab versus Interferon Beta-1a in Relapsing Multiple Sclerosis. *N Engl J Med*, 376(3), 221-234. doi:10.1056/NEJMoa1601277
- Herman, S., Akerfeldt, T., Spjuth, O., Burman, J., & Kultima, K. (2019). Biochemical Differences in Cerebrospinal Fluid between Secondary Progressive and Relapsing(-)Remitting Multiple Sclerosis. *Cells*, 8(2). doi:10.3390/cells8020084
- Herman, S., Khoonsari, P. E., Tolf, A., Steinmetz, J., Zetterberg, H., Akerfeldt, T., . . . Kultima, K. (2018). Integration of magnetic resonance imaging and protein and metabolite CSF measurements to enable early diagnosis of secondary progressive multiple sclerosis. *Theranostics*, 8(16), 4477-4490. doi:10.7150/thno.26249
- Hernandes, V. V., Barbas, C., & Dudzik, D. (2017). A review of blood sample handling and pre-processing for metabolomics studies. *Electrophoresis*, 38(18), 2232-2241. doi:10.1002/elps.201700086
- Hickman, S. J. (2007). Optic nerve imaging in multiple sclerosis. *J Neuroimaging*, 17 Suppl 1, 42S-45S. doi:10.1111/j.1552-6569.2007.00136.x
- Hinsinger, G., Galeotti, N., Nabholz, N., Urbach, S., Rigau, V., Demattei, C., . . . Thouvenot, E. (2015). Chitinase 3-like proteins as diagnostic and prognostic biomarkers of multiple sclerosis. *Mult Scler*, 21(10), 1251-1261. doi:10.1177/1352458514561906
- Hinson, S. R., Pittock, S. J., Lucchinetti, C. F., Roemer, S. F., Fryer, J. P., Kryzer, T. J., & Lennon, V. A. (2007). Pathogenic potential of IgG binding to water channel extracellular domain in neuromyelitis optica. *Neurology*, 69(24), 2221-2231. doi:10.1212/01.WNL.0000289761.64862.ce
- Hirst, C., Ingram, G., Pearson, O., Pickersgill, T., Scolding, N., & Robertson, N. (2008). Contribution of relapses to disability in multiple sclerosis. *J Neurol*, 255(2), 280-287. doi:10.1007/s00415-008-0743-8
- Hooper, D. C., Spitsin, S., Kean, R. B., Champion, J. M., Dickson, G. M., Chaudhry, I., & Koprowski, H. (1998). Uric acid, a natural scavenger of peroxynitrite, in experimental allergic encephalomyelitis and multiple sclerosis. *Proc Natl Acad Sci U S A*, 95(2), 675-680. doi:10.1073/pnas.95.2.675
- Hore, P. J. (2015). *Nuclear magnetic resonance* (Second edition. ed.). Oxford: Oxford University Press.
- Houten, S. M., Te Brinke, H., Denis, S., Ruiter, J. P., Knegt, A. C., de Klerk, J. B., . . . Duran, M. (2013). Genetic basis of hyperlysinemia. *Orphanet J Rare Dis*, 8, 57. doi:10.1186/1750-1172-8-57
- Howell, O. W., Reeves, C. A., Nicholas, R., Carassiti, D., Radotra, B., Gentleman, S. M., . . . Reynolds, R. (2011). Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. *Brain*, 134(Pt 9), 2755-2771. doi:10.1093/brain/awr182
- Hupperts, R., Smolders, J., Vieth, R., Holmoy, T., Marhardt, K., Schluep, M., . . . Group, S. S. (2019). Randomized trial of daily high-dose vitamin D3 in patients with RRMS receiving subcutaneous interferon beta-1a. *Neurology*, 93(20), e1906-e1916. doi:10.1212/WNL.00000000000008445
- Huseby, E. S., Huseby, P. G., Shah, S., Smith, R., & Stadinski, B. D. (2012). Pathogenic CD8 T cells in multiple sclerosis and its experimental models. *Front Immunol*, 3, 64. doi:10.3389/fimmu.2012.00064

- Hyun, J. W., Kim, W., Huh, S. Y., Park, M. S., Ahn, S. W., Cho, J. Y., . . . Kim, H. J. (2019). Application of the 2017 McDonald diagnostic criteria for multiple sclerosis in Korean patients with clinically isolated syndrome. *Mult Scler*, 25(11), 1488-1495. doi:10.1177/1352458518790702
- Hyun, J. W., Woodhall, M. R., Kim, S. H., Jeong, I. H., Kong, B., Kim, G., . . . Kim, H. J. (2017). Longitudinal analysis of myelin oligodendrocyte glycoprotein antibodies in CNS inflammatory diseases. *J Neurol Neurosurg Psychiatry*, 88(10), 811-817. doi:10.1136/jnnp-2017-315998
- Inglese, M., Li, B. S., Rusinek, H., Babb, J. S., Grossman, R. I., & Gonen, O. (2003). Diffusely elevated cerebral choline and creatine in relapsing-remitting multiple sclerosis. *Magn Reson Med*, 50(1), 190-195. doi:10.1002/mrm.10481
- Jarius, S., Paul, F., Franciotta, D., Waters, P., Zipp, F., Hohlfeld, R., . . . Wildemann, B. (2008). Mechanisms of disease: aquaporin-4 antibodies in neuromyelitis optica. *Nat Clin Pract Neurol*, 4(4), 202-214. doi:10.1038/ncpneuro0764
- Jarius, S., Ruprecht, K., Kleiter, I., Borisow, N., Asgari, N., Pitarokoli, K., . . . in cooperation with the Neuromyelitis Optica Study, G. (2016). MOG-IgG in NMO and related disorders: a multicenter study of 50 patients. Part 1: Frequency, syndrome specificity, influence of disease activity, long-term course, association with AQP4-IgG, and origin. *J Neuroinflammation*, 13(1), 279. doi:10.1186/s12974-016-0717-1
- Jia, X., Madireddy, L., Caillier, S., Santaniello, A., Esposito, F., Comi, G., . . . Baranzini, S. E. (2018). Genome sequencing uncovers phenocopies in primary progressive multiple sclerosis. *Ann Neurol*, 84(1), 51-63. doi:10.1002/ana.25263
- Jobard, E., Tredan, O., Postoly, D., Andre, F., Martin, A. L., Elena-Herrmann, B., & Boyault, S. (2016). A Systematic Evaluation of Blood Serum and Plasma Pre-Analytics for Metabolomics Cohort Studies. *Int J Mol Sci*, 17(12). doi:10.3390/ijms17122035
- Johnson, C. H., Ivanisevic, J., & Siuzdak, G. (2016). Metabolomics: beyond biomarkers and towards mechanisms. *Nat Rev Mol Cell Biol*, 17(7), 451-459. doi:10.1038/nrm.2016.25
- Jokubaitis, V. G., Spelman, T., Kalincik, T., Lorscheider, J., Havrdova, E., Horakova, D., . . . Group, M. S. S. (2016). Predictors of long-term disability accrual in relapse-onset multiple sclerosis. *Ann Neurol*, 80(1), 89-100. doi:10.1002/ana.24682
- Jorissen, W., Wouters, E., Bogie, J. F., Vanmierlo, T., Noben, J. P., Sviridov, D., . . . Hendriks, J. J. (2017). Relapsing-remitting multiple sclerosis patients display an altered lipoprotein profile with dysfunctional HDL. *Sci Rep*, 7, 43410. doi:10.1038/srep43410
- Jurynczyk, M., Craner, M., & Palace, J. (2015). Overlapping CNS inflammatory diseases: differentiating features of NMO and MS. *J Neurol Neurosurg Psychiatry*, 86(1), 20-25. doi:10.1136/jnnp-2014-308984
- Jurynczyk, M., Gheraldes, R., Probert, F., Woodhall, M. R., Waters, P., Tackley, G., . . . Palace, J. (2017). Distinct brain imaging characteristics of autoantibody-mediated CNS conditions and multiple sclerosis. *Brain*, 140(3), 617-627. doi:10.1093/brain/aww350
- Jurynczyk, M., Messina, S., Woodhall, M. R., Raza, N., Everett, R., Roca-Fernandez, A., . . . Palace, J. (2017a). Clinical presentation and prognosis in MOG-antibody disease: a UK study. *Brain*. doi:10.1093/brain/aww276
- Jurynczyk, M., Messina, S., Woodhall, M. R., Raza, N., Everett, R., Roca-Fernandez, A., . . . Palace, J. (2017b). Clinical presentation and prognosis in MOG-antibody disease: a UK study. *Brain*, 140(12), 3128-3138. doi:10.1093/brain/aww276
- Jurynczyk, M., Probert, F., Yeo, T., Tackley, G., Claridge, T. D. W., Cavey, A., . . . Palace, J. (2017). Metabolomics reveals distinct, antibody-independent, molecular signatures of MS, AQP4-antibody and MOG-antibody disease. *Acta Neuropathol Commun*, 5(1), 95. doi:10.1186/s40478-017-0495-8
- Jurynczyk, M., Weinshenker, B., Akman-Demir, G., Asgari, N., Barnes, D., Boggild, M., . . . Palace, J. (2016). Status of diagnostic approaches to AQP4-IgG seronegative NMO and NMO/MS overlap syndromes. *J Neurol*, 263(1), 140-149. doi:10.1007/s00415-015-7952-8

- Kalincik, T. (2015). Multiple Sclerosis Relapses: Epidemiology, Outcomes and Management. A Systematic Review. *Neuroepidemiology*, 44(4), 199-214. doi:10.1159/000382130
- Kalm, M., Bostrom, M., Sandelius, A., Eriksson, Y., Ek, C. J., Blennow, K., . . . Zetterberg, H. (2017). Serum concentrations of the axonal injury marker neurofilament light protein are not influenced by blood-brain barrier permeability. *Brain Res*, 1668, 12-19. doi:10.1016/j.brainres.2017.05.011
- Kanehisa, M., Goto, S., Sato, Y., Furumichi, M., & Tanabe, M. (2012). KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res*, 40(Database issue), D109-114. doi:10.1093/nar/gkr988
- Kaneko, K., Sato, D. K., Nakashima, I., Nishiyama, S., Tanaka, S., Marignier, R., . . . Aoki, M. (2016). Myelin injury without astrocytopathy in neuroinflammatory disorders with MOG antibodies. *J Neurol Neurosurg Psychiatry*, 87(11), 1257-1259. doi:10.1136/jnnp-2015-312676
- Kaneko, K., Sato, D. K., Nakashima, I., Ogawa, R., Akaishi, T., Takai, Y., . . . Aoki, M. (2018). CSF cytokine profile in MOG-IgG+ neurological disease is similar to AQP4-IgG+ NMOSD but distinct from MS: a cross-sectional study and potential therapeutic implications. *J Neurol Neurosurg Psychiatry*, 89(9), 927-936. doi:10.1136/jnnp-2018-317969
- Kappos, L., Bar-Or, A., Cree, B. A. C., Fox, R. J., Giovannoni, G., Gold, R., . . . Investigators, E. C. (2018). Siponimod versus placebo in secondary progressive multiple sclerosis (EXPAND): a double-blind, randomised, phase 3 study. *Lancet*, 391(10127), 1263-1273. doi:10.1016/S0140-6736(18)30475-6
- Kapur, B. M., Vandenbroucke, A. C., Adamchik, Y., Lehotay, D. C., & Carlen, P. L. (2007). Formic acid, a novel metabolite of chronic ethanol abuse, causes neurotoxicity, which is prevented by folic acid. *Alcohol Clin Exp Res*, 31(12), 2114-2120. doi:10.1111/j.1530-0277.2007.00541.x
- Kasakin, M. F., Rogachev, A. D., Predtechenskaya, E. V., Zaigraev, V. J., Koval, V. V., & Pokrovsky, A. G. (2019). Targeted metabolomics approach for identification of relapsing-remitting multiple sclerosis markers and evaluation of diagnostic models. *Medchemcomm*, 10(10), 1803-1809. doi:10.1039/c9md00253g
- Katz Sand, I., Krieger, S., Farrell, C., & Miller, A. E. (2014). Diagnostic uncertainty during the transition to secondary progressive multiple sclerosis. *Mult Scler*, 20(12), 1654-1657. doi:10.1177/1352458514521517
- Kean, R. B., Spitsin, S. V., Mikheeva, T., Scott, G. S., & Hooper, D. C. (2000). The peroxynitrite scavenger uric acid prevents inflammatory cell invasion into the central nervous system in experimental allergic encephalomyelitis through maintenance of blood-central nervous system barrier integrity. *J Immunol*, 165(11), 6511-6518. doi:10.4049/jimmunol.165.11.6511
- Kearney, H., Miller, D. H., & Ciccarelli, O. (2015). Spinal cord MRI in multiple sclerosis--diagnostic, prognostic and clinical value. *Nat Rev Neurol*, 11(6), 327-338. doi:10.1038/nrneurol.2015.80
- Keegan, M., Pineda, A. A., McClelland, R. L., Darby, C. H., Rodriguez, M., & Weinshenker, B. G. (2002). Plasma exchange for severe attacks of CNS demyelination: predictors of response. *Neurology*, 58(1), 143-146. doi:10.1212/wnl.58.1.143
- Keeler, J. (2010). *Understanding NMR spectroscopy* (2nd ed.). Chichester, U.K.: John Wiley and Sons.
- Ketelslegers, I. A., Van Pelt, D. E., Bryde, S., Neuteboom, R. F., Catsman-Berrevorts, C. E., Hamann, D., & Hintzen, R. Q. (2015). Anti-MOG antibodies plead against MS diagnosis in an Acquired Demyelinating Syndromes cohort. *Mult Scler*, 21(12), 1513-1520. doi:10.1177/1352458514566666
- Khalil, M., Teunissen, C. E., Otto, M., Piehl, F., Sormani, M. P., Gattringer, T., . . . Kuhle, J. (2018). Neurofilaments as biomarkers in neurological disorders. *Nat Rev Neurol*, 14(10), 577-589. doi:10.1038/s41582-018-0058-z
- Khovidhunkit, W., Kim, M. S., Memon, R. A., Shigenaga, J. K., Moser, A. H., Feingold, K. R., & Grunfeld, C. (2004). Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. *J Lipid Res*, 45(7), 1169-1196. doi:10.1194/jlr.R300019-JLR200

- Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., & Altman, D. G. (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol*, 8(6), e1000412. doi:10.1371/journal.pbio.1000412
- Kim, H. H., Jeong, I. H., Hyun, J. S., Kong, B. S., Kim, H. J., & Park, S. J. (2017). Metabolomic profiling of CSF in multiple sclerosis and neuromyelitis optica spectrum disorder by nuclear magnetic resonance. *PLoS One*, 12(7), e0181758. doi:10.1371/journal.pone.0181758
- Kim, H. J., Paul, F., Lana-Peixoto, M. A., Tenembaum, S., Asgari, N., Palace, J., . . . Biorepository. (2015). MRI characteristics of neuromyelitis optica spectrum disorder: an international update. *Neurology*, 84(11), 1165-1173. doi:10.1212/WNL.0000000000001367
- Kinzel, S., Lehmann-Horn, K., Torke, S., Hausler, D., Winkler, A., Stadelmann, C., . . . Weber, M. S. (2016). Myelin-reactive antibodies initiate T cell-mediated CNS autoimmune disease by opsonization of endogenous antigen. *Acta Neuropathologica*, 132(1), 43-58. doi:10.1007/s00401-016-1559-8
- Kirk, J., Plumb, J., Mirakhur, M., & McQuaid, S. (2003). Tight junctional abnormality in multiple sclerosis white matter affects all calibres of vessel and is associated with blood-brain barrier leakage and active demyelination. *J Pathol*, 201(2), 319-327. doi:10.1002/path.1434
- Kirov, I., Patil, V., Babb, J. S., Rusinek, H., Herbert, J., & Gonen, O. (2009). MR spectroscopy indicates diffuse multiple sclerosis activity during remission. *J Neurol Neurosurg Psychiatry*, 80(12), 1330-1336. doi:10.1136/jnnp.2009.176263
- Kitley, J. L., Leite, M. I., George, J. S., & Palace, J. A. (2012). The differential diagnosis of longitudinally extensive transverse myelitis. *Mult Scler*, 18(3), 271-285. doi:10.1177/1352458511406165
- Kleiter, I., Hellwig, K., Berthele, A., Kumpf, T., Linker, R. A., Hartung, H. P., . . . Neuromyelitis Optica Study, G. (2012). Failure of natalizumab to prevent relapses in neuromyelitis optica. *Arch Neurol*, 69(2), 239-245. doi:10.1001/archneurol.2011.216
- Koch-Henriksen, N., & Sorensen, P. S. (2010). The changing demographic pattern of multiple sclerosis epidemiology. *Lancet Neurol*, 9(5), 520-532. doi:10.1016/S1474-4422(10)70064-8
- Koch, M., & De Keyser, J. (2006). Uric acid in multiple sclerosis. *Neurol Res*, 28(3), 316-319. doi:10.1179/016164106X98215
- Koel-Simmelink, M. J., Vennegoor, A., Killestein, J., Blankenstein, M. A., Norgren, N., Korth, C., & Teunissen, C. E. (2014). The impact of pre-analytical variables on the stability of neurofilament proteins in CSF, determined by a novel validated SinglePlex Luminex assay and ELISA. *J Immunol Methods*, 402(1-2), 43-49. doi:10.1016/j.jim.2013.11.008
- Kremenetzky, M., Rice, G. P., Baskerville, J., Wingerchuk, D. M., & Ebers, G. C. (2006). The natural history of multiple sclerosis: a geographically based study 9: observations on the progressive phase of the disease. *Brain*, 129(Pt 3), 584-594. doi:10.1093/brain/awh721
- Kuchling, J., Ramien, C., Bozin, I., Dorr, J., Harms, L., Rosche, B., . . . Wuerfel, J. (2014). Identical lesion morphology in primary progressive and relapsing-remitting MS--an ultrahigh field MRI study. *Mult Scler*, 20(14), 1866-1871. doi:10.1177/1352458514531084
- Kuhle, J., Barro, C., Andreasson, U., Derfuss, T., Lindberg, R., Sandelius, A., . . . Zetterberg, H. (2016). Comparison of three analytical platforms for quantification of the neurofilament light chain in blood samples: ELISA, electrochemiluminescence immunoassay and Simoa. *Clin Chem Lab Med*, 54(10), 1655-1661. doi:10.1515/cclm-2015-1195
- Kuhle, J., Disanto, G., Dobson, R., Adutori, R., Bianchi, L., Topping, J., . . . Giovannoni, G. (2015). Conversion from clinically isolated syndrome to multiple sclerosis: A large multicentre study. *Mult Scler*, 21(8), 1013-1024. doi:10.1177/1352458514568827
- Kuhle, J., Kropshofer, H., Haering, D. A., Kundu, U., Meinert, R., Barro, C., . . . Kappos, L. (2019). Blood neurofilament light chain as a biomarker of MS disease activity and treatment response. *Neurology*, 92(10), e1007-e1015. doi:10.1212/WNL.0000000000007032
- Kurtzke, J. F. (1983). Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology*, 33(11), 1444-1452. doi:10.1212/wnl.33.11.1444

- Kutzelnigg, A., Lucchinetti, C. F., Stadelmann, C., Bruck, W., Rauschka, H., Bergmann, M., . . . Lassmann, H. (2005). Cortical demyelination and diffuse white matter injury in multiple sclerosis. *Brain*, 128(Pt 11), 2705-2712. doi:10.1093/brain/awh641
- Lassmann, H. (2014). Mechanisms of white matter damage in multiple sclerosis. *Glia*, 62(11), 1816-1830. doi:10.1002/glia.22597
- Lassmann, H. (2018). Pathogenic Mechanisms Associated With Different Clinical Courses of Multiple Sclerosis. *Front Immunol*, 9, 3116. doi:10.3389/fimmu.2018.03116
- Lazzarino, G., Amorini, A. M., Petzold, A., Gasperini, C., Ruggieri, S., Quartuccio, M. E., . . . Tavazzi, B. (2017). Serum Compounds of Energy Metabolism Impairment Are Related to Disability, Disease Course and Neuroimaging in Multiple Sclerosis. *Mol Neurobiol*, 54(9), 7520-7533. doi:10.1007/s12035-016-0257-9
- Lee, G., Hasan, M., Kwon, O. S., & Jung, B. H. (2019). Identification of Altered Metabolic Pathways during Disease Progression in EAE Mice via Metabolomics and Lipidomics. *Neuroscience*, 416, 74-87. doi:10.1016/j.neuroscience.2019.07.029
- Leist, T. P., Comi, G., Cree, B. A., Coyle, P. K., Freedman, M. S., Hartung, H. P., . . . oral cladribine for early, M. S. S. G. (2014). Effect of oral cladribine on time to conversion to clinically definite multiple sclerosis in patients with a first demyelinating event (ORACLE MS): a phase 3 randomised trial. *Lancet Neurol*, 13(3), 257-267. doi:10.1016/S1474-4422(14)70005-5
- Lennon, V. A., Kryzer, T. J., Pittock, S. J., Verkman, A. S., & Hinson, S. R. (2005). IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel. *J Exp Med*, 202(4), 473-477. doi:10.1084/jem.20050304
- Leray, E., Yaouanq, J., Le Page, E., Coustans, M., Laplaud, D., Oger, J., & Edan, G. (2010). Evidence for a two-stage disability progression in multiple sclerosis. *Brain*, 133(Pt 7), 1900-1913. doi:10.1093/brain/awq076
- Levin, L. I., Munger, K. L., O'Reilly, E. J., Falk, K. I., & Ascherio, A. (2010). Primary infection with the Epstein-Barr virus and risk of multiple sclerosis. *Ann Neurol*, 67(6), 824-830. doi:10.1002/ana.21978
- Liberti, M. V., & Locasale, J. W. (2016). The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem Sci*, 41(3), 211-218. doi:10.1016/j.tibs.2015.12.001
- Lim, C. K., Bilgin, A., Lovejoy, D. B., Tan, V., Bustamante, S., Taylor, B. V., . . . Guillemin, G. J. (2017). Kynurenine pathway metabolomics predicts and provides mechanistic insight into multiple sclerosis progression. *Sci Rep*, 7, 41473. doi:10.1038/srep41473
- Lim, C. K., Brew, B. J., Sundaram, G., & Guillemin, G. J. (2010). Understanding the roles of the kynurenine pathway in multiple sclerosis progression. *Int J Tryptophan Res*, 3, 157-167. doi:10.4137/ijtr.s4294
- Lin, C. C., Kuo, C. T., Cheng, C. Y., Wu, C. Y., Lee, C. W., Hsieh, H. L., . . . Yang, C. M. (2009). IL-1 beta promotes A549 cell migration via MAPKs/AP-1- and NF-kappaB-dependent matrix metalloproteinase-9 expression. *Cell Signal*, 21(11), 1652-1662. doi:10.1016/j.cellsig.2009.07.002
- Lin, C. H., Li, C. H., Yang, K. C., Lin, F. J., Wu, C. C., Chieh, J. J., & Chiu, M. J. (2019). Blood NFL: A biomarker for disease severity and progression in Parkinson disease. *Neurology*, 93(11), e1104-e1111. doi:10.1212/WNL.0000000000008088
- Lindon, J. C., Holmes, E., & Nicholson, J. K. (2003). So what's the deal with metabonomics? *Anal Chem*, 75(17), 384A-391A. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/14632032>
- Liu, X. (2012). Classification accuracy and cut point selection. *Stat Med*, 31(23), 2676-2686. doi:10.1002/sim.4509
- Liu, Z., Pardini, M., Yaldizli, O., Sethi, V., Muhlert, N., Wheeler-Kingshott, C. A., . . . Chard, D. T. (2015). Magnetization transfer ratio measures in normal-appearing white matter show periventricular gradient abnormalities in multiple sclerosis. *Brain*, 138(Pt 5), 1239-1246. doi:10.1093/brain/awv065

- Llorens, F., Thune, K., Tahir, W., Kanata, E., Diaz-Lucena, D., Xanthopoulos, K., . . . Zerr, I. (2017). YKL-40 in the brain and cerebrospinal fluid of neurodegenerative dementias. *Mol Neurodegener*, 12(1), 83. doi:10.1186/s13024-017-0226-4
- Lorscheider, J., Buzzard, K., Jokubaitis, V., Spelman, T., Havrdova, E., Horakova, D., . . . Group, M. S. S. (2016). Defining secondary progressive multiple sclerosis. *Brain*, 139(Pt 9), 2395-2405. doi:10.1093/brain/aww173
- Lotsch, J., Schiffmann, S., Schmitz, K., Brunkhorst, R., Lerch, F., Ferreiros, N., . . . Ultsch, A. (2018). Machine-learning based lipid mediator serum concentration patterns allow identification of multiple sclerosis patients with high accuracy. *Sci Rep*, 8(1), 14884. doi:10.1038/s41598-018-33077-8
- Louveau, A., Plog, B. A., Antila, S., Alitalo, K., Nedergaard, M., & Kipnis, J. (2017). Understanding the functions and relationships of the glymphatic system and meningeal lymphatics. *J Clin Invest*, 127(9), 3210-3219. doi:10.1172/JCI90603
- Lu, Y., Zhang, X., Zhao, L., Yang, C., Pan, L., Li, C., . . . Yan, Z. (2018). Metabolic Disturbances in the Striatum and Substantia Nigra in the Onset and Progression of MPTP-Induced Parkinsonism Model. *Front Neurosci*, 12, 90. doi:10.3389/fnins.2018.00090
- Lublin, F. D., Reingold, S. C., Cohen, J. A., Cutter, G. R., Sorensen, P. S., Thompson, A. J., . . . Polman, C. H. (2014). Defining the clinical course of multiple sclerosis: the 2013 revisions. *Neurology*, 83(3), 278-286. doi:10.1212/WNL.0000000000000560
- Lucchinetti, C., Bruck, W., Parisi, J., Scheithauer, B., Rodriguez, M., & Lassmann, H. (2000). Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol*, 47(6), 707-717. doi:10.1002/1531-8249(200006)47:6<707::aid-ana3>3.0.co;2-q
- Lucchinetti, C. F., Guo, Y., Popescu, B. F., Fujihara, K., Itoyama, Y., & Misu, T. (2014). The pathology of an autoimmune astrocytopathy: lessons learned from neuromyelitis optica. *Brain Pathol*, 24(1), 83-97. doi:10.1111/bpa.12099
- Luchetti, S., Fransen, N. L., van Eden, C. G., Ramaglia, V., Mason, M., & Huitinga, I. (2018). Progressive multiple sclerosis patients show substantial lesion activity that correlates with clinical disease severity and sex: a retrospective autopsy cohort analysis. *Acta Neuropathologica*, 135(4), 511-528. doi:10.1007/s00401-018-1818-y
- Lutz, N. W., Fernandez, C., Pellissier, J. F., Cozzone, P. J., & Beraud, E. (2013). Cerebral biochemical pathways in experimental autoimmune encephalomyelitis and adjuvant arthritis: a comparative metabolomic study. *PLoS One*, 8(2), e56101. doi:10.1371/journal.pone.0056101
- Lutz, N. W., Viola, A., Malikova, I., Confort-Gouny, S., Audoin, B., Ranjeva, J. P., . . . Cozzone, P. J. (2007). Inflammatory multiple-sclerosis plaques generate characteristic metabolic profiles in cerebrospinal fluid. *PLoS One*, 2(7), e595. doi:10.1371/journal.pone.0000595
- Mader, S., Gredler, V., Schanda, K., Rostasy, K., Dujmovic, I., Pfaller, K., . . . Reindl, M. (2011). Complement activating antibodies to myelin oligodendrocyte glycoprotein in neuromyelitis optica and related disorders. *J Neuroinflammation*, 8, 184. doi:10.1186/1742-2094-8-184
- Maggi, P., Absinta, M., Grammatico, M., Vuolo, L., Emmi, G., Carlucci, G., . . . Massacesi, L. (2018). Central vein sign differentiates Multiple Sclerosis from central nervous system inflammatory vasculopathies. *Ann Neurol*, 83(2), 283-294. doi:10.1002/ana.25146
- Magistretti, P. J., & Allaman, I. (2015). A cellular perspective on brain energy metabolism and functional imaging. *Neuron*, 86(4), 883-901. doi:10.1016/j.neuron.2015.03.035
- Mahmoud, S., Gharagozloo, M., Simard, C., & Gris, D. (2019). Astrocytes Maintain Glutamate Homeostasis in the CNS by Controlling the Balance between Glutamate Uptake and Release. *Cells*, 8(2). doi:10.3390/cells8020184
- Malm, L., Tybring, G., Moritz, T., Landin, B., & Galli, J. (2016). Metabolomic Quality Assessment of EDTA Plasma and Serum Samples. *Biopreserv Biobank*, 14(5), 416-423. doi:10.1089/bio.2015.0092

- Mandal, R., Guo, A. C., Chaudhary, K. K., Liu, P., Yallou, F. S., Dong, E., . . . Wishart, D. S. (2012). Multi-platform characterization of the human cerebrospinal fluid metabolome: a comprehensive and quantitative update. *Genome Med*, 4(4), 38. doi:10.1186/gm337
- Mane-Martinez, M. A., Olsson, B., Bau, L., Matas, E., Cobo-Calvo, A., Andreasson, U., . . . Zetterberg, H. (2016). Glial and neuronal markers in cerebrospinal fluid in different types of multiple sclerosis. *J Neuroimmunol*, 299, 112-117. doi:10.1016/j.jneuroim.2016.08.004
- Mangalam, A., Poisson, L., Nemutlu, E., Datta, I., Denic, A., Dzeja, P., . . . Giri, S. (2013). Profile of Circulatory Metabolites in a Relapsing-remitting Animal Model of Multiple Sclerosis using Global Metabolomics. *J Clin Cell Immunol*, 4. doi:10.4172/2155-9899.1000150
- Manouchehrinia, A., Piehl, F., Hillert, J., Kuhle, J., Alfredsson, L., Olsson, T., & Kockum, I. (2020). Confounding effect of blood volume and body mass index on blood neurofilament light chain levels. *Ann Clin Transl Neurol*. doi:10.1002/acn3.50972
- Markley, J. L., Bruschiweiler, R., Edison, A. S., Eghbalnia, H. R., Powers, R., Raftery, D., & Wishart, D. S. (2017). The future of NMR-based metabolomics. *Curr Opin Biotechnol*, 43, 34-40. doi:10.1016/j.copbio.2016.08.001
- Markowitz, C. E., Spitsin, S., Zimmerman, V., Jacobs, D., Udupa, J. K., Hooper, D. C., & Koprowski, H. (2009). The treatment of multiple sclerosis with inosine. *J Altern Complement Med*, 15(6), 619-625. doi:10.1089/acm.2008.0513
- Marshall, D. D., & Powers, R. (2017). Beyond the paradigm: Combining mass spectrometry and nuclear magnetic resonance for metabolomics. *Prog Nucl Magn Reson Spectrosc*, 100, 1-16. doi:10.1016/j.pnmrs.2017.01.001
- Martin, S. J., McGlasson, S., Hunt, D., & Overell, J. (2019). Cerebrospinal fluid neurofilament light chain in multiple sclerosis and its subtypes: a meta-analysis of case-control studies. *J Neurol Neurosurg Psychiatry*, 90(9), 1059-1067. doi:10.1136/jnnp-2018-319190
- Matthews, L., Marasco, R., Jenkinson, M., Kuker, W., Luppe, S., Leite, M. I., . . . Palace, J. (2013). Distinction of seropositive NMO spectrum disorder and MS brain lesion distribution. *Neurology*, 80(14), 1330-1337. doi:10.1212/WNL.0b013e3182887957
- Mattsson, N., Cullen, N. C., Andreasson, U., Zetterberg, H., & Blennow, K. (2019). Association Between Longitudinal Plasma Neurofilament Light and Neurodegeneration in Patients With Alzheimer Disease. *JAMA Neurol*, 76(7), 791-799. doi:10.1001/jamaneurol.2019.0765
- Matute-Blanch, C., Villar, L. M., Alvarez-Cermenio, J. C., Rejdak, K., Evdoshenko, E., Makshakov, G., . . . Comabella, M. (2018). Neurofilament light chain and oligoclonal bands are prognostic biomarkers in radiologically isolated syndrome. *Brain*, 141(4), 1085-1093. doi:10.1093/brain/awy021
- Matyszak, M. K., & Perry, V. H. (1995). Demyelination in the central nervous system following a delayed-type hypersensitivity response to bacillus Calmette-Guerin. *Neuroscience*, 64(4), 967-977. doi:10.1016/0306-4522(94)00448-e
- Mehrpour, M., Kyani, A., Tafazzoli, M., Fathi, F., & Joghataie, M. T. (2013). A metabonomics investigation of multiple sclerosis by nuclear magnetic resonance. *Magn Reson Chem*, 51(2), 102-109. doi:10.1002/mrc.3915
- Mikitsh, J. L., & Chacko, A. M. (2014). Pathways for small molecule delivery to the central nervous system across the blood-brain barrier. *Perspect Medicin Chem*, 6, 11-24. doi:10.4137/PMC.S13384
- Miller, D. H., Lublin, F. D., Sormani, M. P., Kappos, L., Yaldizli, O., Freedman, M. S., . . . Wolinsky, J. S. (2018). Brain atrophy and disability worsening in primary progressive multiple sclerosis: insights from the INFORMS study. *Ann Clin Transl Neurol*, 5(3), 346-356. doi:10.1002/acn3.534
- Mills, E. A., Mirza, A., & Mao-Draayer, Y. (2017). Emerging Approaches for Validating and Managing Multiple Sclerosis Relapse. *Front Neurol*, 8, 116. doi:10.3389/fneur.2017.00116

- Min, J. H., Kim, B. J., & Lee, K. H. (2012). Development of extensive brain lesions following fingolimod (FTY720) treatment in a patient with neuromyelitis optica spectrum disorder. *Mult Scler*, 18(1), 113-115. doi:10.1177/1352458511431973
- Moccia, M., Lanzillo, R., Costabile, T., Russo, C., Carotenuto, A., Sasso, G., . . . Brescia Morra, V. (2015). Uric acid in relapsing-remitting multiple sclerosis: a 2-year longitudinal study. *J Neurol*, 262(4), 961-967. doi:10.1007/s00415-015-7666-y
- Modvig, S., Degn, M., Roed, H., Sorensen, T. L., Larsson, H. B., Langkilde, A. R., . . . Sellebjerg, F. (2015). Cerebrospinal fluid levels of chitinase 3-like 1 and neurofilament light chain predict multiple sclerosis development and disability after optic neuritis. *Mult Scler*, 21(14), 1761-1770. doi:10.1177/1352458515574148
- Moffett, J. R., Ross, B., Arun, P., Madhavarao, C. N., & Namboodiri, A. M. (2007). N-Acetylaspartate in the CNS: from neurodiagnostics to neurobiology. *Prog Neurobiol*, 81(2), 89-131. doi:10.1016/j.pneurobio.2006.12.003
- Montalban, X., Hauser, S. L., Kappos, L., Arnold, D. L., Bar-Or, A., Comi, G., . . . Investigators, O. C. (2017). Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis. *N Engl J Med*, 376(3), 209-220. doi:10.1056/NEJMoa1606468
- Moussallieh, F. M., Elbayed, K., Chanson, J. B., Rudolf, G., Piotto, M., De Seze, J., & Namer, I. J. (2014). Serum analysis by 1H nuclear magnetic resonance spectroscopy: a new tool for distinguishing neuromyelitis optica from multiple sclerosis. *Mult Scler*, 20(5), 558-565. doi:10.1177/1352458513504638
- Nagy, A. M., Fekete, R., Horvath, G., Koncsos, G., Kriston, C., Sebestyen, A., . . . Tretter, L. (2018). Versatility of microglial bioenergetic machinery under starving conditions. *Biochim Biophys Acta Bioenerg*, 1859(3), 201-214. doi:10.1016/j.bbabi.2017.12.002
- Newcombe, J., Li, H., & Cuzner, M. L. (1994). Low density lipoprotein uptake by macrophages in multiple sclerosis plaques: implications for pathogenesis. *Neuropathol Appl Neurobiol*, 20(2), 152-162. doi:10.1111/j.1365-2990.1994.tb01174.x
- Newman, T. A., Woolley, S. T., Hughes, P. M., Sibson, N. R., Anthony, D. C., & Perry, V. H. (2001). T-cell- and macrophage-mediated axon damage in the absence of a CNS-specific immune response: involvement of metalloproteinases. *Brain*, 124, 2203-2214. doi:DOI 10.1093/brain/124.11.2203
- NICE. (2020). <https://pathways.nice.org.uk/pathways/multiple-sclerosis>.
- Nicholls, P. (1976). The effect of formate on cytochrome aa3 and on electron transport in the intact respiratory chain. *Biochim Biophys Acta*, 430(1), 13-29. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/4141>
- Nightingale. (2020). <https://nightingalehealth.com/>.
- Noga, M. J., Dane, A., Shi, S., Attali, A., van Aken, H., Suidgeest, E., . . . Hankemeier, T. (2012). Metabolomics of cerebrospinal fluid reveals changes in the central nervous system metabolism in a rat model of multiple sclerosis. *Metabolomics*, 8(2), 253-263. doi:10.1007/s11306-011-0306-3
- Nogueras, L., Gonzalo, H., Jove, M., Sol, J., Gil-Sanchez, A., Hervas, J. V., . . . Brieva, L. (2019). Lipid profile of cerebrospinal fluid in multiple sclerosis patients: a potential tool for diagnosis. *Sci Rep*, 9(1), 11313. doi:10.1038/s41598-019-47906-x
- Norata, G. D., Caligiuri, G., Chavakis, T., Matarese, G., Netea, M. G., Nicoletti, A., . . . Marelli-Berg, F. M. (2015). The Cellular and Molecular Basis of Translational Immunometabolism. *Immunity*, 43(3), 421-434. doi:10.1016/j.immuni.2015.08.023
- Nourbakhsh, B., Bhargava, P., Tremlett, H., Hart, J., Graves, J., & Waubant, E. (2018). Altered tryptophan metabolism is associated with pediatric multiple sclerosis risk and course. *Ann Clin Transl Neurol*, 5(10), 1211-1221. doi:10.1002/acn3.637
- Novakova, L., Zetterberg, H., Sundstrom, P., Axelsson, M., Khademi, M., Gunnarsson, M., . . . Lycke, J. (2017). Monitoring disease activity in multiple sclerosis using serum neurofilament light protein. *Neurology*, 89(22), 2230-2237. doi:10.1212/WNL.0000000000004683

- numares. (2020a). <https://www.numares.com/en/products/cardiovascular/axinon-lipofit-s100/technology>.
- numares. (2020b). <https://www.numares.com/en/products/neurology/multiple-sclerosis-diagnostics>.
- Nurieva, O., Diblik, P., Kuthan, P., Sklenka, P., Meliska, M., Bydzovsky, J., . . . Zakharov, S. (2018). Progressive Chronic Retinal Axonal Loss Following Acute Methanol-induced Optic Neuropathy: Four-Year Prospective Cohort Study. *Am J Ophthalmol*, 191, 100-115. doi:10.1016/j.ajo.2018.04.015
- O'Neill, L. A., Kishton, R. J., & Rathmell, J. (2016). A guide to immunometabolism for immunologists. *Nat Rev Immunol*, 16(9), 553-565. doi:10.1038/nri.2016.70
- Ogawa, R., Nakashima, I., Takahashi, T., Kaneko, K., Akaishi, T., Takai, Y., . . . Fujihara, K. (2017). MOG antibody-positive, benign, unilateral, cerebral cortical encephalitis with epilepsy. *Neurol Neuroimmunol Neuroinflamm*, 4(2), e322. doi:10.1212/NXI.0000000000000322
- Orton, S. M., Wald, L., Confavreux, C., Vukusic, S., Krohn, J. P., Ramagopalan, S. V., . . . Ebers, G. C. (2011). Association of UV radiation with multiple sclerosis prevalence and sex ratio in France. *Neurology*, 76(5), 425-431. doi:10.1212/WNL.0b013e31820a0a9f
- Padden, M., Leech, S., Craig, B., Kirk, J., Brankin, B., & McQuaid, S. (2007). Differences in expression of junctional adhesion molecule-A and beta-catenin in multiple sclerosis brain tissue: increasing evidence for the role of tight junction pathology. *Acta Neuropathologica*, 113(2), 177-186. doi:10.1007/s00401-006-0145-x
- Palace, J., Leite, M. I., Nairne, A., & Vincent, A. (2010). Interferon Beta treatment in neuromyelitis optica: increase in relapses and aquaporin 4 antibody titers. *Arch Neurol*, 67(8), 1016-1017. doi:10.1001/archneurol.2010.188
- Palmer, A. L., & Ousman, S. S. (2018). Astrocytes and Aging. *Front Aging Neurosci*, 10, 337. doi:10.3389/fnagi.2018.00337
- Palsson-McDermott, E. M., Curtis, A. M., Goel, G., Lauterbach, M. A., Sheedy, F. J., Gleeson, L. E., . . . O'Neill, L. A. (2015). Pyruvate kinase M2 regulates Hif-1alpha activity and IL-1beta induction and is a critical determinant of the warburg effect in LPS-activated macrophages. *Cell Metab*, 21(1), 65-80. doi:10.1016/j.cmet.2014.12.005
- Papadopoulos, D., Rundle, J., Patel, R., Marshall, I., Stretton, J., Eaton, R., . . . Reynolds, R. (2010). FTY720 ameliorates MOG-induced experimental autoimmune encephalomyelitis by suppressing both cellular and humoral immune responses. *J Neurosci Res*, 88(2), 346-359. doi:10.1002/jnr.22196
- Park, S. J., Jeong, I. H., Kong, B. S., Lee, J. E., Kim, K. H., Lee, D. Y., & Kim, H. J. (2016). Disease Type- and Status-Specific Alteration of CSF Metabolome Coordinated with Clinical Parameters in Inflammatory Demyelinating Diseases of CNS. *PLoS One*, 11(11), e0166277. doi:10.1371/journal.pone.0166277
- Park, Y., Le, N. A., Yu, T., Strobel, F., Gletsu-Miller, N., Accardi, C. J., . . . Jones, D. P. (2011). A sulfur amino acid-free meal increases plasma lipids in humans. *J Nutr*, 141(8), 1424-1431. doi:10.3945/jn.111.138875
- Patti, G. J., Yanes, O., & Siuzdak, G. (2012). Innovation: Metabolomics: the apogee of the omics trilogy. *Nat Rev Mol Cell Biol*, 13(4), 263-269. doi:10.1038/nrm3314
- Pearce, E. L., & Pearce, E. J. (2013). Metabolic pathways in immune cell activation and quiescence. *Immunity*, 38(4), 633-643. doi:10.1016/j.immuni.2013.04.005
- Peschl, P., Schanda, K., Zeka, B., Given, K., Bohm, D., Ruprecht, K., . . . Reindl, M. (2017). Human antibodies against the myelin oligodendrocyte glycoprotein can cause complement-dependent demyelination. *J Neuroinflammation*, 14(1), 208. doi:10.1186/s12974-017-0984-5
- Petzold, A. (2013). Intrathecal oligoclonal IgG synthesis in multiple sclerosis. *J Neuroimmunol*, 262(1-2), 1-10. doi:10.1016/j.jneuroim.2013.06.014

- Phuan, P. W., Ratelade, J., Rossi, A., Tradtrantip, L., & Verkman, A. S. (2012). Complement-dependent cytotoxicity in neuromyelitis optica requires aquaporin-4 protein assembly in orthogonal arrays. *J Biol Chem*, 287(17), 13829-13839. doi:10.1074/jbc.M112.344325
- Pieragostino, D., Cicalini, I., Lanuti, P., Ercolino, E., di Ioia, M., Zucchelli, M., . . . Del Boccio, P. (2018). Enhanced release of acid sphingomyelinase-enriched exosomes generates a lipidomics signature in CSF of Multiple Sclerosis patients. *Sci Rep*, 8(1), 3071. doi:10.1038/s41598-018-21497-5
- Pieragostino, D., D'Alessandro, M., di Ioia, M., Rossi, C., Zucchelli, M., Urbani, A., . . . Del Boccio, P. (2015). An integrated metabolomics approach for the research of new cerebrospinal fluid biomarkers of multiple sclerosis. *Mol Biosyst*, 11(6), 1563-1572. doi:10.1039/c4mb00700j
- Pinto, J., Domingues, M. R., Galhano, E., Pita, C., Almeida Mdo, C., Carreira, I. M., & Gil, A. M. (2014). Human plasma stability during handling and storage: impact on NMR metabolomics. *Analyst*, 139(5), 1168-1177. doi:10.1039/c3an02188b
- Pittock, S. J., Lennon, V. A., Krecke, K., Wingerchuk, D. M., Lucchinetti, C. F., & Weinshenker, B. G. (2006). Brain abnormalities in neuromyelitis optica. *Arch Neurol*, 63(3), 390-396. doi:10.1001/archneur.63.3.390
- Plantone, D., De Angelis, F., Doshi, A., & Chataway, J. (2016). Secondary Progressive Multiple Sclerosis: Definition and Measurement. *CNS Drugs*, 30(6), 517-526. doi:10.1007/s40263-016-0340-9
- Podbielska, M., Banik, N. L., Kurowska, E., & Hogan, E. L. (2013). Myelin recovery in multiple sclerosis: the challenge of remyelination. *Brain Sci*, 3(3), 1282-1324. doi:10.3390/brainsci3031282
- Poddighe, S., Murgia, F., Loreface, L., Liggi, S., Cocco, E., Marrosu, M. G., & Atzori, L. (2017). Metabolomic analysis identifies altered metabolic pathways in Multiple Sclerosis. *Int J Biochem Cell Biol*, 93, 148-155. doi:10.1016/j.biocel.2017.07.004
- Podlecka-Pietowska, A., Kacka, A., Zakrzewska-Pniewska, B., Nojszewska, M., Zieminska, E., Chalimoniuk, M., & Toczyłowska, B. (2019). Altered Cerebrospinal Fluid Concentrations of Hydrophobic and Hydrophilic Compounds in Early Stages of Multiple Sclerosis-Metabolic Profile Analyses. *J Mol Neurosci*, 69(1), 94-105. doi:10.1007/s12031-019-01336-6
- Poisson, L. M., Suhail, H., Singh, J., Datta, I., Denic, A., Labuzek, K., . . . Giri, S. (2015). Untargeted Plasma Metabolomics Identifies Endogenous Metabolite with Drug-like Properties in Chronic Animal Model of Multiple Sclerosis. *J Biol Chem*, 290(52), 30697-30712. doi:10.1074/jbc.M115.679068
- Pontes, T. A., Barbosa, A. D., Silva, R. D., Melo-Junior, M. R., & Silva, R. O. (2019). Osteopenia-osteoporosis discrimination in postmenopausal women by ¹H NMR-based metabolomics. *PLoS One*, 14(5), e0217348. doi:10.1371/journal.pone.0217348
- Poser, C. M., Paty, D. W., Scheinberg, L., McDonald, W. I., Davis, F. A., Ebers, G. C., . . . Tourtellotte, W. W. (1983). New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol*, 13(3), 227-231. doi:10.1002/ana.410130302
- Pranger, I. G., van Raalte, D. H., Brands, M., Muskiet, M. H. A., Kema, I. P., Serlie, M. J., . . . Muskiet, F. A. J. (2018). Influence of prednisolone on parameters of de novo lipogenesis and indices for stearoyl-CoA- and Delta6- desaturase activity in healthy males: A Post-hoc analysis of a randomized, placebo-controlled, double-blind trial. *Prostaglandins Leukot Essent Fatty Acids*, 132, 8-15. doi:10.1016/j.plefa.2018.03.009
- Preisiche, O., Schultz, S. A., Apel, A., Kuhle, J., Kaeser, S. A., Barro, C., . . . Dominantly Inherited Alzheimer, N. (2019). Serum neurofilament dynamics predicts neurodegeneration and clinical progression in presymptomatic Alzheimer's disease. *Nat Med*, 25(2), 277-283. doi:10.1038/s41591-018-0304-3
- Pryce, G., & Baker, D. (2018). Oligoclonal bands in multiple sclerosis; Functional significance and therapeutic implications. Does the specificity matter? *Mult Scler Relat Disord*, 25, 131-137. doi:10.1016/j.msard.2018.07.030

- Psychogios, N., Hau, D. D., Peng, J., Guo, A. C., Mandal, R., Bouatra, S., . . . Wishart, D. S. (2011). The human serum metabolome. *PLoS One*, 6(2), e16957. doi:10.1371/journal.pone.0016957
- Querol-Vilaseca, M., Colom-Cadena, M., Pegueroles, J., San Martin-Paniello, C., Clarimon, J., Belbin, O., . . . Lleó, A. (2017). YKL-40 (Chitinase 3-like I) is expressed in a subset of astrocytes in Alzheimer's disease and other tauopathies. *J Neuroinflammation*, 14(1), 118. doi:10.1186/s12974-017-0893-7
- Quintana, F. J., Yeste, A., Weiner, H. L., & Covacu, R. (2012). Lipids and lipid-reactive antibodies as biomarkers for multiple sclerosis. *J Neuroimmunol*, 248(1-2), 53-57. doi:10.1016/j.jneuroim.2012.01.002
- R Core Team (R Foundation for Statistical Computing V, A. (2013). R: A language and environment for statistical computing.
- Rae, C. D. (2014). A guide to the metabolic pathways and function of metabolites observed in human brain 1H magnetic resonance spectra. *Neurochem Res*, 39(1), 1-36. doi:10.1007/s11064-013-1199-5
- Ramalho, J., Semelka, R. C., Ramalho, M., Nunes, R. H., AlObaidy, M., & Castillo, M. (2016). Gadolinium-Based Contrast Agent Accumulation and Toxicity: An Update. *AJNR Am J Neuroradiol*, 37(7), 1192-1198. doi:10.3174/ajnr.A4615
- Ramanathan, S., Reddel, S. W., Henderson, A., Parratt, J. D., Barnett, M., Gatt, P. N., . . . Brilot, F. (2014). Antibodies to myelin oligodendrocyte glycoprotein in bilateral and recurrent optic neuritis. *Neurol Neuroimmunol Neuroinflamm*, 1(4), e40. doi:10.1212/NXI.0000000000000040
- Rao, M. V., Campbell, J., Yuan, A., Kumar, A., Gotow, T., Uchiyama, Y., & Nixon, R. A. (2003). The neurofilament middle molecular mass subunit carboxyl-terminal tail domains is essential for the radial growth and cytoskeletal architecture of axons but not for regulating neurofilament transport rate. *J Cell Biol*, 163(5), 1021-1031. doi:10.1083/jcb.200308076
- Rao, M. V., Yuan, A., Campbell, J., Kumar, A., & Nixon, R. A. (2012). The C-terminal domains of NF-H and NF-M subunits maintain axonal neurofilament content by blocking turnover of the stationary neurofilament network. *PLoS One*, 7(9), e44320. doi:10.1371/journal.pone.0044320
- Regenold, W. T., Phatak, P., Makley, M. J., Stone, R. D., & Kling, M. A. (2008). Cerebrospinal fluid evidence of increased extra-mitochondrial glucose metabolism implicates mitochondrial dysfunction in multiple sclerosis disease progression. *J Neurol Sci*, 275(1-2), 106-112. doi:10.1016/j.jns.2008.07.032
- Reich, D. S., Lucchinetti, C. F., & Calabresi, P. A. (2018). Multiple Sclerosis. *N Engl J Med*, 378(2), 169-180. doi:10.1056/NEJMr1401483
- Reindl, M., & Waters, P. (2019). Myelin oligodendrocyte glycoprotein antibodies in neurological disease. *Nat Rev Neurol*, 15(2), 89-102. doi:10.1038/s41582-018-0112-x
- Reinke, S. N., Broadhurst, D. L., Sykes, B. D., Baker, G. B., Catz, I., Warren, K. G., & Power, C. (2014). Metabolomic profiling in multiple sclerosis: insights into biomarkers and pathogenesis. *Mult Scler*, 20(10), 1396-1400. doi:10.1177/1352458513516528
- Robinson, R. R., Dietz, A. K., Maroof, A. M., Asmis, R., & Forsthuber, T. G. (2019). The role of glial-neuronal metabolic cooperation in modulating progression of multiple sclerosis and neuropathic pain. *Immunotherapy*, 11(2), 129-147. doi:10.2217/imt-2018-0153
- Rocca, M. A., Battaglini, M., Benedict, R. H., De Stefano, N., Geurts, J. J., Henry, R. G., . . . Filippi, M. (2017). Brain MRI atrophy quantification in MS: From methods to clinical application. *Neurology*, 88(4), 403-413. doi:10.1212/WNL.0000000000003542
- Rush, C. A., MacLean, H. J., & Freedman, M. S. (2015). Aggressive multiple sclerosis: proposed definition and treatment algorithm. *Nat Rev Neurol*, 11(7), 379-389. doi:10.1038/nrneurol.2015.85
- Saadoun, S., Waters, P., Bell, B. A., Vincent, A., Verkman, A. S., & Papadopoulos, M. C. (2010). Intracerebral injection of neuromyelitis optica immunoglobulin G and human complement

- produces neuromyelitis optica lesions in mice. *Brain*, 133(Pt 2), 349-361. doi:10.1093/brain/awp309
- Saadoun, S., Waters, P., Owens, G. P., Bennett, J. L., Vincent, A., & Papadopoulos, M. C. (2014). Neuromyelitis optica MOG-IgG causes reversible lesions in mouse brain. *Acta Neuropathol Commun*, 2, 35. doi:10.1186/2051-5960-2-35
- Scalfari, A., Neuhaus, A., Degenhardt, A., Rice, G. P., Muraro, P. A., Daumer, M., & Ebers, G. C. (2010). The natural history of multiple sclerosis: a geographically based study 10: relapses and long-term disability. *Brain*, 133(Pt 7), 1914-1929. doi:10.1093/brain/awq118
- Schilling, S., Goelz, S., Linker, R., Luehder, F., & Gold, R. (2006). Fumaric acid esters are effective in chronic experimental autoimmune encephalomyelitis and suppress macrophage infiltration. *Clin Exp Immunol*, 145(1), 101-107. doi:10.1111/j.1365-2249.2006.03094.x
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., . . . Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods*, 9(7), 676-682. doi:10.1038/nmeth.2019
- Schwarzschild, M. A., Macklin, E. A., Bakshi, R., Battacharyya, S., Logan, R., Espay, A. J., . . . Parkinson Study Group, S.-P. D. I. (2019). Sex differences by design and outcome in the Safety of Urate Elevation in PD (SURE-PD) trial. *Neurology*, 93(14), e1328-e1338. doi:10.1212/WNL.0000000000008194
- Scott, L. J. (2013). Glatiramer acetate: a review of its use in patients with relapsing-remitting multiple sclerosis and in delaying the onset of clinically definite multiple sclerosis. *CNS Drugs*, 27(11), 971-988. doi:10.1007/s40263-013-0117-3
- Senanayake, V. K., Jin, W., Mochizuki, A., Chitou, B., & Goodenowe, D. B. (2015). Metabolic dysfunctions in multiple sclerosis: implications as to causation, early detection, and treatment, a case control study. *BMC Neurol*, 15, 154. doi:10.1186/s12883-015-0411-4
- Serafini, B., Rosicarelli, B., Magliozzi, R., Stigliano, E., & Aloisi, F. (2004). Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. *Brain Pathol*, 14(2), 164-174. doi:10.1111/j.1750-3639.2004.tb00049.x
- Shahim, P., Zetterberg, H., Tegner, Y., & Blennow, K. (2017). Serum neurofilament light as a biomarker for mild traumatic brain injury in contact sports. *Neurology*, 88(19), 1788-1794. doi:10.1212/WNL.0000000000003912
- Sharpe, J. A., Hostovsky, M., Bilbao, J. M., & Rewcastle, N. B. (1982). Methanol optic neuropathy: a histopathological study. *Neurology*, 32(10), 1093-1100. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/6889696>
- Shimizu, J., Hatanaka, Y., Hasegawa, M., Iwata, A., Sugimoto, I., Date, H., . . . Tsuji, S. (2010). IFNbeta-1b may severely exacerbate Japanese optic-spinal MS in neuromyelitis optica spectrum. *Neurology*, 75(16), 1423-1427. doi:10.1212/WNL.0b013e3181f8832e
- Shu, Y., Long, Y., Wang, S., Hu, W., Zhou, J., Xu, H., . . . Qiu, W. (2019). Brain histopathological study and prognosis in MOG antibody-associated demyelinating pseudotumor. *Ann Clin Transl Neurol*, 6(2), 392-396. doi:10.1002/acn3.712
- Siegel, G. J. (1999). *Basic neurochemistry : molecular, cellular, and medical aspects* (6th ed.). Philadelphia: Lippincott Williams & Wilkins.
- Simone, I. L., Federico, F., Trojano, M., Tortorella, C., Liguori, M., Giannini, P., . . . Livrea, P. (1996). High resolution proton MR spectroscopy of cerebrospinal fluid in MS patients. Comparison with biochemical changes in demyelinating plaques. *J Neurol Sci*, 144(1-2), 182-190. doi:10.1016/s0022-510x(96)00224-9
- Simoyi, M. F., Falkenstein, E., Van Dyke, K., Blemings, K. P., & Klandorf, H. (2003). Allantoin, the oxidation product of uric acid is present in chicken and turkey plasma. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 135(2), 325-335. doi:10.1016/S1096-4959(03)00086-1
- Singh, J., Cerghet, M., Poisson, L. M., Datta, I., Labuzek, K., Suhail, H., . . . Giri, S. (2019). Urinary and Plasma Metabolomics Identify the Distinct Metabolic Profile of Disease State in Chronic

- Mouse Model of Multiple Sclerosis. *J Neuroimmune Pharmacol*, 14(2), 241-250. doi:10.1007/s11481-018-9815-4
- Sinnecker, T., Clarke, M. A., Meier, D., Enzinger, C., Calabrese, M., De Stefano, N., . . . Group, M. S. (2019). Evaluation of the Central Vein Sign as a Diagnostic Imaging Biomarker in Multiple Sclerosis. *JAMA Neurol*. doi:10.1001/jamaneurol.2019.2478
- Skripuletz, T., Manzel, A., Gropengiesser, K., Schafer, N., Gudi, V., Singh, V., . . . Stangel, M. (2015). Pivotal role of choline metabolites in remyelination. *Brain*, 138(Pt 2), 398-413. doi:10.1093/brain/awu358
- Soininen, P., Kangas, A. J., Wurtz, P., Tukiainen, T., Tynkkynen, T., Laatikainen, R., . . . Ala-Korpela, M. (2009). High-throughput serum NMR metabolomics for cost-effective holistic studies on systemic metabolism. *Analyst*, 134(9), 1781-1785. doi:10.1039/b910205a
- Solomon, A. J. (2019). Instead of tweaking the diagnostic criteria for MS in those with CIS, we should develop diagnostic criteria that distinguish MS from other conditions - Yes. *Mult Scler*, 25(6), 766-768. doi:10.1177/1352458518813107
- Soylu-Kucharz, R., Sandelius, A., Sjogren, M., Blennow, K., Wild, E. J., Zetterberg, H., & Bjorkqvist, M. (2017). Neurofilament light protein in CSF and blood is associated with neurodegeneration and disease severity in Huntington's disease R6/2 mice. *Sci Rep*, 7(1), 14114. doi:10.1038/s41598-017-14179-1
- Spadaro, M., Gerdes, L. A., Krumbholz, M., Ertl-Wagner, B., Thaler, F. S., Schuh, E., . . . Kumpfel, T. (2016). Autoantibodies to MOG in a distinct subgroup of adult multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm*, 3(5), e257. doi:10.1212/NXI.0000000000000257
- Spadaro, M., Gerdes, L. A., Mayer, M. C., Ertl-Wagner, B., Laurent, S., Krumbholz, M., . . . Kumpfel, T. (2015). Histopathology and clinical course of MOG-antibody-associated encephalomyelitis. *Ann Clin Transl Neurol*, 2(3), 295-301. doi:10.1002/acn3.164
- Squadrito, G. L., Cueto, R., Splenser, A. E., Valavanidis, A., Zhang, H., Uppu, R. M., & Pryor, W. A. (2000). Reaction of uric acid with peroxynitrite and implications for the mechanism of neuroprotection by uric acid. *Arch Biochem Biophys*, 376(2), 333-337. doi:10.1006/abbi.2000.1721
- Steinman, L. (2014). Immunology of relapse and remission in multiple sclerosis. *Annu Rev Immunol*, 32, 257-281. doi:10.1146/annurev-immunol-032713-120227
- Stellmann, J. P., Krumbholz, M., Friede, T., Gahlen, A., Borisow, N., Fischer, K., . . . Nemos. (2017). Immunotherapies in neuromyelitis optica spectrum disorder: efficacy and predictors of response. *J Neurol Neurosurg Psychiatry*, 88(8), 639-647. doi:10.1136/jnnp-2017-315603
- Stoessel, D., Stellmann, J. P., Willing, A., Behrens, B., Rosenkranz, S. C., Hodecker, S. C., . . . Pless, O. (2018). Metabolomic Profiles for Primary Progressive Multiple Sclerosis Stratification and Disease Course Monitoring. *Front Hum Neurosci*, 12, 226. doi:10.3389/fnhum.2018.00226
- Svendsen, B., Grytten, N., Bo, L., Aarseth, H., Smedal, T., & Myhr, K. M. (2018). The economic impact of multiple sclerosis to the patients and their families in Norway. *Eur J Health Econ*, 19(9), 1243-1257. doi:10.1007/s10198-018-0971-5
- Tartaglia, M. C., Narayanan, S., De Stefano, N., Arnaoutelis, R., Antel, S. B., Francis, S. J., . . . Arnold, D. L. (2002). Choline is increased in pre-lesional normal appearing white matter in multiple sclerosis. *Journal of Neurology*, 249(10), 1382-1390. doi:10.1007/s00415-002-0846-6
- Tavazzi, B., Batocchi, A. P., Amorini, A. M., Nociti, V., D'Urso, S., Longo, S., . . . Lazzarino, G. (2011). Serum metabolic profile in multiple sclerosis patients. *Mult Scler Int*, 2011, 167156. doi:10.1155/2011/167156
- Teahan, O., Gamble, S., Holmes, E., Waxman, J., Nicholson, J. K., Bevan, C., & Keun, H. C. (2006). Impact of analytical bias in metabolomic studies of human blood serum and plasma. *Anal Chem*, 78(13), 4307-4318. doi:10.1021/ac051972y
- Thevenot, E. A., Roux, A., Xu, Y., Ezan, E., & Junot, C. (2015). Analysis of the Human Adult Urinary Metabolome Variations with Age, Body Mass Index, and Gender by Implementing a

- Comprehensive Workflow for Univariate and OPLS Statistical Analyses. *J Proteome Res*, 14(8), 3322-3335. doi:10.1021/acs.jproteome.5b00354
- Thompson, A. J., Banwell, B. L., Barkhof, F., Carroll, W. M., Coetzee, T., Comi, G., . . . Cohen, J. A. (2018). Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. *Lancet Neurol*, 17(2), 162-173. doi:10.1016/S1474-4422(17)30470-2
- Thouvenot, E., Hisinger, G., Demattei, C., Uygunoglu, U., Castelnovo, G., Pittion-Vouyovitch, S., . . . Lebrun, C. (2019). Cerebrospinal fluid chitinase-3-like protein 1 level is not an independent predictive factor for the risk of clinical conversion in radiologically isolated syndrome. *Mult Scler*, 25(5), 669-677. doi:10.1177/1352458518767043
- Tintore, M., Rovira, A., Rio, J., Otero-Romero, S., Arrambide, G., Tur, C., . . . Montalban, X. (2015). Defining high, medium and low impact prognostic factors for developing multiple sclerosis. *Brain*, 138(Pt 7), 1863-1874. doi:10.1093/brain/awv105
- Torres, A., Luke, J. D., Kullas, A. L., Kapilashrami, K., Botbol, Y., Koller, A., . . . van der Velden, A. W. (2016). Asparagine deprivation mediated by Salmonella asparaginase causes suppression of activation-induced T cell metabolic reprogramming. *J Leukoc Biol*, 99(2), 387-398. doi:10.1189/jlb.4A0615-252R
- Treichel, J. L., Henry, M. M., Skumatz, C. M., Eells, J. T., & Burke, J. M. (2003). Formate, the toxic metabolite of methanol, in cultured ocular cells. *Neurotoxicology*, 24(6), 825-834. doi:10.1016/S0161-813X(03)00059-7
- Tremlett, H., Yinshan, Z., & Devonshire, V. (2008). Natural history of secondary-progressive multiple sclerosis. *Mult Scler*, 14(3), 314-324. doi:10.1177/1352458507084264
- Tremlett, H., & Zhao, Y. (2017). Primary and secondary progressive MS have a similar age at onset of progression - NO. *Mult Scler*, 23(5), 640-642. doi:10.1177/1352458516684559
- Uitdehaag, B. M. J. (2018). Disability Outcome Measures in Phase III Clinical Trials in Multiple Sclerosis. *CNS Drugs*, 32(6), 543-558. doi:10.1007/s40263-018-0530-8
- van der Sar, S. A., Zielman, R., Terwindt, G. M., van den Maagdenberg, A. M., Deelder, A. M., Mayboroda, O. A., . . . Ferrari, M. D. (2015). Ethanol contamination of cerebrospinal fluid during standardized sampling and its effect on (1)H-NMR metabolomics. *Anal Bioanal Chem*, 407(16), 4835-4839. doi:10.1007/s00216-015-8663-9
- van der Vurst de Vries, R. M., Mescheriakova, J. Y., Wong, Y. Y. M., Runia, T. F., Jafari, N., Samijn, J. P., . . . Hintzen, R. Q. (2018). Application of the 2017 Revised McDonald Criteria for Multiple Sclerosis to Patients With a Typical Clinically Isolated Syndrome. *JAMA Neurol*, 75(11), 1392-1398. doi:10.1001/jamaneurol.2018.2160
- Varhaug, K. N., Barro, C., Bjornevik, K., Myhr, K. M., Torkildsen, O., Wergeland, S., . . . Vedeler, C. (2018). Neurofilament light chain predicts disease activity in relapsing-remitting MS. *Neurol Neuroimmunol Neuroinflamm*, 5(1), e422. doi:10.1212/NXI.0000000000000422
- Verde, F., Steinacker, P., Weishaupt, J. H., Kassubek, J., Oeckl, P., Halbgebauer, S., . . . Otto, M. (2019). Neurofilament light chain in serum for the diagnosis of amyotrophic lateral sclerosis. *J Neurol Neurosurg Psychiatry*, 90(2), 157-164. doi:10.1136/jnnp-2018-318704
- Verwaest, K. A., Vu, T. N., Laukens, K., Clemens, L. E., Nguyen, H. P., Van Gasse, B., . . . Dommissie, R. (2011). (1)H NMR based metabolomics of CSF and blood serum: a metabolic profile for a transgenic rat model of Huntington disease. *Biochim Biophys Acta*, 1812(11), 1371-1379. doi:10.1016/j.bbadis.2011.08.001
- Vidaurre, O. G., Haines, J. D., Katz Sand, I., Adula, K. P., Huynh, J. L., McGraw, C. A., . . . Casaccia, P. (2014). Cerebrospinal fluid ceramides from patients with multiple sclerosis impair neuronal bioenergetics. *Brain*, 137(Pt 8), 2271-2286. doi:10.1093/brain/awu139
- Villoslada, P., Alonso, C., Agirrezabal, I., Kotelnikova, E., Zubizarreta, I., Pulido-Valdeolivas, I., . . . Castro, A. (2017). Metabolomic signatures associated with disease severity in multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm*, 4(2), e321. doi:10.1212/NXI.0000000000000321

- Vukusic, S., & Confavreux, C. (2003). Prognostic factors for progression of disability in the secondary progressive phase of multiple sclerosis. *J Neurol Sci*, 206(2), 135-137. doi:10.1016/s0022-510x(02)00426-4
- Wallace, C., Smirl, J. D., Zetterberg, H., Blennow, K., Bryk, K., Burma, J., . . . van Donkelaar, P. (2018). Heading in soccer increases serum neurofilament light protein and SCAT3 symptom metrics. *BMJ Open Sport Exerc Med*, 4(1), e000433. doi:10.1136/bmjsem-2018-000433
- Wang, H., & Eckel, R. H. (2014). What are lipoproteins doing in the brain? *Trends Endocrinol Metab*, 25(1), 8-14. doi:10.1016/j.tem.2013.10.003
- Wang, L., Hu, W., Wang, J., Qian, W., & Xiao, H. (2016). Low serum uric acid levels in patients with multiple sclerosis and neuromyelitis optica: An updated meta-analysis. *Mult Scler Relat Disord*, 9, 17-22. doi:10.1016/j.msard.2016.05.008
- Waters, P., Woodhall, M., O'Connor, K. C., Reindl, M., Lang, B., Sato, D. K., . . . Vincent, A. (2015). MOG cell-based assay detects non-MS patients with inflammatory neurologic disease. *Neurol Neuroimmunol Neuroinflamm*, 2(3), e89. doi:10.1212/NXI.0000000000000089
- Waters, P. J., McKeon, A., Leite, M. I., Rajasekharan, S., Lennon, V. A., Villalobos, A., . . . Pittock, S. J. (2012). Serologic diagnosis of NMO: a multicenter comparison of aquaporin-4-IgG assays. *Neurology*, 78(9), 665-671; discussion 669. doi:10.1212/WNL.0b013e318248dec1
- Watford, M. (2015). Glutamine and glutamate: Nonessential or essential amino acids? *Anim Nutr*, 1(3), 119-122. doi:10.1016/j.aninu.2015.08.008
- Wellen, K. E., Hatzivassiliou, G., Sachdeva, U. M., Bui, T. V., Cross, J. R., & Thompson, C. B. (2009). ATP-citrate lyase links cellular metabolism to histone acetylation. *Science*, 324(5930), 1076-1080. doi:10.1126/science.1164097
- Westerhuis, J. A., Hoefsloot, H.C.J., Smit, S. et al. (2008). Assessment of PLS-DA cross validation. *Metabolomics*, 4(1), 81-89.
- Whittam, D., Bhojak, M., Das, K., & Jacob, A. (2017). Longitudinally extensive myelitis in MS mimicking neuromyelitis optica. *Neurol Neuroimmunol Neuroinflamm*, 4(3), e333. doi:10.1212/NXI.0000000000000333
- Wildemann, B., Jarius, S., Schwarz, A., Diem, R., Viehover, A., Hahnel, S., . . . Korpenthal-Kuhnke, M. (2017). Failure of alemtuzumab therapy to control MOG encephalomyelitis. *Neurology*, 89(2), 207-209. doi:10.1212/WNL.00000000000004087
- Wilke, C., Preische, O., Deusche, C., Roeben, B., Apel, A., Barro, C., . . . Synofzik, M. (2016). Neurofilament light chain in FTD is elevated not only in cerebrospinal fluid, but also in serum. *J Neurol Neurosurg Psychiatry*, 87(11), 1270-1272. doi:10.1136/jnnp-2015-312972
- Wingerchuk, D. M., Banwell, B., Bennett, J. L., Cabre, P., Carroll, W., Chitnis, T., . . . International Panel for, N. M. O. D. (2015). International consensus diagnostic criteria for neuromyelitis optica spectrum disorders. *Neurology*, 85(2), 177-189. doi:10.1212/WNL.00000000000001729
- Wishart, D. S. (2016). Emerging applications of metabolomics in drug discovery and precision medicine. *Nat Rev Drug Discov*, 15(7), 473-484. doi:10.1038/nrd.2016.32
- Wishart, D. S., Feunang, Y. D., Marcu, A., Guo, A. C., Liang, K., Vazquez-Fresno, R., . . . Scalbert, A. (2018). HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Res*, 46(D1), D608-D617. doi:10.1093/nar/gkx1089
- Witte, M. E., Mahad, D. J., Lassmann, H., & van Horssen, J. (2014). Mitochondrial dysfunction contributes to neurodegeneration in multiple sclerosis. *Trends Mol Med*, 20(3), 179-187. doi:10.1016/j.molmed.2013.11.007
- Wolburg, H., Wolburg-Buchholz, K., & Engelhardt, B. (2005). Diapedesis of mononuclear cells across cerebral venules during experimental autoimmune encephalomyelitis leaves tight junctions intact. *Acta Neuropathologica*, 109(2), 181-190. doi:10.1007/s00401-004-0928-x
- Worley, B., & Powers, R. (2013). Multivariate Analysis in Metabolomics. *Curr Metabolomics*, 1(1), 92-107. doi:10.2174/2213235X11301010092
- Worley, B., & Powers, R. (2016). PCA as a practical indicator of OPLS-DA model reliability. *Curr Metabolomics*, 4(2), 97-103. doi:10.2174/2213235X04666160613122429

- Yamauchi, M., & Sricholpech, M. (2012). Lysine post-translational modifications of collagen. *Essays Biochem*, 52, 113-133. doi:10.1042/bse0520113
- Yamout, B. I., Beaini, S., Zeineddine, M. M., & Akkawi, N. (2017). Catastrophic relapses following initiation of dimethyl fumarate in two patients with neuromyelitis optica spectrum disorder. *Mult Scler*, 23(9), 1297-1300. doi:10.1177/1352458517694086
- Yang, W., Chen, Y., Xi, C., Zhang, R., Song, Y., Zhan, Q., . . . Abliz, Z. (2013). Liquid chromatography-tandem mass spectrometry-based plasma metabolomics delineate the effect of metabolites' stability on reliability of potential biomarkers. *Anal Chem*, 85(5), 2606-2610. doi:10.1021/ac303576b
- Yeo, T., Probert, F., Jurynczyk, M., Sealey, M., Cavey, A., Claridge, T. D. W., . . . Palace, J. (2019). Classifying the antibody-negative NMO syndromes: Clinical, imaging, and metabolomic modeling. *Neurol Neuroimmunol Neuroinflamm*, 6(6), e626. doi:10.1212/NXI.0000000000000626
- Yin, P., Lehmann, R., & Xu, G. (2015). Effects of pre-analytical processes on blood samples used in metabolomics studies. *Anal Bioanal Chem*, 407(17), 4879-4892. doi:10.1007/s00216-015-8565-x
- Yin, P., Peter, A., Franken, H., Zhao, X., Neukamm, S. S., Rosenbaum, L., . . . Lehmann, R. (2013). Preanalytical aspects and sample quality assessment in metabolomics studies of human blood. *Clin Chem*, 59(5), 833-845. doi:10.1373/clinchem.2012.199257
- Yuan, A., Rao, M. V., Veeranna, & Nixon, R. A. (2017). Neurofilaments and Neurofilament Proteins in Health and Disease. *Cold Spring Harb Perspect Biol*, 9(4). doi:10.1101/cshperspect.a018309
- Yuan, A., Sasaki, T., Rao, M. V., Kumar, A., Kanumuri, V., Dunlop, D. S., . . . Nixon, R. A. (2009). Neurofilaments form a highly stable stationary cytoskeleton after reaching a critical level in axons. *J Neurosci*, 29(36), 11316-11329. doi:10.1523/JNEUROSCI.1942-09.2009
- Yuan, A., Sershen, H., Veeranna, Basavarajappa, B. S., Kumar, A., Hashim, A., . . . Nixon, R. A. (2015). Neurofilament subunits are integral components of synapses and modulate neurotransmission and behavior in vivo. *Mol Psychiatry*, 20(8), 986-994. doi:10.1038/mp.2015.45
- Zhang, H., Bennett, J. L., & Verkman, A. S. (2011). Ex vivo spinal cord slice model of neuromyelitis optica reveals novel immunopathogenic mechanisms. *Ann Neurol*, 70(6), 943-954. doi:10.1002/ana.22551
- Zhang, S., Zeng, X., Ren, M., Mao, X., & Qiao, S. (2017). Novel metabolic and physiological functions of branched chain amino acids: a review. *J Anim Sci Biotechnol*, 8, 10. doi:10.1186/s40104-016-0139-z
- Zlokovic, B. V. (2011). Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. *Nat Rev Neurosci*, 12(12), 723-738. doi:10.1038/nrn3114