

Metabolic Signatures of Post-Viral Syndromes:
Insights from Long COVID, Experimental
Models, and Comparative Studies



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Abstract

Post-viral syndromes, such as long COVID, represent a significant and growing public health challenge, yet their underlying biological mechanisms remain incompletely understood. This thesis explores the molecular basis of the host response to viral illness and its long-term sequelae through an integrated, multi-modal analysis spanning behaviour, inflammation, and metabolism. In a foundational murine study, distinct sex-dependent behavioural and metabolic responses to TLR-4 and TLR-7 activation were identified, revealing divergent immune-metabolic pathways and highlighting N-acetyl aspartate as a potential marker of resilience. Building on this, a longitudinal human study applied ^1H NMR metabolomics to serum and urine from individuals with long COVID and fully recovered controls. Multivariate analyses uncovered robust metabolic signatures distinguishing long COVID, with subtypes linked to systemic inflammation or altered neuroendocrine stress responses. Amino acid availability emerged as a key determinant of symptom persistence. Expanding this work, a multi-omics analysis of fibromyalgia (FM) revealed shared metabolic disruptions with the non-recovered long COVID patients in amino acid pathways (notably glutamine and histidine), alongside elevated central CRP, supporting a role for neuroinflammation and metabolic dysfunction in pain sensitisation. Quantitative sensory testing further identified biologically distinct FM subtypes based on pain thresholds. Parallel work in osteoarthritis patients used serum and CSF metabolomics to identify glutamine and histidine as consistent markers of pain and disability, strengthening the case for a shared metabolic substrate across chronic pain states. Together, these studies demonstrate that chronic post-viral syndromes are underpinned by measurable metabolic and inflammatory alterations, affecting both central and peripheral systems, and that as these post viral syndromes evolve and for long COVID they seem to move toward a metabolic signature that is similar to FM. By combining metabolomics, proteomics, and sensory phenotyping, this work identifies candidate biomarkers and biological subtypes, laying a foundation for more accurate diagnostics and personalised therapeutic approaches to complex, chronic conditions emerging in the aftermath of viral illness.

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Table of Abbreviations

Abbreviation	Definition
¹ H NMR	Proton Nuclear Magnetic Resonance
AUC	Area Under the Curve
BMI	Body Mass Index
BCAA	Branched-Chain Amino Acids
CNS	Central Nervous System
CRP	C-reactive Protein
CSF	Cerebrospinal Fluid
CXCL	C-X-C Motif Chemokine Ligand
DAMP	Damage-Associated Molecular Pattern
FACIT-F	Functional Assessment of Chronic Illness Therapy – Fatigue
FDR	False Discovery Rate
FEV	Forced Expiratory Volume
FVC	Forced Vital Capacity
FM	Fibromyalgia
GlycA	Glycoprotein Acetylation (N-acetylglycoproteins)
GCP	Good Clinical Practice
HDC	Histidine Decarboxylase
HPX-MRI / XeMRI	Hyperpolarised Xenon Magnetic Resonance Imaging
ISI	Insomnia Severity Index
LC	Long COVID
LPS	Lipopolysaccharide (TLR4 agonist)

Abbreviation	Definition
MRI	Magnetic Resonance Imaging
NAA	N-acetylaspartate
NICE	National Institute for Health and Care Excellence
NMR	Nuclear Magnetic Resonance
OA	Osteoarthritis
OPLS-DA	Orthogonal Partial Least Squares–Discriminant Analysis
PAMP	Pathogen-Associated Molecular Pattern
PCS/MCS	Physical/Mental Component Summary (from SF-12)
PHQ-9	Patient Health Questionnaire-9
PFC	Prefrontal Cortex
QOL	Quality of Life
QST	Quantitative Sensory Testing
REC	Research Ethics Committee
R848	Resiquimod (TLR7 agonist)
ROC	Receiver Operating Characteristic
SAA	Serum Amyloid A
SEM	Standard Error of the Mean
SF-12	Short Form-12 Health Survey
SIQR	Symptom Impact Questionnaire Revised
SLP	Structured Light Plethysmography
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor

Abbreviation	Definition
TXB2	Thromboxane B2
VIMP	Variable Importance

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Chapter 1

1.0. Introduction

Throughout history, human behaviour – and the consequences of human behaviour – have been shaped not only by the intrinsic traits of individuals, such as their genetic makeup, but also by external influences, including environmental stressors and infectious agents such as bacteria, viruses, and fungi (Dominguez-Andres and Netea 2019). These infections do not always resolve cleanly; their effects can linger, influencing cognition, mood, and physical stamina long after the pathogen itself has been cleared (Izquierdo-Condoy et al. 2024; Bowe, Xie, and Al-Aly 2023). For example, President Woodrow Wilson contracted influenza during the 1919 Paris Peace Conference, likely a severe case of the H1N1 “Spanish flu.” Observers at the time noted a sudden decline in his mental sharpness, physical energy, and emotional stability. Some historians have argued that this post-viral state contributed to his uncharacteristically passive stance in critical negotiations – decisions that may have shaped the punitive nature of the Treaty of Versailles and, indirectly, laid the groundwork for future global conflict (Barry 2004). Such episodes underscore how post-infectious syndromes can extend their influence far beyond individual suffering, sometimes altering the course of history itself.

Long COVID has reignited interest in the long-recognised but often marginalised phenomenon of post-viral syndromes. While this emerging condition is now widely acknowledged, its protean symptoms and uncertain mechanisms place it in a category of illness that has long troubled medicine. Historical diagnoses such as *neurasthenia* – popular in the 19th and early 20th centuries – share striking similarities with long COVID and other post-viral fatigue states

(Blomberg, Cox, and Langeland 2022). Characterised by exhaustion, cognitive impairment, and malaise, neurasthenia was often attributed to the strains of modern life, though it may, in retrospect, have represented a post-infection syndrome misunderstood through the lens of prevailing psychosocial theories. As with long COVID, the medicalisation of such poorly understood conditions sometimes led to overdiagnosis, and in some cases, individuals may have embraced the diagnosis in the absence of other explanations. Yet the fact remains that many patients experienced prolonged and disabling symptoms, even after apparent recovery from the initial illness.

This pattern is not unique to SARS-CoV-2. Prolonged recovery periods have been documented following infection with viruses such as *Chikungunya*, *Dengue*, and *Epstein-Barr virus*, where symptoms – especially fatigue and pain – can persist for months or even years despite viral clearance (Bowe, Xie, and Al-Aly 2023). Such chronic manifestations are increasingly understood to arise not from persistent infection, but from complex and individualised host responses to the virus. In this light, long COVID offers a rare scientific opportunity: a large-scale, global “experiment” through which the long-term effects of viral infection on the immune, neurological, and metabolic systems can be studied in real time. It serves as a reminder that viral mass and material are disproportionately small compared to the scale and duration of the physiological disruption they may trigger. Indeed, it has been estimated that the total mass of all SARS-CoV-2 particles at the height of the pandemic would barely fill a teaspoon – yet the consequences have impacted on every aspect of society and human health.

Understanding the molecular basis of these host-driven, post-viral syndromes is now an urgent scientific and clinical priority. The parallels between long COVID, historical entities such as neurasthenia, and post-infectious fatigue following other viral illnesses suggest that a common pathophysiological thread may be waiting to be unravelled.

In this thesis, I explore the molecular underpinnings of the host response to viral illness and its long-term consequences through a series of five interlinked studies, employing behavioural analysis, immunological profiling, and metabolomics. The overarching aim is to investigate how viral infections – and the response of the host to them – can lead to enduring symptoms such as fatigue, pain, and cognitive dysfunction, and to identify measurable biomarkers that may underpin these syndromes. Each chapter contributes a different perspective on this broad question, together forming a coherent narrative from mechanistic animal studies to translational human research.

Chapter 2 begins with a preclinical investigation into the divergent host responses to two pathogen-associated molecular patterns – TLR-4 and TLR-7 agonists – representing bacterial and viral infections, respectively. Using a combination of behavioural assays, cytokine gene expression, and ^1H NMR metabolomics in male and female mice, I demonstrate distinct inflammatory and metabolic pathways downstream of each receptor, with sex-dependent behavioural outcomes. These findings lay the mechanistic foundation for understanding how different innate immune stimuli can produce long-lasting effects on mood and energy, features common to post-infectious syndromes.

Chapters 3, 4, and 5 focus on long COVID, a contemporary example of post-viral illness. Chapter 3 presents the protocol for a longitudinal, multi-omics study of non-hospitalised

individuals with confirmed SARS-CoV-2 infection, contrasting those with persistent symptoms against those who recovered fully. Chapter 4 details the results of this study, identifying metabolic signatures in serum and urine that not only differentiate long COVID from recovered controls but also reveal biologically distinct subtypes linked to either neuroendocrine disturbance or persistent inflammation. Chapter 5 investigates the clotting and inflammatory profiles of individuals with long COVID, recruited from a hospital-based study, and explores how these profiles relate to metabolic signatures. By integrating coagulation markers with metabolomic data, this chapter identifies distinct subgroups within the long COVID cohort, altogether pointing towards the need for tailored therapeutic approaches for affected individuals.

Chapter 6 investigates fibromyalgia, a prototypical central sensitisation disorder. Here, I combine metabolomic and neuroinflammatory profiling with quantitative sensory testing to identify metabolic subtypes that align with pain thresholds. The findings provide evidence that metabolic dysfunction and neuroinflammation are closely tied to symptom expression and offer a potential pathway towards precision medicine in chronic pain and fatigue syndromes.

In Chapter 7, I examine another chronic pain condition – osteoarthritis (OA) – using integrated CSF and serum metabolomics. I identify glutamine and histidine as key metabolic correlates of pain intensity and disability, findings that resonate with those seen in Long COVID and fibromyalgia, suggesting shared molecular mechanisms across different chronic conditions.

Together, these studies build a multi-layered picture of how viral illness can trigger lasting changes in host metabolism and immunity, offering insights into shared biological mechanisms across post-viral and chronic conditions.

1.1. Post-viral syndromes

Following the 1918 influenza pandemic, that killed 50 million people worldwide, physicians such as Dr. Constantin von Economo and Jean-René Cruchet described a condition known as *encephalitis lethargica* - affecting President Wilson - which typically began with flu-like symptoms such as fever and malaise, followed by acute neurological manifestations including profound sleep disturbances, oculomotor abnormalities, and psychiatric symptoms (Hoffman and Vilensky 2017). In many individuals, a chronic phase developed for months or even years, characterised most notably by parkinsonian-like features, profound lethargy and catatonia. Although a definitive viral cause was never confirmed, the condition was widely believed to be related to influenza or another infectious agent, due to its close temporal association with the pandemic and the frequent presence of respiratory symptoms during the acute phase (Hoffman and Vilensky 2017). Another example emerged a few decades later during a 1948–49 outbreak of a febrile illness in Iceland, where many individuals experienced persistent fatigue, muscle pain, and cognitive difficulties following a flu-like illness (Acheson 1959). Termed “Iceland disease,” the condition was also known as *benign myalgic encephalomyelitis* (ME) in the UK (Galpine and Brady 1957) and *epidemic neuromyasthenia* in the US (Henderson and Shelokov 1959), where similar outbreaks with overlapping clinical features occurred within a relatively short period. It is now recognised as one of the earliest clinical descriptions of what is now known as myalgic encephalitis/chronic fatigue syndrome (ME/CFS).

Other, currently relevant viruses, including Epstein–Barr virus, Ebola, Polio, SARS, and Coxsackie B, have also been implicated in long-term complications, underscoring the fact that a wide range of infectious agents can give rise to persistent post-viral illness (Choutka et al. 2022). Dengue virus has been linked to chronic fatigue, neurological complications, and an increased risk of new-onset inflammatory disorders (Hertanti, Nguyen, and Chuang 2025; Tiga et al. 2016). Chikungunya virus frequently results in long-lasting arthralgia, likely due to ongoing immune activation and the persistence of viral RNA in affected tissues (Schilte et al. 2013). These syndromes may emerge sporadically or follow epidemic waves, and they often lack clear diagnostic markers or consistent treatment pathways (Behan and Bakheit 1991). The COVID-19 pandemic has drawn increased attention to these conditions. Many individuals infected with SARS-CoV-2 report a range of symptoms, including fatigue, cognitive impairment, anxiety, and depression, that persist well beyond the acute phase of illness, developing a condition that is now widely known as long COVID (Penninx et al. 2022).

Long COVID has also provided new opportunities to investigate chronic conditions with similar clinical profiles. Many of the symptoms reported by individuals with long COVID – such as persistent fatigue, cognitive dysfunction, orthostatic intolerance, widespread pain, and post-exertional malaise – closely resemble those seen in ME/CFS and indeed, many long COVID patients meet diagnostic criteria for ME/CFS (Wong and Weitzer 2021). Unlike chronic complications associated with viruses such as Dengue or Chikungunya, individuals with long COVID or ME/CFS often show no structural abnormalities on conventional imaging or laboratory tests. The absence of demonstrable pathology has posed significant challenges to diagnosis and management, and many patients have reported dismissal from healthcare services (Ladds et al. 2020; McManimen et al. 2019). Although recognition of post-viral

syndromes is improving, the mechanisms that lead to persistent symptoms in a subset of individuals remain poorly understood. To effectively address these conditions, it is crucial to investigate the pathophysiology across both the acute and chronic phases of illness, and to identify the factors that determine why only some individuals transition to a chronic state. This is essential for the development of targeted treatment strategies and for improving long-term outcomes in affected patients.

1.1.1. Metabolic consequences of infection

Post-viral syndromes are increasingly recognised to involve persistent metabolic disturbances, including insulin resistance, dyslipidaemia, and impaired regulation of lipid and glucose metabolism (Palmer 2022). Metabolic comorbidities such as type 2 diabetes and obesity not only increase susceptibility to severe viral infections but also raise the likelihood of long-term complications, as seen in COVID-19 (Palmer 2022). Viral infections trigger profound metabolic reprogramming, driven by the increased energy demands of the host during immune activation and by the virus exploiting host metabolic pathways to support replication. One of the hallmarks of a viral infection is an increase in aerobic glycolysis, primarily (but not exclusively) in immune cells. This metabolic shift enables rapid ATP generation and provides precursors for macromolecular synthesis (Sanchez and Lagunoff 2015; Sumbria et al. 2020). In addition to fuelling energy-intensive processes, metabolites themselves have non-canonical functions that influence the course of infection. For example, itaconate, produced by activated macrophages, has been shown to modulate inflammatory responses and exert antiviral effects, implicating metabolites in preventing harmful excess inflammation (Hooftman et al. 2020; Sohail et al. 2022).

A further example of the metabolic and immune interplay in the context of a viral infection involves the use of NAD⁺ as a cofactor. Following pattern recognition receptor (PRR) activation, the mTOR–JAK–STAT signalling cascade is induced, which not only supports glycolytic flux but also promotes the expression of interferon-stimulated genes (ISGs), including NAD⁺-consuming enzymes such as members of the PARP family (Figure 1.1). These PARPs utilise NAD⁺ as a substrate for ADP-ribosylation of viral proteins, contributing to antiviral defence (Li, Shi, et al. 2021). Because NAD⁺ is essential for both glycolysis and immune signalling, its depletion – as seen in ageing and metabolically impaired individuals – may compromise host defences and help explain the increased vulnerability of these groups to both severe and chronic infections. The JAK–STAT pathway also leads to type I interferon-mediated suppression of fatty acid synthase (FASN), thereby limiting de novo lipid synthesis required for viral assembly. Indeed, overexpression of FASN has been shown to increase SARS-CoV-2 replication, while knocking it out has the opposite effect (Aliyari et al. 2022). This highlights again the key role of metabolism in anti-viral defence.

However, the glycolytic shift can also favour viral replication. In SARS-CoV-2 infection, mitochondrial disruption leads to increased reactive oxygen species (ROS), which in turn stabilise HIF-1 α . This transcription factor promotes the expression of glycolytic genes, reinforcing the metabolic switch. HIF-1 α -mediated glycolysis under high-glucose conditions has been shown to enhance both SARS-CoV-2 replication and pro-inflammatory cytokine production in primary human monocytes (Codo et al. 2020). These metabolic interactions are complex and context-dependent, involving dynamic feedback between immune signalling, cellular energetics, and viral manipulation of host pathways. Nevertheless, understanding these processes is critical for identifying new therapeutic targets and for elucidating why

some individuals experience a transition into post-viral syndromes. Indeed, it is possible that such rewiring of metabolism, alongside chronic inflammation leads to mitochondrial dysfunction that may underlie long-lasting symptoms such as fatigue, pain, and cognitive dysfunction.

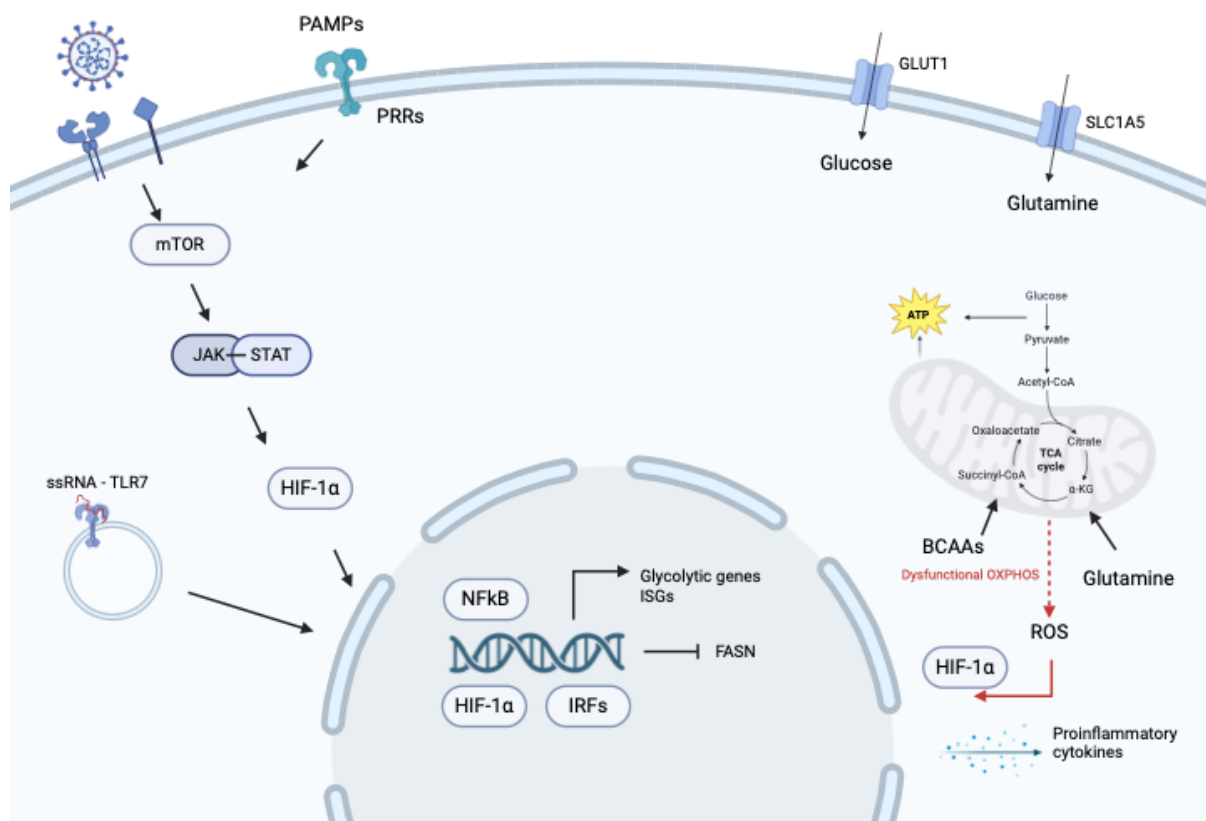


Figure 1.1: Viral infection reprograms cellular metabolism and inflammatory signalling through HIF-1α. Viral components such as single-stranded RNA (ssRNA) are detected by pattern recognition receptors (PRRs) at the cell surface or in endosomes (e.g., TLR7), triggering downstream pathways via mTOR and JAK-STAT signalling. This activates transcription factors including HIF-1α, NF-κB, and IRFs, which in turn drive the expression of glycolytic genes and interferon-stimulated genes (ISGs), including PPARs that support antiviral responses. Concurrently, fatty acid synthase (FASN) activity is suppressed, reducing de novo lipogenesis and thereby limiting viral replication. Viral infection also enhances cellular uptake of glucose, which feeds into glycolysis and the tricarboxylic acid (TCA) cycle. Increased glutamine uptake also contributes to the increased energetic demands via anaplerosis, feeding into the TCA cycle, in addition to host-derived branched-chain amino acids (BCAAs), which are catabolised to provide TCA intermediates. This enhanced metabolic flux can lead to dysfunctional mitochondrial oxidative phosphorylation (OXPHOS), leading to reactive oxygen species (ROS) production and further stabilisation of HIF-1α. This positive feedback loop

promotes the expression of proinflammatory cytokines, contributing to the immunopathology of viral infection. Figure created using BioRender.

1.1.2. Behavioural consequences of infection

Alongside metabolic changes, immune activation leads to profound changes in behaviour. Indeed, the increased metabolic demands along with inflammatory signalling likely contributes to a state characterised by low energy or low mood. Mood disorders are increasingly recognised as both a risk factor for, and a consequence of, post-viral syndromes (Bowe, Xie, and Al-Aly 2023). Individuals with pre-existing depression or anxiety may be more susceptible to persistent symptoms following infection, while those recovering from viral illness show elevated rates of mood disturbances, cognitive impairment, and fatigue (Mazza et al. 2020). However, the mechanisms driving these behavioural changes remain poorly understood. The following section explores the hypothesis that low-grade, chronic inflammation and immune mediators play a central role, introducing the concept of sickness behaviour and how it can be modelled in rodents.

1.2. Sickness behaviour

Sickness behaviour is a coordinated set of behavioural changes that occur during infection, characterised by reduced activity, social withdrawal, and decreased appetite (Dantzer 2004, 2001). Rather than being mere byproducts of illness, these behaviours are thought to be adaptive, conserving energy to support the host immune response while also limiting the spread of infection. By reducing movement and other energetically expensive activities, sickness behaviour is proposed to limit heat loss, thereby facilitating fever generation and

enhancing immune function (Dantzer 2004). This response is strikingly conserved across species with an innate immune system, potentially reflecting its evolutionary importance, but also facilitating its mechanistic study in model organisms (Dantzer 2023). While these mechanisms have not yet been fully elucidated, most theories propose that sickness behaviour is driven by immune-to-brain signalling, primarily mediated by pro-inflammatory cytokines such as IL-1 β and IL-6, which influence neural activity in regions like the hypothalamus and limbic system to orchestrate the characteristic behavioural changes (Dantzer 2004). Importantly, these inflammatory mediators are also implicated in chronic conditions such as depression, post-viral syndromes, and other inflammatory disorders, where persistent immune activation can drive prolonged behavioural changes that mirror those seen in acute sickness (Dantzer et al. 2008). Understanding these pathways therefore, not only sheds light on the evolution of host defence mechanisms but also provides insight into conditions where these behaviours become maladaptive, such as in chronic inflammatory diseases or mood disorders.

1.2.1. Possible neuroimmune mechanisms underlying sickness behaviour

Historically, reductionist approaches using models such as lipopolysaccharide (LPS) injection have been employed to study the cytokine-driven mood disturbances of 'sickness behaviour', with the implicit assumption that these models mimic aspects of major depressive disorder (MDD) (Dantzer et al. 2008). LPS, a component of the gram-negative bacterial cell wall, triggers an inflammatory response by activating the toll-like receptor 4 (TLR4), which then induces the production of several downstream proinflammatory cytokines via the nuclear

factor kappa B (NFκB) pathway (Dantzer et al. 2008). The way in which these inflammatory mediators then propagate the innate immune signalling to the brain to alter behaviour is still a matter of debate, although multiple neuroimmune pathways have been put forward (Figure 1.2) (Dantzer 2004). In the neural route, cytokines activate primary afferent nerves, such as the vagus nerve, whereas in the humoral route, cytokines enter the brain directly via volume diffusion, potentially at the choroid plexus and circumventricular organs where the blood-brain barrier (BBB) is thought to be less intact. This leads to microglial activation and the production of further cytokines and chemokines, also known as glial mediators. These chemotactic signals can facilitate immune cell recruitment across the BBB, where the infiltrating cells adopt an activated phenotype indistinguishable from brain resident macrophages (microglia), contributing to neuroinflammation (Dantzer 2004). Experimental models show that LPS administration induces gliosis, reduces hippocampal spine density, and decreases synaptic markers such as PSD-95, BDNF, synaptophysin, and NMDAR subunits, highlighting inflammation-induced synaptic defects (Lin et al. 2019; Li, Ali, et al. 2021). These molecular and structural changes are thought to impair neural circuits involved in motivation, cognition, and mood regulation – providing a mechanistic basis for the behavioural alterations observed during systemic inflammation. Consistently, direct injection of cytokines such as IL-1β into the brain reproduces the cardinal features of sickness behaviour, while their blockade with IL-1 receptor antagonists (IL-1RA) can prevent these effects, reinforcing the role of central cytokine signalling in mediating peripheral immune-to-brain communication (Kent et al. 1992).

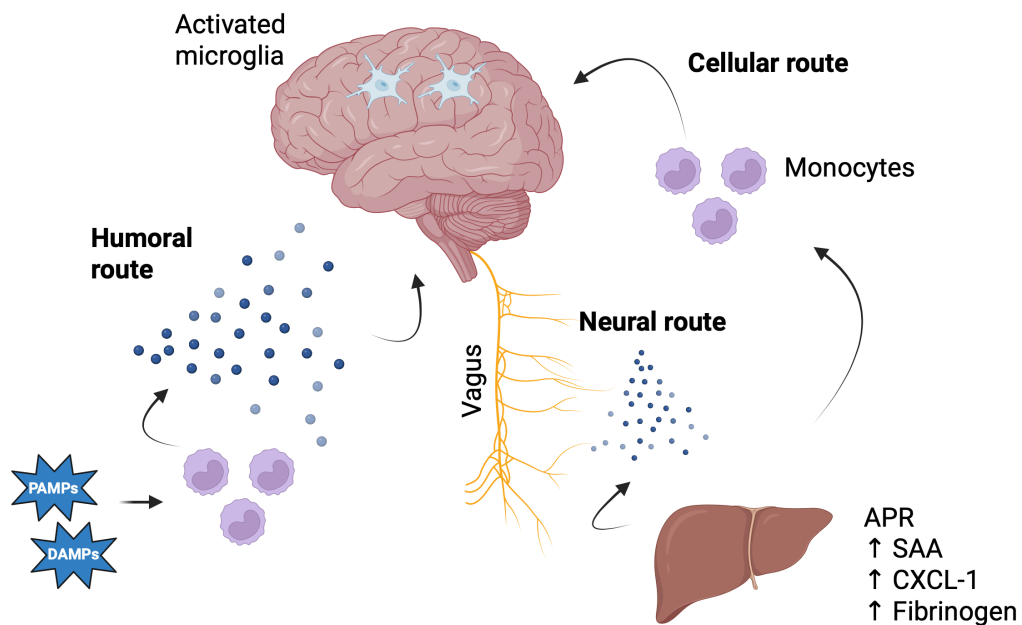


Figure 1.2: Neuroimmune pathways through which the periphery communicates with the brain. The figure illustrates a simplified representation of three pathways through which peripheral immune activation may induce sickness behaviour: the humoral route, the neural route, and the cellular route. **Humoral route:** Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) in the periphery stimulate the production of inflammatory mediators (e.g., cytokines) that can cross the blood-brain barrier or act at brain regions with permeable barriers. **Neural route:** Inflammatory signals stimulate peripheral nerves, such as the vagus nerve, which transmit signals to the brain, resulting in neuroinflammation. **Cellular route:** Peripheral monocytes migrate into the brain parenchyma, where they differentiate and contribute to neuroinflammation. The acute phase response (APR) contributes to all three pathways, being activated by peripheral immune signals and is also involved in the response downstream of microglial activation. It is characterised by increased production of serum amyloid A (SAA), CXCL-1, and fibrinogen, among other pro-inflammatory mediators.

1.2.2. The link to chronic conditions

Chronic immune activation has been linked to long-term behavioural changes in both animal and human studies. Monkeys given prolonged interferon (IFN) therapy exhibit increased

anxiety and reduced exploration (Felger et al. 2007). More broadly, chronic low-grade inflammation is implicated in depression and other chronic diseases, with many inflammatory conditions showing high comorbidity with mood disorders (de Melo et al. 2017). Given the overlap in symptoms between sickness behaviour and depression, these pathways may provide a common mechanistic link, explaining the increased incidence of depression in physically ill individuals (Dantzer et al. 2008). Meta-analyses confirm that patients with depression have elevated circulating pro-inflammatory cytokines and acute-phase proteins such as C-reactive protein (CRP) (Gimeno et al. 2009; Chamberlain et al. 2019). A causal relationship is further suggested by clinical observations; patients receiving IFN therapy for cancer or hepatitis C frequently develop depression, which can be reversed by antidepressants, while anti-inflammatory drugs have been shown to alleviate depressive symptoms (Hauser et al. 2002; Kappelmann et al. 2018). These findings highlight the importance of understanding shared neuroimmune mechanisms rather than focusing solely on specific disease triggers, as acute and chronic illness-related behaviours, though seemingly distinct, may stem from common underlying inflammatory processes. It should also be noted that activation of innate immune receptors is not solely dependent on pathogens; endogenous danger-associated molecular patterns (DAMPs) can also trigger sterile inflammation. Stress, for example, activates the HPA axis, elevating cortisol and engaging pathways that parallel those seen in infection-induced sickness behaviour (Miller, Chen, and Zhou 2007). Taken together, these findings underscore the relevance of sickness behaviour as a conceptual framework for understanding the neuroimmune basis of persistent symptoms following illness – a perspective now gaining renewed attention in clinical investigations of post-viral syndromes such as long COVID.

1.3. Long COVID

1.3.1. Definitions of long COVID

Shortly after the COVID-19 pandemic began, reports of lingering symptoms started to accumulate. Due to the lack of understanding and patient support, online communities started to emerge and played a large role in the support and recognition of the newly termed 'long COVID'. There is no universally accepted definition of long COVID. The term itself has been coined by patient groups to describe persistent symptoms following COVID-19 infection, yet the duration of these symptoms varies among definitions by different organisations. The World Health Organization (WHO) describes 'post-COVID condition' occurring 3 months after SARS-CoV-2 infection with symptoms lasting at least 2 months (Soriano et al. 2022), while The National Institute for Health and Care Excellence (NICE) defines 'ongoing symptomatic COVID-19' as symptoms lasting 4-12 weeks and 'post-COVID-19 syndrome' as symptoms persisting beyond 12 weeks (NICE 2022). Notably, none of the definitions requires any positive laboratory or lateral flow test. The lack of consistency even defining or naming the condition reflects the confusion around the underlying disease process. Indeed, there is a consensus building that it may not be one condition but a host of different entities. As research progresses, patient stratification may allow more formal definitions of subgroups, leading to more directed trials and therapeutic avenues. Due to the inconsistent use of names and definitions of the disease that have been used across many of the studies that are about

to be discussed, the term 'long COVID' to encompass all definitions will be used in the remainder of this thesis.

1.3.2. Prevalence of long COVID

The lack of an established diagnostic test, in addition to inconsistent disease definitions, contributes to the difficulty in estimating the prevalence of long COVID. Generally, it is agreed that ~10% of SARS-CoV-2 infections give rise to long COVID, but estimations vary between 2.9% and 85%, with the higher end largely owing to unvaccinated and hospitalised groups. Methodological differences across studies also contribute to such inconsistencies. Among the lowest reported prevalence, estimations of long COVID cases are gained from electronic health records, yet many individuals do not seek medical advice or begin new prescriptions, possibly because the lack of clarity around therapeutic advice. In a meta-analysis of 194 studies covering approximately 0.75 million COVID-19 cases in total, 45% of patients still experienced symptoms at 4 months, regardless of hospitalisation status (O'Mahoney et al. 2023). Similarly, the REACT-2 study from Imperial College, UK, which is a longitudinal, community-based study of self-reported symptoms, found around 40% of participants reported at least one symptom at 4 weeks, falling only to 38% at 12 weeks and remaining relatively consistent for 6 months thereafter (Whitaker et al. 2022). However, it is important to note these higher estimates do not account for the severity of symptoms. According to the Office for National Statistics (ONS), there are ~2 million people in the UK alone with self-reported long COVID (~3% of the population) and only around 20% of these individuals described these symptoms as severe, reporting their day-to-day activities had been "limited a lot" (ONS 2023a).

1.3.3. Risk factors for long COVID

Several risk factors for developing long COVID have been identified, including female sex, older age, higher BMI, and pre-existing conditions such as type 2 diabetes, obesity, asthma, and mood disorders. Although a more severe acute infection is associated with a higher risk of developing long COVID, many cases, and in fact the majority of current cases, arise from mild or even asymptomatic acute infections. Similar to reports of long COVID prevalence, investigations into risk factors are limited by sampling biases. Many were conducted by the US Department of Veterans Affairs (skewed towards older men) (Bowe, Xie, and Al-Aly 2023; Xie, Choi, and Al-Aly 2024), those who were hospitalised or died in the acute phase of COVID-19 (and are therefore, skewed towards more severe acute infection) (Huang et al. 2022; Sigfrid et al. 2021), or community-based surveys (representing those who respond to such questionnaires) (Whitaker et al. 2022; Goldhaber et al. 2022). Several studies have shown that long COVID was more common following infection with the alpha (B.1.1.7) and delta (B.1.617.2) variants compared to the omicron (B.1.1.529) variant (Diexer et al. 2023; Agergaard et al. 2023). However, it is difficult to disentangle the effect of the higher number of omicron cases that would have led to a larger number of long COVID cases, as well as psychosocial factors at play since much less was known about the virus during earlier waves. Moreover, higher vaccination levels and improved disease management during the later waves could have also had a great impact on outcomes. While these studies reveal certain risk factors for long COVID, it remains unclear why some individuals go on to develop the

disease; particularly those with a mild or even asymptomatic infection and those who were physically fit and young.

1.3.4. Symptoms of long COVID

Symptoms of long COVID are highly variable, frequently relapsing and remitting in nature, and affect multiple body systems. The most common symptoms include fatigue, dyspnoea, cognitive impairment, headache, and muscle pain (Yong 2021; Aiyegbusi et al. 2021). Other reported symptoms include chest pain, altered smell and taste, gastrointestinal issues, and sleep disturbances (Michelen et al. 2021). Figure 1.3 shows the most frequent symptoms as reported by the ONS COVID-19 and Respiratory Infections Survey (CRIS) 2023. Indeed, the varied nature of these multi-system symptoms have led to different attempts to classify long COVID patients into different symptom clusters. These have generated inconsistent results, perhaps owing to different methodologies and sample sizes, with many overlapping or generating only one cluster (Greenhalgh et al. 2024). If not due to methodological differences, one possible explanation is that there is only one overarching disease mechanism to explain all long COVID symptoms. An alternative explanation is that there are indeed different pathologies, but these give rise to overlapping phenotypes in patients. It is also important to note the dynamic nature of long COVID, as patients often describe symptoms changing and evolving over time. Despite these challenges, further research into the pathophysiological variation within long COVID is necessary for developing appropriate treatment pathways.

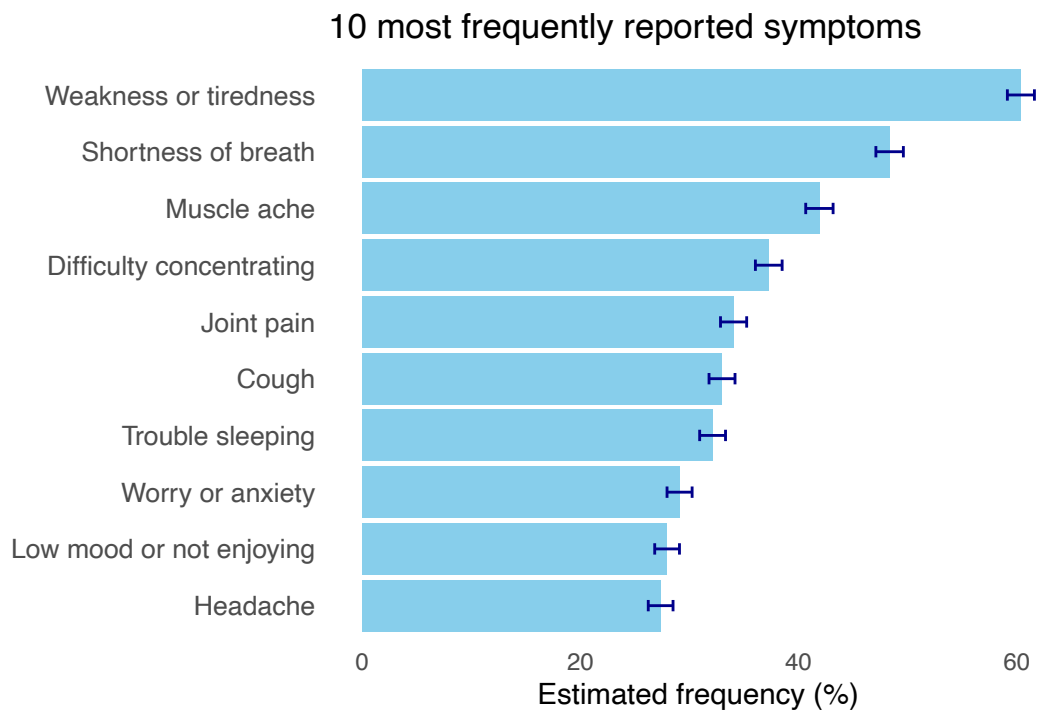


Figure 1.3. Most frequently reported symptoms of long COVID. The bar chart shows the top 10 symptoms reported by individuals with long COVID, as identified in the COVID-19 and Respiratory Infections Survey (CRIS) conducted by the Office for National Statistics (ONS). Symptoms are displayed in descending order of frequency, with weakness or tiredness being the most commonly reported, followed by shortness of breath and muscle ache. Error bars represent the standard error of the mean (SEM). The data were formatted using R version 4.3.3. Frequencies reflect responses from the survey participants and may not be fully representative of the UK population or beyond (ONS 2023b). This figure highlights the range of symptoms that individuals with long COVID may experience, illustrating both physical (e.g., muscle ache, joint pain) and cognitive/psychological (e.g., difficulty concentrating, low mood) effects.

1.3.5. The burden on the healthcare system

The substantial burden that long COVID places on healthcare systems and the broader economy further underscores the urgency of advancing our understanding of this condition. Patients with long COVID are more likely to use healthcare resources, with an estimated 43% increase in primary care consultation costs compared to those without long COVID symptoms

(Tufts et al. 2023). The majority of long COVID management occurs in primary care, with referrals to specialised long COVID outpatient clinics for complex cases (Wolf, Zechmeister-Koss, and Erdos 2022). However, these secondary long COVID services vary widely within healthcare systems and are often described as insufficient by patients (Greenhalgh et al. 2024). This is likely because of the lack of evidence-based therapeutic strategies for long COVID. There is considerable uncertainty among clinicians in terms of how to evaluate and manage patients with long COVID (Greenhalgh et al. 2024). To solve this, clear biomarkers of disease for diagnosis and patient monitoring are urgently required. This will also serve to inform our understanding of the underlying pathobiology of long COVID. Currently, many putative mechanisms have been proposed, with many inconsistencies between studies.

1.3.6. Proposed mechanisms

1.3.6.1. Organ damage

Several studies have shown SARS-CoV-2 infection can cause damage across multiple organ systems. The theory that organ damage resulting from the acute phase of infection causes persistent multisystem symptoms is consistent with reports that severe COVID-19 increases the risk of long COVID. Interestingly, radiological abnormalities across multiple organs have also been found in patients with asymptomatic COVID-19 (Long et al. 2020). Longitudinal studies corroborate this. A community-based study of 201 individuals recovering from COVID-19 assessed organ impairment using the coverscan multiparametric MRI assessment (Dennis et al. 2021). The study found 70% of individuals had impairment in one or more organs 4 months after initial COVID-19 symptoms, including inflammation and ectopic fat accumulation as measured by quantitative T1 mapping and proton density fat fraction. A

more recent study by the same research group substantiated these findings in 536 individuals with long COVID, showing organ impairment was present in 69% at baseline and persisted in 59% of those individuals at 1 year follow-up (Dennis et al. 2023).

1.3.6.1.1. The heart

Investigations of cardiac abnormalities have yielded mixed findings. While some studies report no significant differences in myocardial structure, function, or energetics between long COVID and healthy controls (Gorecka et al. 2022; Joy et al. 2021), others demonstrate a wide range of cardiac abnormalities in convalescent individuals (Puntmann et al. 2020; Boparai et al. 2022). Cardiac magnetic resonance (CMR) abnormalities included myocardial inflammation, tissue changes, and functional impairment (Petersen et al. 2022). However, most pathology appears to resolve over time and there is a lack of a direct association with long COVID symptoms (Boparai et al. 2022). In a UK biobank study, COVID-19 infection was associated with an increased risk of cardiovascular outcomes, highlighting functional consequences downstream of COVID-19 that ought to be monitored in patients (Hilser et al. 2024), but again, this appears to be relevant to post-COVID-19 broadly, and not specifically those reporting long COVID symptoms.

1.3.6.1.2. The lungs

An alternative hypothesis to structural damage causing persisting symptoms post-COVID-19 is that functional abnormalities may be responsible. Hyperpolarised pulmonary xenon 129 (^{129}Xe) MRI has been used to investigate gas transfer in patients 3 months after hospital discharge from their initial infection (Grist et al. 2021). Despite normal or near-normal CT scans and lung function tests, impaired gas exchange was demonstrated in the post-COVID

patients compared to healthy controls (Grist et al. 2021). These findings were then replicated in non-hospitalised patients, with longer gas-blood exchange times and lower red blood cell-to-tissue plasma ratios compared to healthy controls (Grist et al. 2022). Interestingly, non-hospitalised patients showed lower gas transfer than post-hospitalised patients (Grist et al. 2022). However, these studies are limited by their sample size and follow up investigations are underway to see whether these findings hold up in larger cohorts of individuals. Chapter 5 of this thesis describes the results of a sub-study of this large scale follow up, which aimed to identify blood biomarkers that associate with radiological findings. The limitations of availability and cost of ^{129}Xe MRI makes this impractical as a diagnostic tool, and therefore, blood biomarkers from a simple blood test that correspond to such findings would be a valuable tool.

1.3.6.1.3. The brain

In light of the neurological symptoms of long COVID, and the neurotropic potential of SARS-CoV-2, many investigations into subsequent damage to the brain have been conducted. Indeed, studies have shown that SARS-CoV-2 infection is associated with significant changes in brain structure and function. A large-scale UK Biobank study found reduced grey matter thickness in the orbitofrontal cortex and parahippocampal gyrus, along with increased markers of tissue damage in regions connected to the primary olfactory cortex (Douaud et al. 2022). These changes were observed even in mild cases and persisted after the exclusion of hospitalised patients. While not a report on long COVID *per se*, the study found increased cognitive decline in individuals post-COVID-19 compared to those who had not yet been infected. Moreover, this cognitive decline correlated with changes in cerebellum volume. It is possible that SARS-CoV-2 may enter the central nervous system through the olfactory mucosa

and could explain the observed brain changes (Douaud et al. 2022). Despite this, subsequent investigations into patients with long COVID have shown the presence of symptoms is possible in the absence of radiological damage and conversely, the presence of damage has been demonstrated despite lack of symptoms (Altmann et al. 2023).

1.3.6.2. *Persistent viral reservoirs or antigens*

There is evidence to suggest that replicating virus or viral-derived RNA that is then translated into viral proteins may persist in individuals with long COVID (Lupi et al. 2024; Proal et al. 2023; Goh et al. 2022; Zuo et al. 2024). It is inherently difficult to detect viral reservoirs – most studies have either used post-mortem samples or accessible biological samples such as stool. The former studies are limited by their skew to a more severe form of COVID-19, while the latter are limited by a proxy-measurement, which has been shown to be inconsistent with findings from colon biopsies (Zollner et al. 2022). Studies have shown that SARS-CoV-2 RNA can persist in stool samples of COVID-19 patients after respiratory samples test negative (Natarajan et al. 2022; Kipkorir et al. 2020). Viral shedding in faeces has been shown to continue for up to 7 months after respiratory clearance in patients with mild to moderate disease (Natarajan et al. 2022). Despite only being in the minority of patients (only ~4% of the 113 individuals shed at 7 months), viral RNA was associated with gastrointestinal (GI) symptoms, for example abdominal pain, that are characteristic of many long COVID cases. Several other studies have demonstrated evidence of ongoing viral presence in the GI tract months after initial symptom presentation by detection of nucleic acids as well as antigens (Gaebler et al. 2021; Cheung et al. 2022). However, the majority of studies do not specifically look at individuals with long COVID; observations have been in both symptomatic and asymptomatic individuals at the time of sampling (Bussani et al. 2023; Cheung et al. 2022; Zuo

et al. 2024). In a convincing endoscopy study of inflammatory bowel disease (IBD) patients recovering from COVID-19 (approximately 219 days after acute infection), viral antigen persistence in the gut was found in only those who reported long COVID symptoms (Zollner et al. 2022). The inability to cultivate virus from biopsy tissue highlights possible incomplete viral clearance rather than subclinical persistent infection. It would be helpful for longitudinal studies of patients 2-3 years into their long COVID diagnosis to investigate whether detection of virus is possible, or whether this finding is limited to a subset of individuals, such as those with pre-existing GI disease. This highlights again the possibility that different patient subgroups could explain the discrepancy across the literature.

Using ultra-sensitive single molecule array assays (SIMOA), Swank *et al.* (2023) detected either the full-length Spike protein, its S1 subunit, or Nucleocapsid (N) protein in plasma of 60% of long COVID participants (n=37) for up to 12 months after infection. In comparison, viral antigens were detected in 0% of the control group, i.e. participants who had tested positive for COVID-19, but had not gone on to develop long COVID (Swank et al. 2023). Interestingly, the detection of the spike protein was the most common, with N protein only present in one patient. Repeat samples were taken over several months and showed sustained Spike levels in the long COVID group, whereas these rapidly fell in controls soon after COVID-19 diagnosis. Further investigations are necessary to see if these findings are replicated in larger cohorts and with more consistent matching, as many of the assays were carried out at different time points between groups. Nevertheless, these findings highlight an interesting property of the Spike protein and raise the question of why Spike protein persists beyond Nucleocapsid. It is possible that Spike evades degradation more effectively; indeed, studies have shown it is incorporated into circulating extracellular vesicles, although the same

data is missing for Nucleocapsid (Troyer et al. 2021; Craddock et al. 2023). This raises the possibility that circulating spike protein rather than the virus per se may give rise to long COVID symptoms.

1.3.6.3. Latent virus reactivation

Recent studies suggest that long COVID may be associated with the reactivation of latent viruses, particularly Epstein-Barr virus (EBV). Several papers report serological evidence of EBV reactivation in both acute COVID-19 and long COVID (Gold et al. 2021; Rohrhofer et al. 2023; Bernal and Whitehurst 2023). However, reports have been inconsistent. In one study, EBV viral capsid-antigen (VCA) IgM, IgG, and EBV nuclear antigen 1 (EBNA) IgG screens, the gold standard tests for EBV infection, showed no differences between long COVID and control (Hoeggerl et al. 2023). This work was driven by studies suggesting that respiratory tract infections, particularly viral infections like influenza or Epstein-Barr virus (EBV) reactivation, are more commonly associated with multiple sclerosis relapses. The most influential studies examining the relationship between infection and relapse in multiple sclerosis were conducted by Sibley and colleagues in 1965 (Sibley and Foley 1965). Subsequently, many have demonstrated that bacterial and viral infections frequently preceded relapse in MS, highlighting a temporal association that supported the hypothesis of infection-induced immune activation as a trigger for disease exacerbation. A notable, more recent study, from an Argentinian research group showed that there is a significant association between systemic infections and risk of MS relapse, increased MRI activity, and T cells activation which happens a few weeks later (Correale, Fiol, and Gilmore 2006). This finding is consistent with the idea that systemic immune activation following infection can lead to reactivation of CNS inflammation, potentially by enhancing the trafficking of autoreactive lymphocytes across the

blood-brain barrier (1). The underlying mechanisms remain an area of active investigation. Infections may trigger relapse through multiple pathways: by increasing pro-inflammatory cytokine production (e.g., IL-6, TNF- α), altering blood-brain barrier permeability, or activating peripheral immune cells that subsequently infiltrate the CNS. Additionally, molecular mimicry, where microbial antigens resemble myelin proteins, could prime the immune system to target CNS components. Interestingly, not all infections appear to have the same propensity to trigger relapse, and individual variability in immune responses may modulate this risk.

In addition to EBV, human endogenous retroviruses (HERVs) are being investigated for their role in long COVID. HERVs are abundant sequences within the human genome that were integrated during ancient retrovirus infections and comprise about 8% of human DNA (Mao, Zhang, and Cong 2021). Inherited through generations, HERVs are mostly defective due to accumulated mutations (Lower, Lower, and Kurth 1996). However, some retain functional coding and non-coding sequences that are tightly regulated and maintain various physiological roles in healthy individuals (Parseval and Heidmann 2005). Infections can influence the expression of HERV proteins, and this has been associated with autoimmune diseases and cancers, potentially through superantigen production or molecular mimicry (Nelson et al. 2003; Balada, Vilardell-Tarres, and Ordi-Ros 2010). More recently, SARS-CoV-2 infection has been shown to activate the HERV-W envelope (ENV) protein expression in some individuals. HERV-W ENV was found to be highly expressed in leukocytes of COVID-19 patients, correlating with disease severity and respiratory outcomes (Balestrieri et al. 2021; Charvet et al. 2023). If such expression is responsible for ongoing symptoms post-COVID-19, then neutralising HERV-W by monoclonal antibodies such as temelimab ought to improve

long COVID. Indeed, the results of an ongoing phase 2 clinical trial to assess the safety and efficacy of temelimab in W-ENV-positive long COVID patients with neuropsychiatric symptoms should be published soon.

1.3.6.4. Autoimmunity

Symptoms of long COVID, such as fatigue, joint, and muscle pain overlap with those of autoimmune conditions, such as Rheumatoid Arthritis and Lupus. While not unique to the SARS-CoV-2 virus, studies have demonstrated the production of a wide array of autoantibodies during the acute stage of COVID-19 (Wang et al. 2021; Franke et al. 2021). Additionally, pre-existing autoantibodies against type I interferons have been linked to more severe COVID-19 outcome (Bastard et al. 2020). Severe COVID-19 is characterized by extrafollicular B cell activation, similar to that seen in lupus (Woodruff et al. 2020; Woodruff et al. 2022). This pathway is associated with the production of neutralizing antibodies, but also leads to the generation of autoreactive antibodies (Woodruff et al. 2022; Woodruff et al. 2020). These autoantibodies may contribute to disease pathology, including thrombosis and cytokine signalling disruption (Knight et al. 2021). The autoreactivity typically resolves upon recovery, but may persist in some patients with post-COVID sequelae (Woodruff et al. 2022). Beyond the acute phase, electronic health records suggest an increased incidence of new-onset autoimmune disorders following COVID-19 infection (Chang et al. 2023; Tesch et al. 2023). However, the relationship between autoimmunity and long COVID remains unclear, particularly as most studies focused on a severe form of the acute disease, while many long COVID patients previously experienced a milder version (Altmann et al. 2023). Still, some studies have found anti-nuclear antibodies one year after infection in COVID-19 convalescents (higher compared to controls) (Son et al. 2023). Moreover, the levels of these antibodies were

positively associated with ongoing symptoms and cytokine levels (Son et al. 2023). However, similar investigations have yielded negative results. It is unlikely that this is a result of methodological inconsistencies, as comparable methods such as those defining antigen conformation and therefore, epitope exposure still show conflicts (see (Peluso et al. 2022) and (Seessle et al. 2022) for example.

Autoantibodies have also been linked to dysautonomia in long COVID. Two thirds of patients with long COVID have reported moderate-severe autonomic dysfunction, with the most common phenotype being postural orthostatic tachycardia syndrome (POTS). While the underlying mechanisms are not fully elucidated, several autoantibodies have been associated with POTS, including anti-angiotensin II receptor antibodies, those against structural cardiac proteins, and antiphospholipid antibodies (El-Rhermoul et al. 2023; Schofield 2021). Other studies have focussed on investigating a link between autoantibodies and neurological symptoms. For example, IgG isolated from long COVID patients experiencing neurological symptoms bound to epitopes on mouse CNS tissue and, when injected into receiver mice, increased pain sensitivity and muscle weakness (Santos Guedes de Sa et al. 2024). Current trials including immunoadsorption and plasmapheresis to improve symptoms of long COVID are ongoing and will inform whether these findings are responsible for symptoms, although these strategies do not provide a cure for underlying disease (i.e. the ongoing production of antibodies or the failure of negative selection at immune checkpoints) and are limited in terms of scalability.

1.3.6.5. Endothelial dysfunction

Endothelial dysfunction has emerged as a key factor in COVID-19 pathophysiology and long-term sequelae. SARS-CoV-2 can directly (through tropism of ACE-2-positive endothelial cells) or indirectly (through inflammatory mediators) damage the endothelium, leading to widespread vascular injury and inflammation (Xu, Ilyas, and Weng 2023). Endothelial damage and subsequent activation are precursors to thrombi generation, which has been associated with various complications, including cardiovascular issues, in both acute and long COVID (. Research has identified multiple markers of endothelial dysfunction in long COVID patients, including raised D-dimer and von Willebrand Factor (vWF) (Ambaglio et al. 2022; Townsend et al. 2021), the latter of which has been associated with impaired exercise capacity (Prasannan et al. 2022). A study on the Post-hospitalisation COVID-19 (PHOSP-COVID) cohort identified high fibrinogen and D-dimer to C-reactive protein ratios at the time of hospital admission could predict cognitive deficits at 6 and 12 months post-COVID-19 (Taquet et al. 2023). If a hypercoagulable state persists into long COVID, then impaired oxygen delivery to tissues and local hypoxia may provide a link to metabolic perturbations and be responsible for symptoms such as brain fog, fatigue, and post-exertional malaise. Figure 1.4 provides more detail on the proposed mechanisms of SARS-CoV-2-induced endothelial dysfunction.

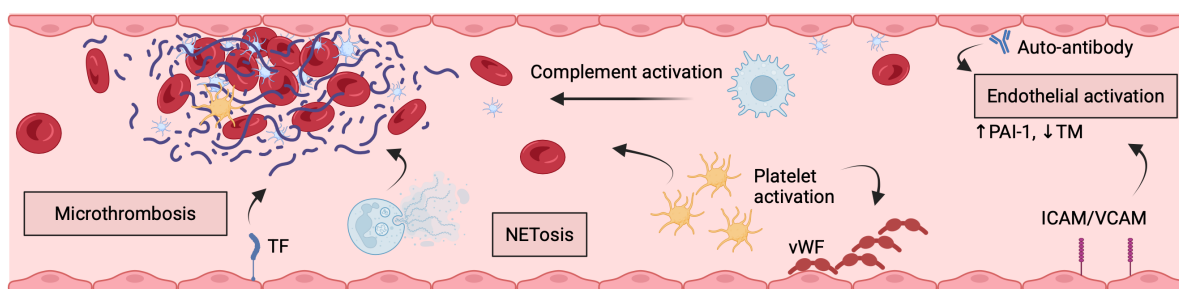


Figure 1.4: Proposed events leading to endothelial dysfunction and microthrombosis in long COVID. Endothelial activation is driven by inflammatory mediators, such as cytokines, as well as the potential presence of autoantibodies. This activation is characterized by increased expression of adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), which promote leukocyte recruitment to the endothelium. Additionally, activated endothelial cells upregulate tissue factor (TF), initiating the coagulation cascade and leading to fibrin clot formation. Endothelial cells also release von Willebrand factor (vWF), contributing to platelet adhesion and aggregation. A pro-thrombotic environment is established due to an imbalance between pro-thrombotic and anticoagulant factors. This imbalance includes increased levels of plasminogen activator inhibitor-1 (PAI-1) and decreased levels of thrombomodulin (TM), reducing fibrinolysis and anticoagulation capacity. Platelet activation contributes to endothelial dysfunction, with vWF released from both endothelial cells and platelets, promoting further platelet aggregation and adhesion. This results in the formation of platelet-rich thrombi. Complement activation occurs through the involvement of activated macrophages and immune complexes, further amplifying endothelial activation and platelet aggregation. Neutrophil activation, driven by endothelial dysfunction and platelet interactions, leads to the process of neutrophil extracellular trap formation (NETosis). During NETosis, activated neutrophils release web-like DNA structures known as neutrophil extracellular traps (NETs). These NETs trap platelets and red blood cells, contributing to the stabilisation of thrombi. The culmination of these events is the formation of microthrombi, consisting of a complex network of fibrin, platelets, red blood cells, and NETs. These microthrombi can occlude small blood vessels, leading to impaired tissue perfusion and persistent vascular dysfunction. This figure highlights the self-amplifying feedback loops that perpetuate endothelial activation and a pro-thrombotic state, contributing to the chronic vascular complications seen in long COVID. Figure created in <https://BioRender.com>.

1.3.6.6. Metabolic dysregulation

The bidirectional relationship between metabolism and immune function was discussed earlier on in this chapter. Indeed, metabolic comorbidities such as obesity and diabetes are associated with increased risk of long COVID (Scherer, Kirwan, and Rosen 2022). Early work

during the COVID-19 pandemic found SARS-CoV-2 can infect adipocytes and adipose tissue-resident macrophages (Martinez-Colon et al. 2022), and that it can also hijack host mitochondrial function to evade immunity and facilitate replication (Singh et al. 2020). However, whether mitochondrial perturbation remains in the long phase in some individuals is contested. Guarnieri et al. (2023) demonstrated downregulation of mitochondrial genes, particularly those involved in oxidative phosphorylation, during and up to 30 days after infection in both hamsters and mice (Guarnieri et al. 2023). Similar transcriptional changes were observed in human nasopharyngeal swabs and autopsy tissues. However, human samples were limited to acute severe COVID-19, and the study did not assess symptoms or outcomes related to long COVID. Notably, a placebo-controlled trial of a combination of amino acids and N-acetylcysteine (named AXA1125) was shown to significantly reduce subjective fatigue in individuals with long COVID. This suggests that supporting mitochondrial function may provide an effective therapeutic strategy. However, there was no measurable improvement in skeletal muscle phosphocreatine recovery times, which was the primary endpoint (Finnigan et al. 2023) and therefore, further investigations into the mechanisms and connection with mitochondrial function are warranted. Nevertheless, the link between metabolism and ongoing immune perturbation highlights the likelihood for metabolome-wide disruptions that may be able to track progression of patients; a theme that will be discussed later in this chapter.

1.3.7. The biopsychosocial model of medicine

There seems little doubt that '*the mind*' can influence health outcomes; indeed, any novel investigational drug must evidence superiority over placebo. The biopsychosocial model of medicine emphasises the integration of biological (physiological) and psychosocial factors (emotion; fear, stress) and is often used in the field of chronic pain (Engel 1977; Sarzi-Puttini et al. 2020). As discussed previously, pre-infection psychological stressors, including depression, anxiety, and perceived stress, in addition to socioeconomic status, have been linked to increased risk of long COVID (Greenhalgh et al. 2024; Wang, Quan, et al. 2022). During the initial wave of the COVID-19 pandemic, limited knowledge about the virus and the widespread enforcement of isolations and PPE measures created significant stress. While factors such as viral variants and vaccination status likely play a role, the majority of long COVID cases appear to have originated during this uncertain early phase of the pandemic (Greenhalgh et al. 2024). This combination of psychological stress and biological threat may have shaped immune responses in ways that predisposed some individuals to prolonged recovery. Stress, for example, is known to alter immune function and has been implicated in the reactivation of latent viruses – both potential contributors to long COVID (Glaser et al. 1991). Long term, chronic stress may also contribute to the development of hypocortisolemia, a feature reported in long COVID patients (Klein et al. 2023). Taken together, these factors may help explain why some people are more susceptible to post-viral syndromes than others. As such, many experts advocate a holistic approach to recovery – one that integrates self-management strategies, psychological support, and conventional medical treatment.

1.3.8. Lessons from similar conditions

As previously discussed, long COVID is not the first post-viral syndrome to be studied. It is therefore essential to integrate emerging findings with the substantial body of pre-existing research on related conditions. Notably, the symptoms of long COVID overlap significantly with those seen in other chronic illnesses of uncertain aetiology, such as myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) and fibromyalgia. One prominent shared feature is the experience of debilitating fatigue following minimal exertion (Hui et al. 2009; Lam et al. 2009; Moldofsky and Patcai 2011). Another is the clear female predominance observed across these conditions, with female sex consistently associated with increased susceptibility (Hamlin et al. 2024; Silva et al. 2024; Yunus 2002), and, in some cases, greater severity of symptoms (Silva et al. 2024; Sylvester et al. 2022). The higher incidence of autoimmune conditions among women suggests a potential role for sex-specific immune responses and hormonal influences in driving vulnerability to these syndromes, although the mechanisms underlying this increased female susceptibility remain incompletely understood (Takahashi et al. 2020; Silva et al. 2024). Research into ME/CFS has pointed to dysfunctional energy metabolism as a central contributor to persistent fatigue. For instance, lower levels of systemic amino acids, lipids, and metabolites involved in purine synthesis have been identified in patients with ME/CFS (Fluge et al. 2016). Similarly, studies in fibromyalgia have described altered metabolic signatures, including impaired mitochondrial function and disrupted lipid and amino acid metabolism (Clos-Garcia et al. 2019). Indeed, while this thesis focuses on characterising the metabolome of long COVID, it also draws comparisons with fibromyalgia to highlight shared and distinct metabolic features. In later chapters, the aim is to situate these findings within the broader literature on post-viral and chronic fatigue

syndromes, and to explore how future studies might be extended to include related conditions such as ME/CFS. With this in mind, the next section of the introduction turns to the concept of pain – its basic molecular underpinnings – and examines how, much like immune function, stress and emotional states can modulate the transition from acute to chronic illness.

1.4. Chronic pain

This thesis investigates chronic pain through two patient cohorts: individuals with osteoarthritis, where pain is linked to identifiable joint damage, and those with fibromyalgia, where pain arises without clear structural pathology. It also explores pain in long COVID, addressing the unmet need to understand post-viral chronic pain syndromes and their potential mechanistic overlap with established chronic pain conditions.

1.4.1. Types of pain

Pain is an unpleasant sensation, evolved as a protective mechanism to warn against and prevent further bodily damage. The protective function of pain is demonstrated by individuals with genetic conditions that cannot perceive pain and suffer from repeated and often life-threatening injuries as a result (Peddareddygari, Oberoi, and Grewal 2014). Despite this, pain can often become deleterious as it transitions to a chronic, pathological state. Chronic pain is defined as pain lasting more than 3 months and is estimated to affect 20% of the adult population globally (Cohen, Vase, and Hooten 2021). There are three main types of chronic pain: nociceptive, neuropathic, and nociplastic (Cohen, Vase, and Hooten 2021). Nociceptive pain arises from ongoing tissue damage or inflammation, activating peripheral nociceptors

that relay pain signals to the central nervous system. It is typically associated with conditions such as osteoarthritis (OA) and rheumatoid arthritis, presenting as an aching or throbbing sensation. Neuropathic pain, in contrast, results from nerve damage or dysfunction, leading to spontaneous pain or hypersensitivity, often described as burning, shooting, or electric-shock-like. Conditions such as diabetic neuropathy, postherpetic neuralgia, and sciatica exemplify neuropathic pain. Nociceptive pain occurs without evidence of tissue or nerve damage and is thought to be driven by altered pain processing in the central nervous system. This type of pain is characteristic of fibromyalgia syndrome (FMS), and is typically diffuse, widespread, and often resistant to conventional analgesics (Sarzi-Puttini et al. 2020).

Pain is also a key component of the acute sickness response and remains a prominent feature during the prolonged phase of illness, with many individuals with long COVID experiencing diffuse musculoskeletal pain and arthralgia (Khoja et al. 2022). Although the underlying mechanisms are not yet fully understood, pain may arise from direct tissue damage caused by the virus or from the ensuing immune response, aligning with nociceptive or neuropathic pathways (Khoja et al. 2022). The presence of joint pain resembling arthritis has led to comparisons with autoimmune conditions (Zacharias et al. 2021). However, the most commonly reported pain is diffuse and widespread in nature (Khoja et al. 2022). When considered alongside the hallmark fatigue and cognitive difficulties, this symptom profile bears a striking resemblance to fibromyalgia, suggesting possible overlapping mechanisms that merit further investigation. In both conditions, an emerging body of evidence implicates chronic low-grade inflammation and dysregulated neuroimmune signalling as contributors to persistent symptoms. However, the precise molecular pathways linking immune activation to this constellation of multisystem features remain poorly defined (Clauw and Calabrese 2024).

1.4.2. Mechanisms of pain

The mechanisms of pain are complex and vary depending on the underlying cause. Nociceptive pain is initiated when nociceptors, specialised sensory neurons, detect tissue damage caused by mechanical, thermal, or chemical stimuli (Ji, Xu, and Gao 2014). Following injury, immune cells such as macrophages, neutrophils, and mast cells infiltrate the damaged area, releasing inflammatory mediators including ATP, bradykinin, prostaglandins, histamine, and protons (H^+). These mediators bind to receptors such as G-protein coupled receptors (GPCRs) and transient receptor potential (TRP) channels, lowering the threshold for nociceptor activation and increasing sensitivity to pain (Ji, Xu, and Gao 2014). This process, known as peripheral sensitisation, occurs when repeated or prolonged exposure to inflammatory mediators lowers the threshold for nociceptor activation, making them more responsive to subsequent stimuli (Ronchetti, Migliorati, and Delfino 2017). This can result from an increase in receptor expression, post-translational modifications that enhance receptor function, or changes in ion channel kinetics that facilitate depolarisation. Additionally, inflammatory mediators such as prostaglandins can modulate voltage-gated sodium channels (Nav1.7, Nav1.8, Nav1.9), further increasing nociceptor excitability (Ronchetti, Migliorati, and Delfino 2017). As a consequence, even mild stimuli that would not typically evoke pain may now trigger action potentials, contributing to hyperalgesia (heightened pain sensitivity) and allodynia (pain from normally non-painful stimuli). This sensitisation may mediate the transition from acute to chronic pain, as persistent nociceptor activation maintains input to the dorsal horn of the spinal cord, reinforcing pain pathways

and increasing sensitivity to pain (Sarzi-Puttini et al. 2020). Once pain signals reach the central nervous system, they are processed through a complex network of brain regions responsible for sensory discrimination, affective integration, and modulation, as illustrated in Figure 1.5.

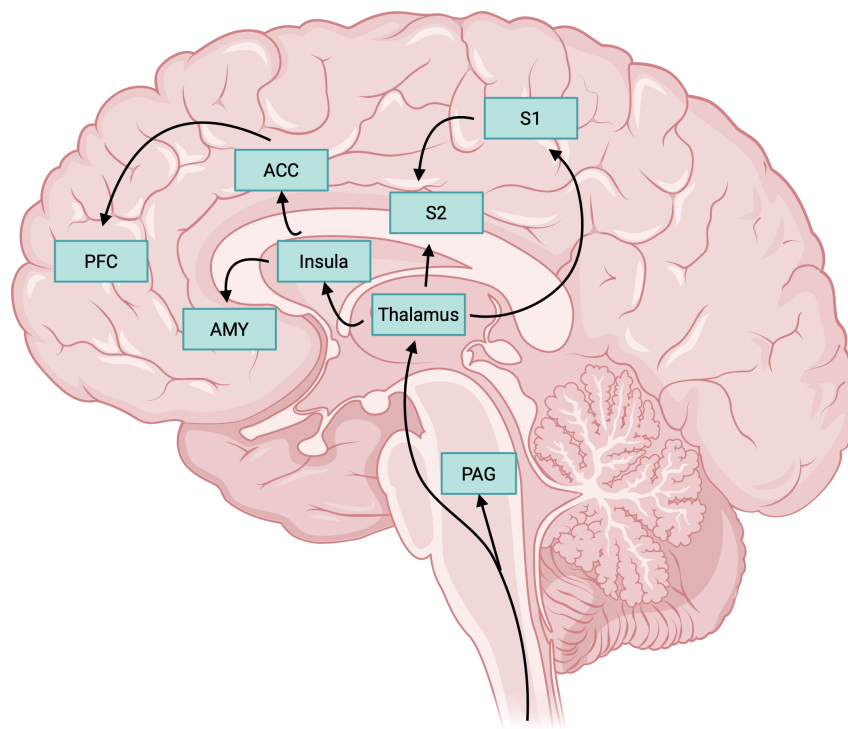


Figure 1.5. Pain signals are transmitted to the dorsal horn of the spinal cord via A δ fibers, which conduct fast, sharp pain, and C fibers, which conduct slow, burning pain. These signals are then relayed through ascending pathways to the brain, as shown here. Among these pathways, the spinothalamic tract (STT) transmits pain signals to the thalamus, which serves as a relay station to the somatosensory cortex (S1), where pain is localised and its intensity determined. The spinomesencephalic tract (SMT) sends signals to the periaqueductal gray (PAG) in the midbrain, a key structure in pain modulation. Beyond sensory processing, other brain regions shown here integrate various aspects of pain. The anterior cingulate cortex (ACC) is involved in the affective and cognitive processing of pain, while the insula integrates sensory and emotional aspects. The amygdala (AMY) plays a role in the emotional response to pain, contributing to pain-related anxiety and stress. The prefrontal cortex (PFC) is implicated in the evaluation and modulation of pain experiences, particularly in chronic pain states. The secondary somatosensory cortex (S2) participates in higher-order processing of pain signals and sensory discrimination. Notably, the relationship between pain and inflammation is not linear, as both inhibitory and excitatory mechanisms modulate pain transmission at the spinal level. It has become increasingly clear that different brain regions are innervated by multiple afferents from different sources, and it is overly simplistic to assume a direct line between peripheral nociceptors and the final perception of pain.

Dysfunction in these pathways contributes to chronic pain conditions such as fibromyalgia, where pain persists in the absence of identifiable peripheral damage. Figure adapted from (Bushnell, Ceko, and Low 2013) and created in <https://BioRender.com>.

1.4.3. Emotional and cognitive regulation of pain

Pain perception is not solely dictated by nociceptive input but is significantly influenced by cognitive and emotional factors, including anticipation, attention, and emotional state. The well-documented placebo analgesia effect demonstrates how positive expectations of pain relief can reduce pain perception via activation of descending pain inhibitory pathways. Conversely, the nocebo effect, where negative expectations or anxiety about pain exacerbate its intensity, highlights the bidirectional relationship between cognition and pain processing (Blasini et al. 2017). Neuroimaging studies have demonstrated that attention can modulate the intensity of pain experienced, with heightened anticipation of pain often exacerbating perceived pain intensity (Ploghaus et al. 1999). Similarly, when individuals focus on a painful stimulus, activity in the primary somatosensory cortex (S1), insula, and ACC, has shown to be increased, correlating with greater pain perception (Sawamoto et al. 2000). In contrast, (Fairhurst et al. 2007) found that distraction through engaging cognitive tasks reduced pain perception, with increased activation in the lateral prefrontal cortex and periaqueductal gray (PAG), areas involved in descending pain inhibition. Emotional state also plays a distinct role in shaping pain perception. For example, participants shown emotionally expressive faces or unpleasant stimuli demonstrate increased ACC activation, which correlates with reports of increased pain unpleasantness. These findings are particularly relevant for chronic pain conditions such as fibromyalgia, where altered pain processing networks are widely believed

to contribute to widespread pain along with the cognitive and emotional aspects of the disease (Sarzi-Puttini et al. 2020). However, while neuroimaging studies have been essential in identifying key brain regions involved in these processes, the underlying neurobiological mechanisms remain less well defined. Understanding these mechanisms is critical for improving pain management strategies, particularly for conditions where standard analgesic treatments are ineffective.

1.4.4. Neuroimmune interactions in chronic pain

Many of the mechanistic insights into pain dysregulation have been derived from animal models, which have provided crucial evidence for the role of central sensitisation, glial activation, and neuroimmune interactions in chronic pain. These studies have demonstrated that persistent nociceptive input leads to long-term changes in the central nervous system (CNS), amplifying pain signals through mechanisms such as temporal summation ("wind-up"), where repeated activation of nociceptors results in increased excitability of spinal cord neurons (Hachisuka et al. 2018). This process is thought to be mediated by the release of glutamate, which binds to N-methyl-D-aspartate (NMDA) receptors, strengthening synaptic transmission and reinforcing chronic pain states (Guirimand et al. 2000). Additionally, glial cell activation plays a pivotal role, with microglia and astrocytes releasing pro-inflammatory cytokines that sensitise central nociceptive neurons, further maintaining pain hypersensitivity (Ikeda, Kiritoshi, and Murase 2012). While these mechanisms are well characterised in preclinical models, evidence in human chronic pain conditions remains less well established. Studies have reported elevated markers of neuroinflammation in the cerebrospinal fluid (CSF) and blood of chronic pain patients (Backryd, Lind, et al. 2017; Backryd, Tanum, et al. 2017), but findings vary across different pain conditions, making it difficult to draw definitive

conclusions. One of the major challenges in translating these findings is the lack of biomarkers of pain, which limits the ability to accurately characterise the underlying pathophysiology and develop targeted therapeutics. However, many human studies suffer from small sample sizes, inconsistent methodologies, and the use of suboptimal control groups, where reference populations may have underlying health conditions that confound comparisons (Blichfeldt-Eckhardt et al. 2023). Addressing these limitations is essential for advancing our understanding of neuroimmune interactions in chronic pain and refining diagnostic criteria to improve patient stratification and treatment approaches.

1.4.5. Fibromyalgia and Long COVID

Fibromyalgia and long COVID exhibit intriguing parallels in their clinical presentation, suggesting potential overlaps in underlying mechanisms that warrant further investigation. Both conditions are commonly associated with widespread pain, fatigue, cognitive dysfunction ("fibro fog" in fibromyalgia and "brain fog" in long COVID), and autonomic disturbances, raising the possibility that central nervous system dysregulation plays a role in their symptom persistence (Clauw and Calabrese 2024). Notably, a significant proportion of long COVID patients fulfil the 2016 diagnostic criteria for fibromyalgia (Clauw and Calabrese 2024; Khoja et al. 2022), suggesting that the two conditions may exist along a continuum of post-infectious syndromes rather than being entirely separate entities.

Several shared risk factors between fibromyalgia and long COVID further support the possibility of overlapping mechanisms. Both conditions are more prevalent in women, a pattern observed in many chronic pain and fatigue syndromes, potentially linked to hormonal influences on immune function and pain processing (Clauw and Calabrese 2024). Other

common risk factors include pre-existing mental health disorders (anxiety, depression, PTSD), metabolic syndromes, obesity, and autonomic dysfunction (Lacourt et al. 2018; Davis et al. 2023). Additionally, it has long been hypothesised that fibromyalgia can be triggered by viral infections (Buchwald et al. 1987). However, this association appears to be based on the similarities in symptom presentation between fibromyalgia, EBV infection and ME/CFS, but has failed to gather direct causal evidence (Ruiz-Pablos et al. 2021). Unlike the current pandemic, where the onset of long COVID can be directly linked to SARS-CoV-2 infection, the difficulty in retrospectively attributing fibromyalgia to a viral trigger may have made this theory more difficult to validate. Despite these common risk factors, the mechanisms by which these factors lead to susceptibility remain poorly understood. The psychosocial factors associated with both conditions, including hypervigilance, chronic stress, and anxiety disorders have been implicated in heightened pain and autonomic dysfunction in fibromyalgia (Sarzi-Puttini et al. 2020). Indeed, long COVID patients often report psychological distress related to the uncertainty of their condition, fear of symptom persistence, and medical dismissal, which may influence disease severity and recovery (Ladds et al. 2020). However, further research into common mechanisms and biomarkers of disease is required.

Both fibromyalgia and long COVID have been proposed to involve low-grade chronic inflammation, which, as discussed earlier in relation to sickness behaviour, may contribute to overlapping symptom presentations (Liew et al. 2024; Backryd, Tanum, et al. 2017). Chronic, systemic inflammation has been linked to fatigue, cognitive dysfunction, and autonomic dysregulation, raising the possibility that persistent immune activation may be a shared pathophysiological feature (Misiak et al. 2018; Metyas et al. 2017; Bellocchi et al. 2022). However, the precise biological underpinnings of both conditions remain poorly understood,

and crucially, neither has a definitive biomarker. This diagnostic uncertainty may also lead to heterogeneous patient populations in research studies, making it difficult to identify clear mechanistic drivers or targeted therapeutic strategies.

Given the lack of reliable biomarkers, it is possible that both fibromyalgia and long COVID represent multiple overlapping clinical entities rather than singular conditions. In the case of long COVID, excluding patients with clearly defined organ damage following severe acute infection may help identify a subgroup with a phenotype more closely resembling fibromyalgia or other post-viral syndromes. Improved patient stratification could enhance the rigor of research studies, helping to uncover distinct and shared pathophysiological pathways. Furthermore, defining objective biomarkers for these conditions would be crucial in addressing the widespread medical dismissal that many patients face, improving both diagnosis and treatment strategies. Understanding the mechanistic overlaps between fibromyalgia and long COVID may not only clarify their biological basis but also lead to personalised therapeutic approaches that target shared drivers of symptom persistence. Indeed, this thesis explores biomarkers across both fibromyalgia and long COVID cohorts.

1.4.6. Importance of a diagnostic test

A diagnostic test that accurately identifies individuals with Long COVID and fibromyalgia or differentiates between them along a pathophysiological spectrum could have significant clinical utility, enabling appropriate referral to services and facilitating more targeted recruitment into clinical trials for tailored therapies. Moreover, many patients report experiencing dismissal and disbelief from healthcare professionals, colleagues, and even friends and family, as there is no objective way to confirm their diagnosis (Ladds et al. 2020).

This scepticism not only has occupational and functional implications, potentially leading to reduced work participation or disability, but also negatively impacts mental health and overall well-being (Ladds et al. 2020). Furthermore, evaluating whether long COVID is a distinct post-viral syndrome or part of a broader group of conditions with overlapping symptoms, such as fibromyalgia, may provide crucial insights into underlying pathophysiological mechanisms and therefore, potential risk factors, therapeutic approaches and prognostic factors.

1.5. An introduction to metabolomics

Given the unmet need for objective biomarkers in long COVID and chronic pain, where diagnosis and monitoring rely heavily on subjective reports, there is an urgent need for better tools to track disease progression and treatment response. Metabolomics, which captures biochemical changes reflective of immune activation, inflammation, and metabolic dysfunction, offers a powerful approach to addressing this gap (Reo 2002). This section will explore the rationale for using NMR metabolomics, highlighting its ability to provide a comprehensive snapshot of disease pathology rather than relying on single biomarkers. By integrating metabolic and immune system alterations, NMR can capture the complex, systemic nature of post-viral conditions and chronic pain.

1.5.1. ^1H NMR theory

Proton nuclear magnetic resonance (^1H NMR) metabolomics is a powerful, non-destructive analytical technique used to quantify and identify small molecules (metabolites) in biological

samples. The underlying principle of NMR is that atomic nuclei with an odd number of protons or neutrons, such as hydrogen-1 (^1H), possess an intrinsic property called spin (Hore 2015). When placed in a strong external magnetic field, these nuclei align either with or against the field, creating discrete energy states. By applying a radiofrequency (RF) pulse at a specific resonance frequency, the nuclei can absorb energy and transition between these states. As they relax back to equilibrium, they emit RF signals, which are detected and transformed into a frequency-domain spectrum via Fourier transform (Hore 2015). The chemical environment surrounding each proton influences its resonance frequency, producing distinct peaks that correspond to different metabolites, or indeed different chemical groups within a metabolite. The resulting spectrum provides a metabolic fingerprint of a sample, enabling identification of biomarkers, insights into disease mechanisms, and the ability to monitor physiological changes in response to treatment or environmental factors (Hore 2015).

1.5.2. A comparison to other techniques

^1H NMR metabolomics offers several advantages over other metabolomic techniques, such as mass spectrometry (MS)-based approaches, including liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS). Unlike MS, which requires extensive sample preparation, derivatisation (for GC-MS), and ionisation, NMR is non-destructive, highly reproducible, and requires minimal processing. However, NMR has lower sensitivity and detects fewer metabolites than MS, which can identify thousands of compounds at much lower concentrations (Emwas 2015). The performance of MS depends heavily on the specific instrument, ionisation method, and acquisition settings, necessitating the use of internal standards to account for variability in ionisation efficiency

and detector response (Emwas 2015). Additionally, LC-MS and GC-MS suffer from batch effects, ion suppression, and variations between runs, which complicate the quantification process. In contrast, NMR provides highly reproducible data because its signals are directly proportional to metabolite concentration, independent of instrument-specific variations (Emwas et al. 2019). This makes NMR particularly well-suited for longitudinal and clinical studies, where robust and standardised measurements are critical for reliable comparisons across samples, cohorts, and time points (Emwas et al. 2019).

1.5.3. Previous findings

Changes in immune function and homeostasis can have a profound effect on the peripheral metabolome. Indeed, NMR metabolomics analysis of blood can detect subtle differences in immune dysfunction in patients with inflammatory diseases of the central nervous system (Yeo et al. 2020; Yeo et al. 2019; Jurynczyk et al. 2017). Furthermore, alterations in blood metabolite abundances can predict worsening of inflammatory activity and treatment response (Probert et al. 2018). In addition, perturbations in the urea cycle, glutathione cycle and amino acid metabolic pathways have been reported in patients with chronic fatigue which are detectable by serum NMR metabolomics (Chou et al. 2021; Armstrong et al. 2012). Together, this provides strong evidence for the use of NMR metabolomics as a method of identifying long COVID patients who suffer similar symptoms to those seen in other post-viral fatigue syndromes.

1.6. Aims of this thesis

The overarching aim of this thesis is to investigate the metabolic and immunological underpinnings of post-viral syndromes, with a particular focus on long COVID, and how this

contrasts to chronic pain syndromes including fibromyalgia and osteoarthritis. Despite the significant clinical impact of post-viral syndromes, the underlying pathophysiological mechanisms remain poorly understood, with many conflicting results in the literature. Indeed, the lack of validated biomarkers of disease contributes to the confusion surrounding these syndromes. Identifying biomarkers would not only enhance our understanding of disease mechanisms but also serve as critical diagnostic tools to address the disbelief faced by patients and to facilitate appropriate patient triaging, a clear unmet clinical need. Furthermore, these biomarkers would direct the design of more meaningful research studies by accounting for patient subgroups, allowing disease progression to be tracked longitudinally and objectively, and informing the development of targeted therapeutic strategies. The aims of each specific chapter are outlined as follows.

Chapter 2: Sex-dependent responses to TLR activation: To identify the sex-specific behavioural, cytokine, and metabolic consequences of activating distinct innate immune pathways through Toll-like Receptor 4 (TLR-4) and Toll-like Receptor 7 (TLR-7) in murine models. The aim is to determine how these immune stimulants may lead to long-term alterations in mood and metabolism, providing foundational insights into post-infectious syndromes.

Chapters 3&4: Multi-omics profiling of long COVID: To develop and implement a multi-omics approach to characterise the metabolic signatures of long COVID, comparing individuals with persistent symptoms to fully recovered controls. This chapter aims to identify metabolite markers associated with ongoing symptomatology and to establish metabolic subtypes within the long COVID cohort.

Chapter 5: Coagulation profiles and metabolic signatures in a hospital-based long COVID

study: To investigate the role of coagulation abnormalities in long COVID by combining metabolomic data with coagulation markers to identify hypercoagulable subgroups. This chapter aims to explore whether these subgroups exhibit distinct inflammatory and metabolic profiles, thereby providing insights into mechanistic heterogeneity within the long COVID population.

Chapter 6: Metabolic and Neuroinflammatory Profiles in Fibromyalgia: To explore the metabolic and neuroinflammatory profiles of fibromyalgia, integrating quantitative sensory testing to identify pain subtypes. The aim is to determine whether altered metabolism and inflammation correlate with pain severity and to assess potential overlaps between fibromyalgia and long COVID.

Chapter 7: Metabolomic Characterisation of Osteoarthritis Pain: To investigate the metabolic signatures of pain in osteoarthritis by comparing serum and cerebrospinal fluid (CSF) profiles. The aim is to identify metabolic markers that correlate with pain severity and to examine whether these profiles share features with long COVID and fibromyalgia.

Chapter 8: Discussion: To bring the individual results from each chapter together, discussing the similarities and differences, and to speculate on potential pathological mechanisms. I will also address the limitations of the work presented and discuss the further studies that should be carried out based on the findings. Together, these aims reflect a comprehensive approach to understanding the complex interplay between immune activation, metabolism, and chronic symptoms in post-viral and chronic pain conditions. The findings are expected to contribute to the development of more targeted diagnostic and therapeutic strategies.

Chapter 2: Unique pathways downstream of TLR-4 and TLR-7 activation: sex-dependent behavioural, cytokine and metabolic consequences.

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Chapter summary

To investigate the metabolic and immunological drivers of post-viral syndromes, this chapter explores the role of innate immune activation via distinct Toll-like receptors (TLRs) in shaping behavioural and metabolic outcomes. While prior work in this field has largely centred on TLR-4 activation and its downstream cytokine cascades, this chapter introduces a comparative approach by examining TLR-7 activation, which has received less mechanistic attention despite its relevance to viral sensing. Using mice, we explore how stimulation of these two TLR pathways alters behaviour, cytokine expression, and metabolic profiles in a sex-dependent manner. Using this approach, we sought to offer a mechanistic perspective on how differential immune signalling may contribute to the symptoms observed in post-viral syndromes. Notably, it was discovered that TLR-4 and TLR-7 activation decreased distance travelled and rearing in the Open Field, but activation of each receptor induced distinct cytokine responses and metabolome profiles. TLR-4 activation increased IL-1 β expression and CXCL1 in the prefrontal cortex (PFC), but TLR-7 activation did not and strongly induced PFC CXCL10 expression. Thus, TLR-7 induced sickness behaviour is independent of IL-1 β expression. In both cases, the behavioural response to TLR activation was sexually dimorphic: females were more resilient. However, dissociation was observed between the resilient female mice behaviour and the levels of gene cytokine expression, which was, in general, higher in the female mice. However, the metabolic shifts induced by immune activation were

better correlated with the sex-dependent behavioural dimorphisms; increased levels of antioxidant potential in the female brain are intrinsic male/female metabolome differences. A common feature of both TLR-4 and TLR-7 activation was an increase in N-acetyl aspartate (NAA) in the PFC, which is likely to be an allostatic response to the challenges as sickness behaviour is inversely correlated with NAA levels.

2.1. Introduction

The risk of developing neuropsychiatric conditions, such as anxiety and mood disorders is increased following viral infection, and the recent COVID-19 pandemic has highlighted this phenomenon; one in eight COVID-19 patients received a neuropsychiatric diagnosis within 6 months of infection (Taquet et al. 2023). Many aspects of the reported post-viral syndromes are consistent with the adaptive sickness behaviours that are exhibited by both humans and rodents in the aftermath of a bacterial or viral infection, which are associated with an alteration in mood, anxiety, and cognition (Dantzer et al. 2008). They are typically transient and designed to assist in the recovery process and are clearly evident in individuals with SARS-CoV-2 infection (Davis et al. 2021). Once an infection has been successfully cleared, most individuals and animals gradually return to their normal behaviours and levels of activity. However, the specific duration and persistence of sickness behaviours can vary depending on the circumstances and the nature of the infection or illness and we, as have others, have shown that changes in behaviour in rodents persist beyond the acute phase in a manner that might be relevant to long COVID (Couch et al. 2015).

To date, the activation of Toll-Like Receptor (TLR)-4 by the endotoxin lipopolysaccharide (LPS) has been a prominent focus in the study of sickness behaviour in rodents owing to its

central role in gram-negative bacterial recognition, but other TLRs also have the capacity to induce behavioural changes when activated (Yates et al. 2022; Cunningham et al. 2007). RNA viruses, such as SARS-CoV-2, are detected by TLR-7, a pattern recognition receptor (PRR) that recognises single-stranded RNA (ssRNA) (Lund et al. 2004; Salvi et al. 2021). The inflammatory response to TLR-7 stimulation induces a type-1 interferon response (Lund et al. 2004) and is initiated by the transcription factor IRF7. Activation of TLR-7 triggers a MyD88-dependent cascade, involving interleukin-1 receptor-associated kinase 1 (IRAK-1), IRAK-4, and TNF receptor-associated factor 6 (TRAF6), which leads to NFkB and IRF7 activation (Cao et al. 1996; Deng et al. 2000; Kawai et al. 2004; Suzuki et al. 2002), which, on the face of it, ought to generate a similar cytokine and, downstream, metabolic response to TLR-4 activation that also activates a MyD88-dependent pathway (Hemmi et al. 2002). However, the differences in the underlying mechanisms are large enough to justify exploration of the consequence of a TLR7-mediated event.

A further, often overlooked issue is that most of the LPS-based rodent studies are performed in male mice. However, sex is known to have a profound effect on immunological outcomes (Klein et al. 2016). It is widely reported that the females generally respond more strongly to an inflammatory challenge, leading to overall better vaccine efficacies and faster clearance of pathogens with less severe resulting disease, for example (Cook and 2008). Concordantly, 80% of autoimmune disorders occur in females (Klein et al. 2016). These differences are largely attributed to the actions of sex steroids, the receptors of which are found on most leukocytes (Kadel et al. 2018). However, germline and other factors must contribute to these outcomes as differences are found throughout different stages of life and can be resilient to gonadectomy (Smith-Bouvier et al. 2008). Despite the striking influence of sex on immunity,

many studies fail to take this factor into consideration (Klein et al. 2016). This issue is especially important in the current study, as TLR-7 is an X-linked gene as is IRAK, which is also pertinent to this study (Fish 2008). Differences in expression and the downstream responses are known to arise owing to incomplete X chromosome inactivation in females in leukocyte populations especially (Souyris et al. 2018). In certain contexts, this has been reported to result in differences in susceptibility to infections, autoimmune diseases, or responses to immunomodulatory therapies that target TLR-7 (Jenks et al. 2018; Shen et al. 2010; Spiering and de Vries 2021).

Immunity and metabolism are inextricably linked, and the energetic demands following immune activation are heightened as a result of cell differentiation, proliferation, and the execution of effector functions (Jung, Zeng, and Horng 2019). Shifts in central metabolism following peripheral immune activation have been proposed to contribute to the behavioural effects of LPS challenge and can be detected using ¹H NMR metabolomics (Chan et al. 2020b). However, the metabolic effects of innate immune activation are complex and cannot be generalised across bacterial and viral infections. A study by (Wang et al. 2016) used murine models to investigate the effect of anorexia, a common sickness behaviour, on infection outcomes. They demonstrated that fasting metabolism, characterised by ketogenesis, was protective in bacterial disease models, but lethal in viral models. Conversely, glucose utilisation was essential for survival of viral infection. These distinct metabolic requirements have direct implications for the development of treatment strategies and highlight the need to study both.

Here, we sought to discover how TLR-7 activation results in sickness-associated behaviours in comparison to TLR-4 activation. We included males and females in the study design to investigate a sex-specific relationship between inflammation, metabolism, and behaviour.

2.2. Methods

2.2.1. Animals and treatments

All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, and were approved by the Animal Welfare and Ethical Review Body of the University of Oxford. Animals were kept in standard laboratory housing conditions (12 hour light/dark cycle, lights on at 7am; $21 \pm 1^\circ \text{C}$; humidity $50 \pm 5\%$) with access to both food and water ad libitum. Male and female 8-week-old CD1 mice (26-32 g; Charles River, UK) were administered i.p with R848 (5mg/kg, ApexBio, Cat No. B1054), LPS (0.83 mg/kg, Sigma Aldrich) from *Escherichia coli* serotype O111:B4, or an equivalent volume of sterile saline.

2.2.2. Behaviour

The Open Field Test (OFT) was used to measure behaviour at 20 hours post-injection, prior to tissue collection. The OFT apparatus consisted of four quadrants of equal dimensions ($l \times w \times h$; $50 \times 50 \times 50 \text{ cm}^3$) to enable four mice to be tested simultaneously. The open field was defined as 25% of the total area of each quadrant ($l \times w$; $25 \times 25 \text{ cm}^2$). Mice were placed in a randomised order in the top left corner of each quadrant and were video recorded for 5 minutes. 75% ethanol was used to wipe the floors and walls in between experiments to prevent the influence of odorants and coprophagy on mouse behaviour. ANY-Maze software (Stoelting Co., Illinois, U.S.) was used to measure all behavioural parameters except rearing,

which was counted manually whilst blinded and was defined as any time a mouse was raised on its two hindlegs (supported by walls or free-standing).

2.2.3. Tissue collection

All animals were anaesthetised under isoflurane gas at 24 hours post-injection. After checking for the absence of a pedal reflex, cardiac puncture was used to collect whole blood in EDTA (0.1M, pH8)-coated tubes and centrifuged after 30 minutes at RT (10 min, 2000xg). Tissue was transcardially perfused with heparinised saline until the liver was clear of blood. Whole brains were extracted and snap-frozen in isopentane on dry ice and liver samples were collected and immediately snap-frozen on dry ice. All tissue and plasma was stored at -80°C for subsequent gene expression and metabolomics analysis.

2.2.4. RNA extraction and reverse transcription

RNA was isolated from 10-15mg of the left prefrontal cortex (PFC) and liver samples according to manufacturer's instructions (RNeasy® Mini Kit (Cat No./ID: 74106). Liver and PFC RNA was eluted in nuclease-free water and 1000ng was converted to cDNA with the Applied Biosystems High Capacity cDNA conversion kit, following manufacturer's instructions. The reverse transcription step was carried out using the High-Capacity cDNA Reverse Transcription Kit (Thermofisher, cat. 4368813) using manufacturer's instructions.

2.2.5. qPCR

Real-time qPCR was performed with samples in duplicate (25ng/well) using the SsoAdvanced Universal SYBR Green Supermix (BioRad) with the Roche LightCycler 480. Transcripts of the acute phase response have previously been shown to be translated to protein (Campbell et al., 2003). Upon reaction completion, melt curve analysis was carried out to ensure primer

specificity. The Ct values were normalised to the housekeeping gene, GAPDH, and were represented as the fold change relative to saline controls using the delta-delta CT ($2^{-\Delta\Delta CT}$) method. Primer sequences can be found in Table 1 below.

Table 2.1: Primer sequences for qPCR

Target gene	Primer sequences
BDNF	F: GGATATTGCGAAGGGTTATTAGATT R: GGAAGGTAATGTGTCTTGTGTTGAA
CXCL-1	F: GCTGGGATTACCTCAAGAAC R: TGTGGCTATGACTTCGGTTTG
CXCL-10	F: CATCCCGAGCCAACCTTCC R: CACTCAGACCCAGCAGGAT
GAPDH	F: AACGACCCCTTCATTGAC R: TCCACGACATACTCAGCAC
IL-1B	F: CAACCAACAAGTGATATTCTCCAT R: GGGTGTGCCGTCTTTCATTA
TLR4	F: CTGGCTAGGACTCTGATCATG R: GCATTGGTAGGTAATATTAGGAACTA
TLR7	F: GTACCAAGAGGCTGCAGATTAGAC R: AGCCTCAAGGCTCAGAAGATG
SAA1	F: CCATGCTCGGGGGAACCTATG R: GCCCCAGCACAACTACT
SAA-2	F: TGGCTGGAAAGATGGAGACAA R: AAAGCTCTCTTTGCATCACTG
SAA3	F: CTGTTTCAGAAGTTCACGGGAC R: AGCAGGTCGGAAGTGGTT

2.2.6. ¹H NMR Metabolomics

2.2.6.1. Sample processing

Metabolites were extracted from brain and liver samples as previously described (Radford-Smith et al., 2022). Briefly, a pestle and mortar was used to homogenise 100mg (+/- 10%) fresh tissue on dry ice before adding 8uL/mg sample of ice-cold acetonitrile (50%). Samples were then vortexed for mixing and centrifuged at 5060xg for 5 minutes at 4°C. Supernatants

were snap frozen on dry ice, lyophilized overnight and stored at -80 °C until the day of NMR. When ready for analysis, lyophilized samples were placed at room temperature and resuspended in 550uL of NMR buffer (75mM sodium phosphate buffer prepared in D₂O, pH 7.4). The plasma samples were also allowed to thaw at RT and 150uL was added to 400uL NMR buffer.

2.2.6.2. NMR experiments

All prepared samples were then placed in a 5mm NMR tube and measured using a 700-MHz Bruker AVII spectrometer operating at 16.4 T, equipped with a ¹H (¹³C/¹⁵N) TCI cryoprobe. Plasma and liver spectra were acquired using a spin-echo sequence (Carr-Purcell-Meiboom-Gill [CPMG]), 32 data collections, an acquisition time of 1.5s, a relaxation delay of 2s, and a fixed receiver gain. Brain spectra were acquired with a Nuclear Overhauser Effect Spectroscopy (NOSEY) sequence as previously described (Chan et al., 2020). All samples were run within 9-hours of being thawed. A more detailed description of NMR experiments can be found in Chapter 3, methods section.

2.2.6.3. NMR data processing

Spectra were phased, baseline-corrected and referenced to the lactate doublet at $\delta=1.33$ ppm in Topspin 4.1.4 (Bruker). ACD/Labs Spectrus Processor Academic Edition 12.01 (Advanced Chemistry Development, Inc.) was used to manually bin each resonance signal, excluding all noise from analysis. The integrals of these bins were sum normalised and exported to R. Metabolites assignment was conducted through a mix of in-house databases, literature reviews, and 2D total correlation spectroscopy (TOCSY) experiments.

2.2.7. Statistical analysis

2.2.7.1. Univariate

All statistical analyses were carried out using the GraphPad Prism 9 software. Any outliers were identified using the GraphPad Outlier Calculator and were removed accordingly. The Shapiro-Wilk normality test was used to test for normality and log transformation was applied for the statistical analysis in instances that the test was not passed. Two-way analysis of variance (ANOVA) was used to assess a main effect of treatment (TLR agonist administration) on behaviour, relative mRNA expression, and spectral intensity. Post-hoc Tukey tests were then used for multiple comparisons if there was a significant interaction between treatment and sex. The significance threshold was set to $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, $p < 0.0001$ **** and all results were presented as mean $\pm$ standard error of the mean (SEM). All correlations were performed via Pearson correlational analyses and were corrected for false discovery rate (FDR).

2.2.7.2. Multivariate

The ROPLS package in R was used for orthogonal partial least squares discriminant analysis (OPLS-DA) of the metabolomes to determine whether there were any between group differences. Variable importance in projection (VIP) scores were used to detect which metabolites were most important for driving separation between groups. Bins with highest VIP scores (before a point of inflection when comparing all VIP scores) then underwent univariate analyses to understand the direction of change of metabolites, as described above.

2.3. Results

2.3.1. Female mice are resistant to Toll-like Receptor (TLR)-induced sickness behaviour

At 24 hours, following the administration of the TLR-7 agonist R848 or the TLR-4 agonist LPS, two-way ANOVA confirmed that there was a main effect of treatment on the distance travelled in the OFT (Figure 2.1A, $F_{2,52} = 5.367$, $p=0.0076$) and that there was a significant interaction between treatment and sex ($F_{2, 52} = 12.02$, $p<0.0001$). However, post-hoc Tukey testing revealed that while both LPS and R848 treatment decreased the distance travelled in the OFT in male mice (LPS $p<0.001$; R848 $p<0.01$), distance travelled was not reduced in female mice (LPS $p=0.93$; R848 $p=0.80$). Further analysis of the OFT data revealed that there was also a main effect of treatment on the number of entries into the centre zone of the open field (Figure 2.1B, $F_{2, 51} = 3.5187$, $p=0.0368$) and, again, a significant interaction between treatment and sex ($F_{2, 53} = 5.5$, $p=0.0064$). Tukey post-hoc comparisons revealed that the number of entries into the centre zone was decreased by LPS only in males ($p<0.001$). Two-way ANOVA also revealed a significant effect of treatment on total immobility time and the total number of exploratory rears during the OFT (immobility $F_{2, 54} = 20$, $p<0.0001$; rearing $F_{2, 53} = 9.672$, $p<0.001$). For these parameters there was no significant interaction between treatment and sex, but this result reveals that the female mice also exhibit sickness behaviours in response to the challenges (Figure 2.1C and D).

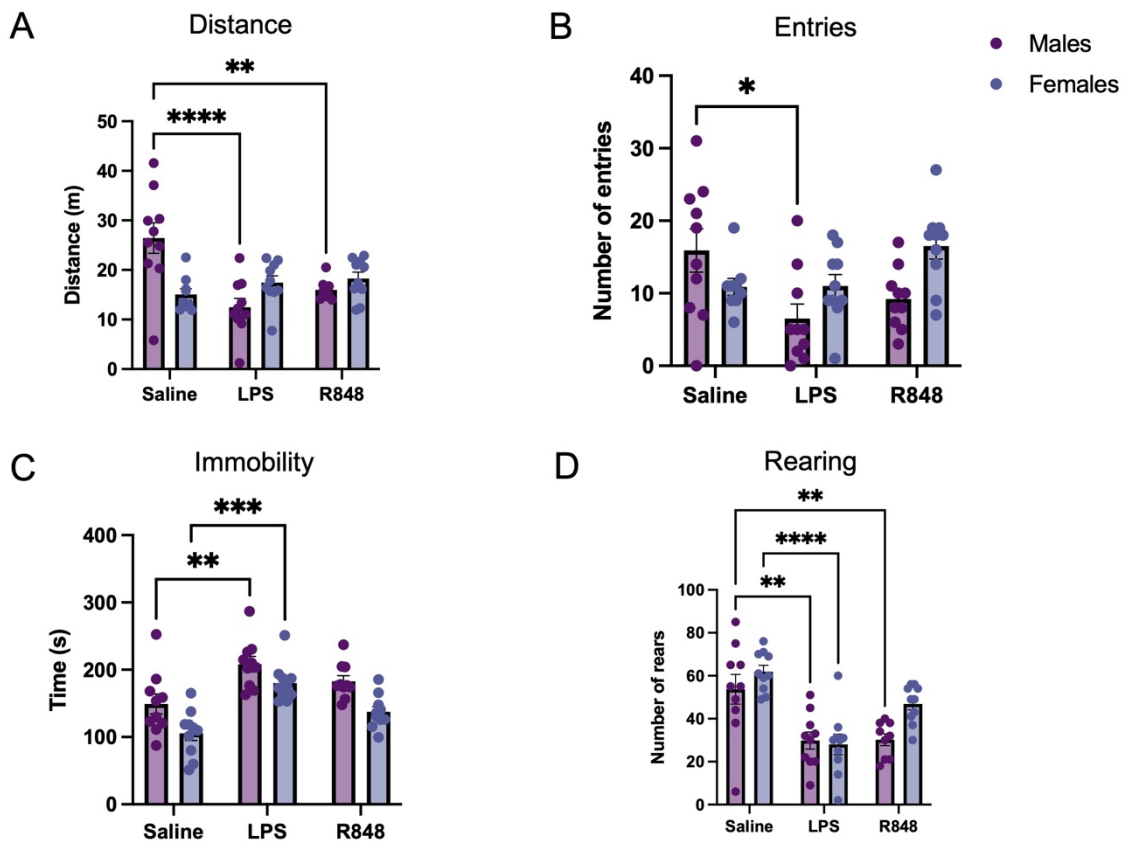


Figure 2.1: TLR activation caused sickness behaviour in mice 24 hours post-injection. (A) Distance travelled during a 5-minute open field test (OFT), (B) Number of entries to the centre zone, (C) Total time spent immobile, (D) Number of rears. Data represented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicating significant post-hoc Tukey comparisons following a significant main effect of TLR activation as determined by two-way ANOVA, $n=9-10$ /group.

2.3.2. The classical TLR-4 induced cytokine response is absent following TLR-7 activation despite the induction of sickness behaviours

TLR-4 and TLR-7 activation induced the mRNA expression of proinflammatory genes both in the liver, as part of the acute phase response (Figure 2.2), and in the prefrontal cortex (Figure 2.3) in male and female mice. Two-way ANOVA revealed a main effect of treatment on hepatic

CXCL-1, CXCL-10, and IL-1 β expression ($F_{2, 50} = 3.964$, $p=0.0252$, $F_{2, 51} = 41.71$, $p<0.0001$, and $F_{2, 54} = 13.57$, $p<0.0001$, respectively) and, for these transcripts, there was a significant interaction between treatment and sex (CXCL1 $F_{2, 50} = 3.682$, $p=0.0322$; CXCL10 $F_{2, 51} = 4.313$, $p=0.0186$; IL1b $F_{2, 54} = 6.248$, $p=0.0036$). Somewhat counterintuitively, Tukey post-hoc comparisons revealed that hepatic CXCL-1 expression was elevated relative to controls only in females 24 hours post-LPS injection ($p<0.001$). CXCL-10 expression was raised at 24 hours following LPS in both males ($p<0.01$) and females ($p<0.0001$), although significantly higher in females ($p<0.001$), whereas liver IL-1 β was higher in females at baseline ($p<0.01$) and thus, only induced in males by LPS ($p<0.01$). Interestingly, there was no significant effect of R848 on the expression of CXCL1 or CXCL10, in males or in females and IL-1 β was lower in female R848 mice alone relative to controls. Two-way ANOVA revealed a main effect of treatment on receptor expression in the liver (TLR-4 $F_{2, 50} = 11.69$, $p<0.0001$; TLR-7 $F_{2, 53} = 2.661$, $p=0.0792$) and an interaction between treatment and sex for TLR-7 alone ($F_{2, 53} = 4.384$, $p=0.0173$). Post hoc analysis indicated that females have a higher baseline expression of TLR-7 in the liver compared to males ($p<0.01$) yet no increase in expression following either challenge in females. Oddly, LPS increased TLR-7 expression in males ($p<0.01$), in a similar manner to the expression of IL-1 β . Both challenges induced three isoforms of the classical acute phase protein, Serum Amyloid A (SAA) (N.B. SAA is an apolipoprotein that is known to bind LPS (Cheng et al., 2018)) (SAA1 $F_{2, 54} = 90.16$ $p<0.0001$; SAA2 $F_{2, 48} = 61.69$ $p<0.0001$; $F_{2, 54} = 65.47$ $p<0.0001$). Furthermore, there was a significant interaction between treatment and sex for SAA2 and SAA3 ($F_{2, 48} = 3.94$, $p=0.026$ and $F_{2, 54} = 6.101$, $p=0.0041$, respectively), with females inducing a greater acute phase response (APR) following LPS in both cases ($p<0.01$).

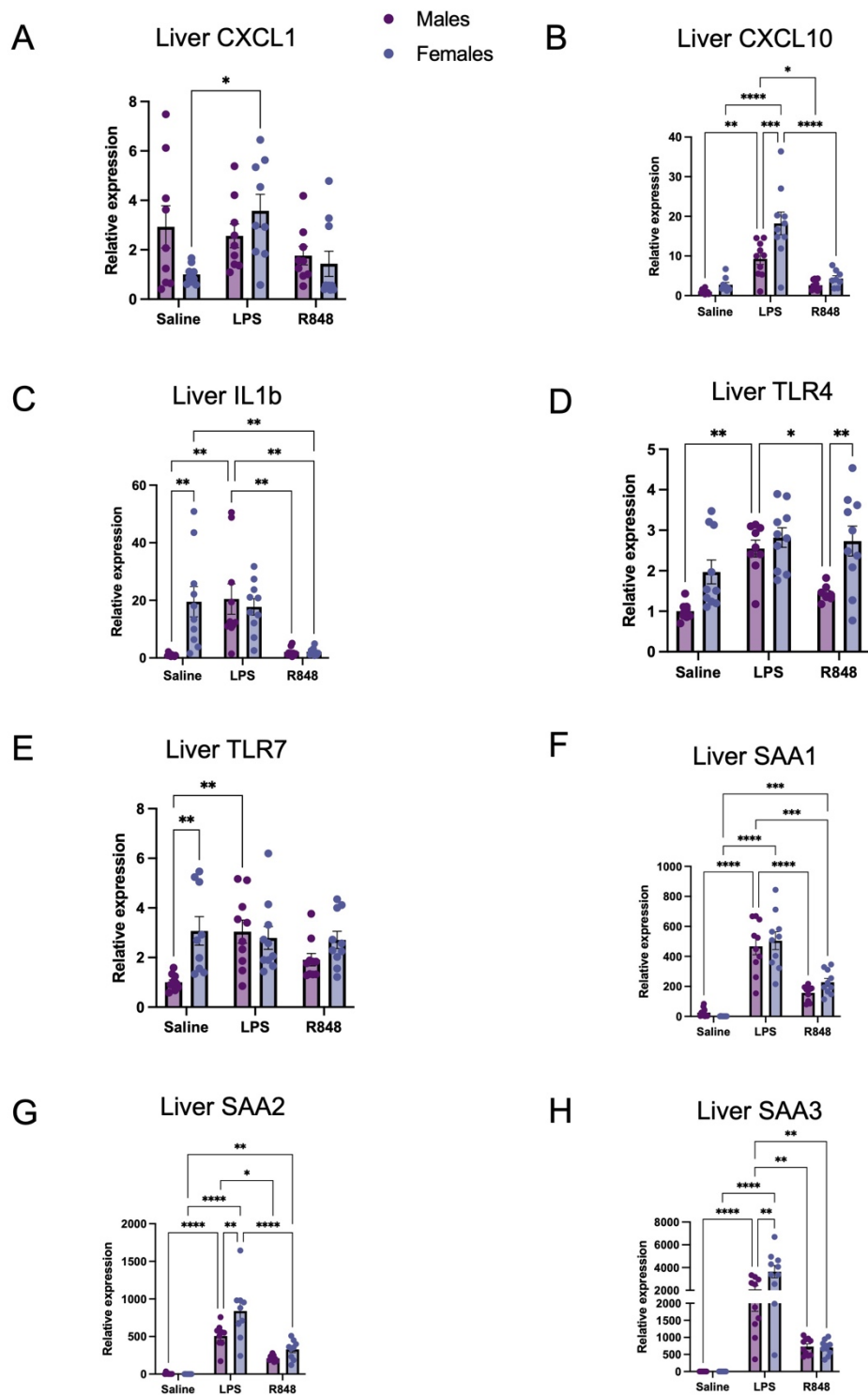


Figure 2.2: TLR-4 and TLR-7 activation by LPS and R848, respectively, increased expression of inflammatory genes in the liver 24 hours post-injection. *Relative expression of hepatic (A) CXCL-1, (B) CXCL-10, (C) IL-1b, (D) TLR-4, (E) TLR7, (F) SAA1, (G) SAA2, and (H) SAA3 mRNA. Results are presented as mean +/- SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicating*

significant post-hoc Tukey comparisons following a significant main effect of TLR activation as determined by two-way ANOVA, n=9-10/group.

In the brain, two-way ANOVA revealed a main effect of treatment on the mRNA expression of proinflammatory genes in the prefrontal cortex 24 hours post-challenge (Figure 2.3, CXCL1 $F_{2, 52} = 31.31$, $p < 0.0001$; CXCL10 $F_{2, 54} = 42.04$, $p < 0.0001$; IL-1 β $F_{2, 53} = 31.85$, $p < 0.0001$; BDNF $F_{2, 53} = 12.66$, $p < 0.0001$), but there were no significant interactions. However, both challenges provoked a different pattern of cytokine/chemokine expression. As confirmed by post-hoc Tukey analysis, TLR-4 activation by LPS increased prefrontal cortex CXCL-1 (males $p < 0.001$; females $p < 0.0001$) and IL-1 β expression (males $p < 0.0001$, females $p < 0.0001$) at 24 hours, but did not alter CXCL10 expression. Conversely, TLR-7 activation by R848 increased prefrontal cortex CXCL-10 expression (male $p < 0.0001$; female $p < 0.0001$) and did not significantly affect the expression of CXCL1 or, intriguingly, IL-1 β . Checking receptor expression in this brain region revealed a main effect of treatment on TLR-4 ($F_{2, 54} = 16.36$, $p < 0.0001$) and TLR-7 ($F_{2, 53} = 3.907$, $p < 0.05$) and no interactions between LPS and sex. It was of interest to note, that no male/female differences were detectable for these transcripts. As a consequence, we sought to discover whether metabolic changes in the blood, liver and brain might underpin the observed differences in behaviour.

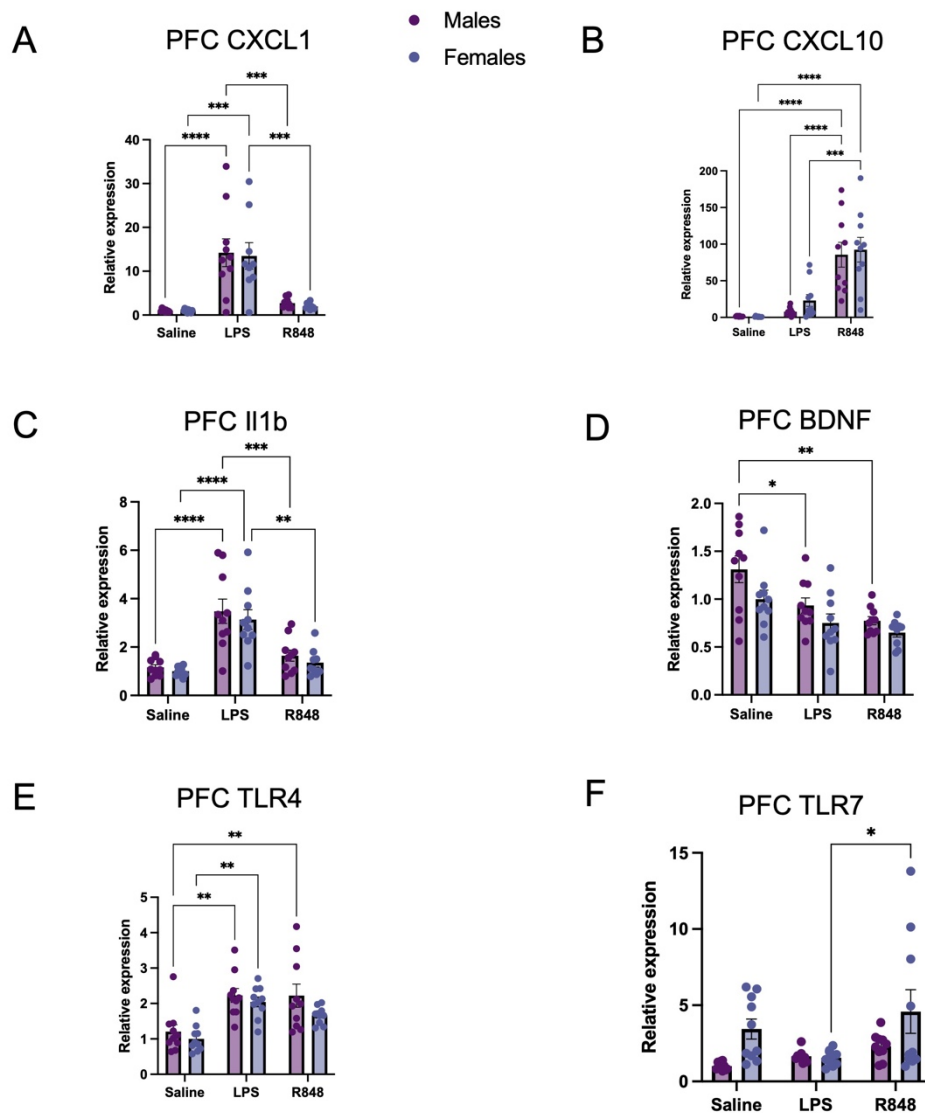


Figure 2.3: TLR-4 and TLR-7 activation by LPS and R848, respectively, increased expression of inflammatory genes in the prefrontal cortex (PFC) 24 hours post-injection. *Relative expression of (A) CXCL-1, (B) CXCL-10, (C) IL-1b, and (D) BDNF, (E) TLR4, and (F) TLR7 mRNA. Results are presented as mean $\pm$ SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 indicating significant post-hoc Tukey comparisons following a significant main effect of TLR activation as determined by two-way ANOVA, $n=9-10$ /group.*

2.3.3. TLR-4 and TLR-7 activation give rise to distinct metabolic signatures in the brain, plasma, and liver

Analysis of the metabolomic data from aqueous NMR spectra using supervised orthogonal partial least squares discriminant analysis (OPLS-DA) revealed the presence of distinct metabolic profiles for the groups treated with R848 and LPS for all tissue types (Figure 2.4). A range of metabolites differed, reflecting the impact of the precise nature of inflammation on host metabolism. Figure 2.4D summarises the direction of change of the most discriminatory metabolites relative to the saline controls and the box plots for metabolites are presented in Appendix A, Figure S2.1-3. It was of interest to note that brain N-acetyl aspartate (NAA) was a conserved marker of TLR challenge, increasing in the LPS and R848 groups relative to controls. To determine whether this change could account for the shared behavioural response in males, we performed a Pearson correlational analysis (Figure 2.4G and H). Indeed, brain NAA was found to correlate highly with distance ($r = -0.63$, $p = 0.0003$) and rearing ($r = -0.59$, $p = 0.0007$), indicating that this conserved central marker of a peripheral TLR inflammatory challenge is associated with stereotypical sickness behaviours.

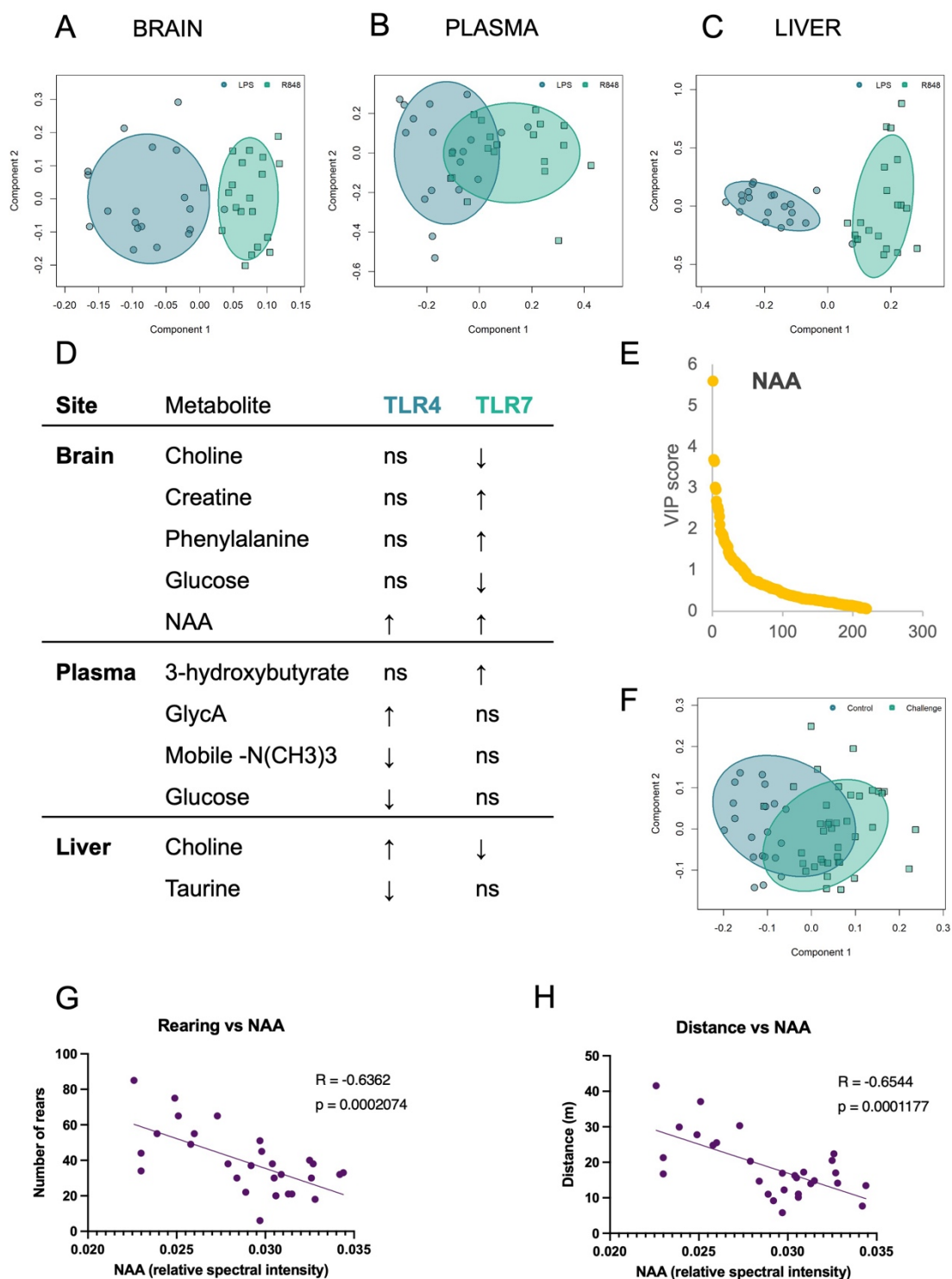


Figure 2.4: TLR-4 and TLR-7 activation gave rise to distinct metabolome profiles, yet both increased brain NAA, which correlated with the sickness behaviour displayed by males. OPLS-DA scores plot of A) brain, B) plasma, and C) liver metabolites demonstrate that the

metabolic shift following immune activation depends on the TLR agonist. D) The direction of change of the discriminatory metabolites for each tissue sites, relative to saline controls. Metabolites were selected based on Variable Importance in Projection (VIP) scores. E) VIP plot and F) associated OPLSDA scores plot of the combined TLR groups ('challenge') vs saline controls brain metabolites, confirming that brain NAA consistently increases following TLR activation, regardless of TLR7 or 4, and scores as the most discriminatory metabolite between inflammatory challenge and control. G) Pearson correlation of rearing behaviour and H) distance travelled in the OFT by males against brain NAA levels. All groups were composed of 50/50 male/female CD1 mice. Individual box plots for these key metabolites are all shown in Supplementary figures S2.1 (brain), S2.2 (plasma), and S2.3 (liver).

2.3.4. Males and females have a distinct metabolic response to TLR challenge that is driven by changes in the brain antioxidant system and is associated with behaviour

Given that females displayed more resistant behaviour to the TLR challenges, we sought to explore whether the brain metabolic response to challenge depended on sex. First, brain metabolite bins of TLR-challenged males and females were normalised to their corresponding saline control groups to avoid identifying baseline sex differences in the metabolome. OPLS-DA of these normalised groups revealed that the metabolic changes resulting from TLR challenge were distinct for males and females (Figure 2.5A). Variable importance in projection (VIP) scores revealed the most discriminatory metabolites were brain fumarate, uridine, glutathione (GSH) and cysteine (Cys). Of these discriminatory metabolites, the distribution of GSH and its precursor, Cys, were particularly distinct and non-overlapping between sexes (Figure 2.5D and E) and were found to strongly correlate with behaviour (Figure 2.5F). The increase in female antioxidant response in the brain could therefore, account for the resilient behavioural phenotype.

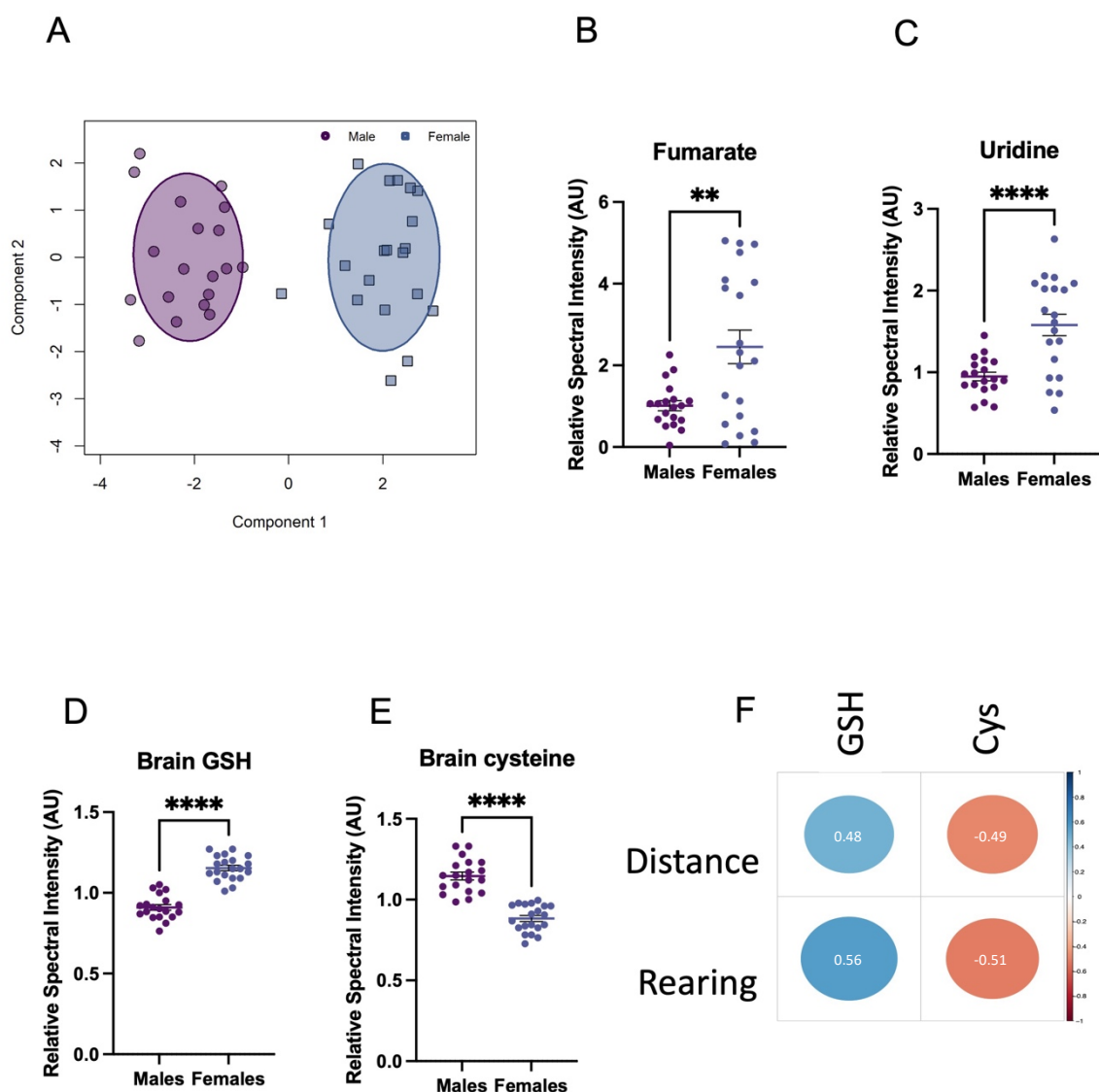


Figure 2.5: TLR challenge induces a sex-dependent metabolic response in the brain 24 hours after i.p. TLR agonist administration. A) OPLS-DA scores plot of male vs female brain metabolites after a TLR challenge, normalized to their respective male/female saline control metabolites. Normalisation avoided the influence of baseline sex differences in brain metabolomes, as we were interested in how males and females respond differently to the TLR challenge. B – E) Top VIP metabolites, selected before a point of inflection when comparing all VIP scores. These metabolites are the most discriminatory between the male vs female inflammatory metabolomes and are presented normalized to baseline i.e. male challenge vs female challenge (TLR4 and TLR7 groups combined)/saline bins. F) Pearson correlational analysis between sickness behaviours displayed by males and the corresponding GSH and Cys levels in the brain, R values shown in the centre of each circle.

2.4. Discussion

Here we sought to compare the acute phase response and changes to the metabolome downstream of systemic TLR-4 and TLR-7 activation in male and female mice. Analysis of the sickness behaviours induced by TLR challenges revealed that the female mice, compared to their male counterparts, were more resilient but cytokine production in the brain and liver in the females was either the same or elevated compared to the males. Given that the central proinflammatory cytokine response is viewed as a dose-dependent regulator of sickness behaviour this was an unexpected result. However, perhaps more striking, were the distinct metabolomic and cytokine responses to each challenge. TLR7-induced sickness behaviour was independent of increased central IL-1 β expression. The metabolic shifts induced by immune activation were better correlated with the sex-dependent behavioural dimorphisms; increased levels of antioxidant potential in the female brain are likely to govern the intrinsic male/female metabolome differences. Among the distinct challenge-specific changes observed, a notable common feature of both TLR4 and TLR7 activation were marked changes in NAA in the PFC that were inversely correlated with the sickness behaviours. These observations will be discussed in turn below.

Female mice have been shown in a few cases to be more resilient, in terms of behaviour, to an LPS challenges (Millett, Phillips, and Saunders 2019). Previous studies of LPS challenge in CD1 have similarly found females to demonstrate more resilient behaviours in terms of recovery time (Meneses et al. 2018). These studies also identified more profound cytokine responses in females, further demonstrating an inconsistency in the classical paradigm of inflammation-induced negative affect (Cai et al. 2016; Murray et al. 2019). In the current study, we found that female resilience was accompanied by a more marked hepatic acute

phase response (SAA2/3 and CXCL10) following the administration of LPS especially. The increase in basal TLR7 and IL-1 β expression in the females is likely to be a consequence of the location of TLR-7 on the X-chromosome. Biallelic B lymphocytes from women display greater TLR7 transcriptional expression than the monoallelic cells, and this was correlated with higher TLR7 protein expression in female than in male leukocyte populations (Souyris et al. 2018). Thus, TLR7 escape from X inactivation, which endows the B cell compartment with added responsiveness to TLR7 ligands, might have been expected to generate greater responses in the female versus the male mice. While this was true to some extent, it should be noted that hepatic IL-1 β expression was suppressed in the male and female mice in response to the TLR7 agonist, and, in the prefrontal cortex, despite the resilient behaviour exhibited by the female mice in response to TLR4 and TLR7 activation the induction of each cytokine was comparable between sexes.

In addition to the observed sex-specific cytokine response, the type of TLR ligand gave rise to distinct cytokine profiles. Despite both TLR-4 and TLR-7 activation inducing sickness behaviour, the expression of prefrontal cortex (PFC) CXCL-1 and IL-1 β was only elevated by the TLR-4 ligand, LPS. Conversely, PFC CXCL-10 was only altered by TLR-7 activation. Central IL-1 β has long been recognised as a mediator of inflammation-associated behaviours, as direct injection of IL-1 β into the brain generates a sickness behaviour (reduced activity and exploratory behaviour, fever, and reduced food intake), while administration of the IL-1 β receptor antagonist (IL-1RA) reverses these behaviours (Kent et al. 1992). Thus, the induction of CNS IL-1 β by LPS was not unexpected; we and many others have used a peripheral injection of LPS to induce central IL-1 β expression over many years (Layd et al. 1994; Radford-Smith et al. 2023; Van Dam et al. 1992). Other studies have found a correlation between elevated

levels of IL-1 β in the blood or cerebrospinal fluid and the severity of sickness behaviour in animals (Dantzer 2001). This further supports the idea that IL-1 β is a key mediator of these behaviours. In the PFC mRNA encoding IL-1R1 and IL-1AcP has been reported to be present in pyramidal cells and interneurons and IL-1 β was shown to induce excitatory/inhibitory imbalances at the network level (Mittli et al. 2023). In human studies, elevated levels of IL-1 β have been observed in individuals with major depressive disorder and other mood disorders. These findings suggest a link between IL-1 β and symptoms of depression, which are often part of sickness behaviour. Thus, it was a surprise that that TLR-7 activation, similar to TLR-4, induces stereotypical sickness behaviours but without the induction of PFC IL-1 β . Together, these sex- and TLR-specific effects on behaviour suggest that the relationship between inflammation and behaviour is more complex and that other factors contribute to the behavioural effects of TLR challenge. Here CXCL10, rather than IL-1 β , was strongly induced by R848, which we previously reported 6 hours after an R848 challenge in male mice (Yates et al. 2022). Others have shown that at 24h post-LPS injection, using a comparable dose of LPS, CCL2 and CXCL10 were elevated in the brain and CXCL10 expression in the brain was most positively and significantly correlated with sickness behaviour (Davis, Stevens, and Thomas 2017). More relevant to TLR7 activation, in mice changed with vesicular stomatitis virus (VSV)-M2, CXCL10 from brain endothelia was shown to disrupt synaptic plasticity and generate virus-induced sickness behaviour (Blank et al. 2016). In their compelling paper, mice lacking CXCR3 or CXCL10 and subjected to IFN- β treatment or infection with the virus were protected from depressive-like behaviour. Thus, brain CXCL10, as for IL-1 β , is clearly able to initiate sickness behaviours, but it remains unclear whether the action is directly on neurons

or via an intermediate cell population such as microglia or astrocytes (Van Weering et al. 2011).

To explore potential unknown mediators of sickness behaviour, we turned to the metabolome as it is influenced by both intrinsic and extrinsic factors and is the closest of the omics to the phenotype. Surprisingly, TLR-4 and TLR-7 activation gave rise to distinct metabolic profiles in the liver, plasma, and brain at 24 hours. Most notably, the decrease in plasma glucose following LPS administration was not observed following R848. Hypoglycaemia resulting from endotoxemia is often recognised for its sickness-inducing properties (Chan et al. 2020b), yet this shows this is not the cause of all post-infection related fatigue and mood alterations. Pertinently, following a more viral-like challenge by R848 compared to LPS, glucose remained unchanged and reveals the metabolic response is sensitive to the nature of the inflammatory challenge. Despite the accuracy of the OPLSDA models, indicating a clear separation between TLR-4 and TLR-7 groups, brain NAA increased consistently across all groups at 24 hours post-injection and was the most discriminatory metabolite when comparing the TLR challenge (LPS and R848 groups combined) to controls. The role of NAA in the brain remains elusive; however, it is the precursor of the most abundant neuropeptide, NAAG, and may be involved in myelin synthesis and neuronal metabolism. It has been demonstrated to reach high concentrations in the brain following acute inflammatory insult and has been associated with white matter destruction in such cases (Moffett et al. 2008). Here, we show NAA is a non-specific marker of peripheral PRR activation, which associates with the sickness behaviour displayed by male mice.

Next, we sought to determine whether the brain metabolic response to TLR challenge depended on sex. After accounting for baseline sex differences, OPLS-DA analysis revealed the male 'inflammatory' metabolome was distinct from that of the females. This shows that the males and females presented a divergent metabolic response to the challenge. While sex differences in immune responses have clearly been established in both rodents and humans, reports on sexual dimorphisms in immunometabolism are comparatively scarce. When testing the difference between the male and female brain metabolites after TLR challenge, we found comparatively lower brain glutathione (GSH) and respective raised cysteine (Cys) in the males. Depleted brain GSH has been used to model neuronal loss and cognitive decline in rodents (González-Fraguela et al. 2018), is associated with cognitive performance in humans and has been demonstrated, via magnetic resonance spectroscopy, to be higher in female than male brains (Mandal, Tripathi, and Sugunan 2012). The association between brain GSH and its precursor, Cys, with rearing and distance travelled in the OF could underly the sex-specific behavioural phenotypes in the current study. In humans, depression, anxiety, and cognitive decline have been associated with oxidative stress (Correia, Cardoso, and Vale 2023). Moreover, lower antioxidant potential in the brain following COVID-19 has been identified in those suffering from convalescing, illustrating that this is also a phenomenon of post-viral mood disorders, and may explain the more resilient behaviours of the female mice in the current study (Al-Hakeim et al. 2023).

2.5. Conclusion

Males and females respond differently to an immune challenge. The current study demonstrates that the relationship between cytokines and behaviour is not dose-dependent but is sex-specific. This was demonstrated by the higher levels of cytokines induced by TLR-4

and TLR-7 activation in females relative to males, despite the more resilient behaviour demonstrated by females. Additionally, the cytokine and corresponding metabolic response is sensitive to the nature of the inflammatory challenge. Specifically, the LPS-induced IL-1 β and glucose responses may not be extrapolated to other TLR challenges. The metabolic shift following TLR challenge was also dependent on the agonist, as well as sex. A striking commonality between the TLR challenges was the response of brain NAA, which was increased in both groups relative to saline controls and correlated strongly with the common sickness behaviours in the TLR male mice. Finally, most disparate changes in the brain metabolomes of male and female mice included a higher antioxidant response in females, which may underly behavioural resilience.

Chapter 3: Protocol: A Metabolomic Analysis of Long COVID

(published, in part, as <https://doi.org/10.12688/wellcomeopenres.23340.1>)

Chapter Summary

Having established in Chapter 2 that immune activation elicits sex-dependent behavioural and metabolic responses in animal models, this chapter marks a transition from preclinical findings to human studies by introducing the first clinical investigation in this thesis with the dual aims of developing a diagnostic test and uncovering potential pathophysiological mechanisms underpinning long COVID.

This chapter serves not only to introduce the first clinical study in this thesis but also to detail the trial design and protocol, which I have chosen to include owing to the extent of my involvement in its conception and execution. The complexity and novelty of studying long COVID, combined with the need for control of potential confounders in metabolomic analysis, necessitated careful study design. I was responsible for developing the trial protocol, obtaining ethical approval, and overseeing all stages of participant recruitment, consent, and sample collection. I also carried out the processing and storage of biological samples in accordance with strict pre-analytical standards to ensure the reliability of downstream metabolomic analysis.

Given the inherent sensitivity of the metabolome to environmental and demographic variables – including age, sex, ethnicity, and BMI – particular care was taken during participant recruitment to ensure appropriate matching between individuals with long COVID and

recovered controls. Such methodological precision allowed us to ascribe observed metabolic differences to disease processes with greater confidence.

In addition to outlining the technical and analytical pipeline – from recruitment through to statistical modelling – this chapter offers a comprehensive description of the clinical groups, enabling a deeper understanding of the risk factors associated with long COVID susceptibility and chronicity. By integrating symptom profiles and patient narratives, we also contextualise the metabolomic data within the lived experience of those affected, thereby linking biological signatures with functional impact. In doing so, this chapter not only lays the groundwork for the development of diagnostic and prognostic tools, but also provides a deeper understanding of the patient experience.

3.1. Introduction

This chapter presents a longitudinal diagnostic accuracy and mechanistic study employing ^1H NMR-based metabolomic profiling of blood and urine to address three interrelated aims: the identification of individuals with long COVID, the stratification of distinct symptom patterns within the condition, and the elucidation of its underlying pathophysiological mechanisms. In parallel with the metabolomic analyses, qualitative data relating to symptoms, illness narratives, and potential confounding influences are incorporated to provide a richer, more holistic understanding of the patient experience. The integration of quantitative and qualitative approaches reflects the complex, multifactorial nature of long COVID and ensures that the biological findings are interpreted within the context of lived experience.

The study was sponsored by the University of Oxford and received ethical approval from the London – Camden and Kings Cross Research Ethics Committee and the UK Health Research

Authority (REC reference 21/PR/0838, December 2021), with approval for subsequent amendments. All participants provided both written and verbal informed consent in accordance with approved procedures. Importantly, in the design phase, input was sought from individuals living with long COVID to ensure that data collection methods were appropriate and acceptable, with particular attention paid to minimising participant burden and avoiding exacerbation of symptoms.

The primary objective of the study was to generate a diagnostic test capable of identifying long COVID using metabolomic data derived from serum and urine. Participants included individuals with a confirmed history of SARS-CoV-2 infection who had either recovered fully within six weeks or continued to experience symptoms beyond this timeframe, without having been hospitalised during the acute phase. By applying multivariate pattern recognition methods to the metabolomic data, we aimed to develop mathematical models capable of distinguishing between these clinical trajectories based solely on biofluid composition.

In addition to the primary diagnostic goal, the study pursued a series of secondary objectives aimed at understanding the heterogeneity of long COVID. These included the development of predictive algorithms for disease severity and chronicity, the identification of distinct phenotypic subgroups, and the exploration of underlying biological mechanisms. Specifically, we examined variations in metabolic pathways, inflammatory markers, and immune profiles, alongside qualitative assessments of symptoms and confounders. Finally, narrative data from participants were analysed to provide deeper insight into the longitudinal experience of illness and recovery. Together, these strands of investigation are designed to produce a

comprehensive characterisation of long COVID, integrating mechanistic understanding with real-world clinical relevance.

3.2. Participant Recruitment and Selection

Figure 3.1 outlines the study flow from patient identification to the analysis. Participants include two cohorts: individuals who had received a positive diagnosis of COVID-19 infection (any type of test) who were never hospitalised and reported persistent symptoms beyond 6 weeks and an age, sex, and BMI-matched cohort of similar individuals who recovered within this timeframe. This 6-week cut-off limit was chosen in line with the duration NICE specified in their guideline for the time after which acute symptoms following COVID-19 infection should have resolved (NICE 2024).

Participants were required to have had the capacity to give informed consent, be aged over 18, and have a sufficient level of English to allow them to fully understand the study information and processes. Hospitalised individuals, those who were pregnant or breastfeeding, known carriers of bloodborne infections or with needle phobias, or those taking part in another study that might have affected the reliability of their samples were not eligible.

Recruitment was conducted via social media advertisements, University Departments and Colleges, and through two patient identification centres: Eynsham Medical Group and the Long COVID Clinic at Churchill Hospital, Oxford University Hospitals. The advertisements included a study-specific university email address for interested individuals to contact. The study team then sent out a Patient Information Sheet (PIS) and the link to the study website, which contained further information.

Potential participants were contacted by email by a member of the study team. They were asked to reconfirm their interest and provide a contact number. A GCP-trained member of the research team then called the patient to confirm eligibility, answer any questions, and arrange a convenient time for a face-to-face visit. Verbal consent was also sought to take notes on the patient narrative of long COVID. Following this, the participant was emailed a link to a questionnaire, which contained a mandatory consent form.

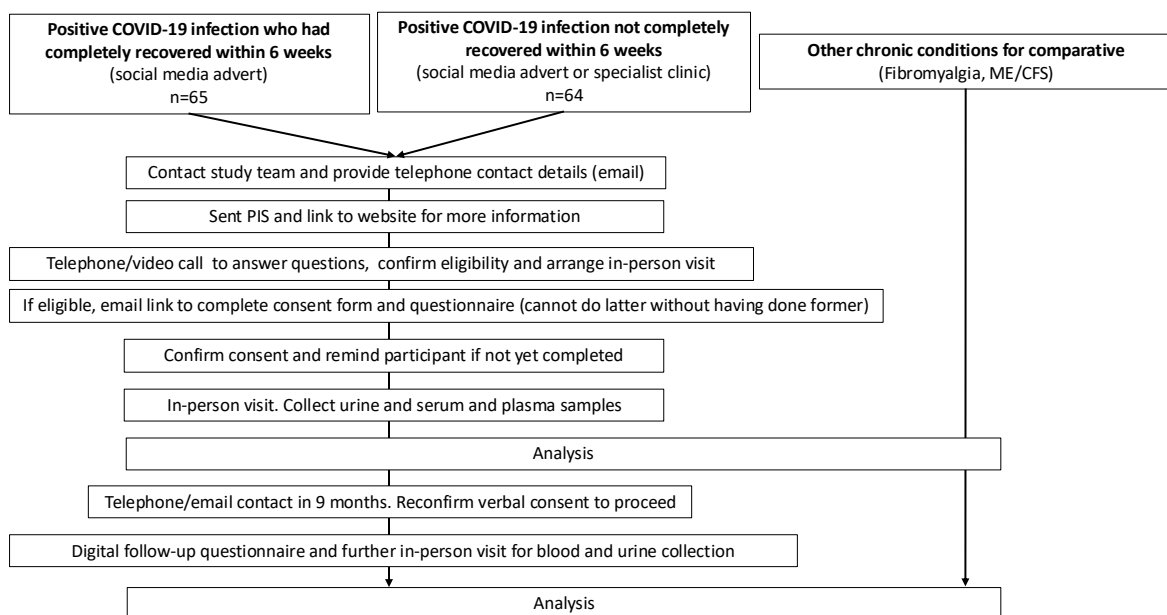


Figure 3.1: The study flow, illustrating the process from study advertisement through to the analysis. The column on the right (other chronic conditions) is not complete, but samples remain for downstream comparative analyses against Myalgic Encephalitis/Chronic Fatigue Syndrome, for example.

3.3. Data Collection

3.3.1. Demographic and Symptom Questionnaire

Feedback was specifically sought from patients with long COVID regarding how best to conduct the study. Given the level of fatigue many continued to experience, it was felt that

an online questionnaire that could be completed over multiple sittings would be most appropriate. Participants therefore had the option of completing this in advance of the first study visit, either as a hard copy mailed to them or online.

The questionnaire was designed to collect demographic information as well as self-reported symptoms throughout the acute and chronic illness phases. It included a number of quantitative metrics, such as the Patient Health Questionnaire-9 (PHQ-9) and Fatigue Severity Score (FSS), in order to objectively assess baseline differences between the cohorts and any longitudinal changes. The results from the baseline questionnaire are summarised in the cohort description in Table 3.1 and Figure 3.2.

3.3.2. Narrative data

During their first telephone call, participants were invited to describe their experience of long COVID. Many individuals did so in an unprompted manner. However, prompting to elucidate further details was used where required. Such approaches have been shown to highlight particularly salient and emotive moments in patients' illness journeys (Ladds et al. 2020). Such reflections were noted, with direct quotes used where possible. Any free text information provided by patients on the questionnaires was also included in this dataset. The aim of this data collection was to understand whether the patient narrative of disease was associated with recovery at the 9-12 month follow up.

3.4. Biological sample collection

Face-to-face visits will take place either at the patient's home (if within the Thames Valley region) or study site (if outside it) according to patient preference. At these visits written consent will be re-confirmed and blood samples will be taken. Participants will also be sent a

urine specimen bottle in the post prior to the visit and will be asked to collect a first void sample to bring with them.

Blood samples (approximately 35ml in total) will be collected. Serum and plasma tubes will be stored for 30 minutes at room temperature prior to centrifugation at 3,000 xg at room temperature for downstream metabolomic analysis. Urine samples will be rendered acellular prior to storage at -80°C.

3.5. Data Management

The study complied with the UK General Data Protection Regulation (GDPR) and the Data Protection Act 2018, which required data to be de-identified as soon as it was practical to do so. All samples were labelled with an individual's unique study code number immediately after collection. The identification list containing participant identifiers and unique study code numbers was kept in softcopy on a password-protected computer, along with copies of the signed consent forms. This information was not and will not be made public.

Acellular material and anonymised patient data were stored at the Department of Pharmacology, University of Oxford, for use in further research studies. Both are scheduled to be destroyed 25 years after the end of the study or at the participant's request up to 3 years from study completion, at which point the consent forms will also be destroyed in accordance with university policy.

Participants gave consent for re-contact by the study team at 9-12 months. At that point, they were asked to provide verbal consent to continue in the study. They were invited to complete a further questionnaire and attend or receive a follow-up visit for a second batch of samples.

Should individuals have chosen not to participate in this follow-up, they remained eligible for inclusion in the study.

3.6. Data Analysis

3.6.1. ¹H NMR Metabolomic analysis

Serum (150 µL) and urine (100µL) were mixed with 400µL or 500µL of NMR buffer (75mM or 0.2M sodium phosphate buffer prepared in D₂O, pH 7.4 for serum and urine), respectively. All samples were then transferred to a 5mm NMR tube using a glass pipette and loaded into a rack cooled at 278 K. A 950 MHz spectrometer equipped with an Oxford Instruments Company magnet, a Bruker Avance III HD console, and a TCI cryoprobe was used to collect spectra at 298 K. Serum was analysed with a Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence (with a τ interval of 400 µs, 80 loops, 32 scans, 1.5 s acquisition time, 2 s relaxation delay), while urine was processed using a 1D ¹H NOESY presaturation scheme (2 s pre-saturation) for water suppression. A fixed receiver gain was used throughout. Pooled plasma samples spread throughout the run were used to monitor technical variation for quality control purposes.

For processing, ¹H NMR spectra were phased, baseline corrected, and referenced to the lactate methyl resonance at $\delta = 1.33$ ppm and baseline-corrected in TopSpin 4.1.4 (Bruker). ACD/Labs Spectrus Processor Academic Edition 12.01 (Advanced Chemistry Development, Inc. <https://www.acdlabs.com/products/spectrus-platform/spectrus-processor/>) was used to manually bin each resonance signal to exclude noise and the integrals were sum normalised and exported to R version 4.3.0 (R_Core_Team 2024) for multivariate analysis. Resonances

were assigned by reference to literature values (Lenz et al. 2003; Tang et al. 2004; Snytnikova et al. 2019) and validated through 2D spectral analysis.

3.6.2. Multivariate Analysis

Random Forest Classification models were used to compare groups across z-scaled serum and urine metabolites and proteins using the Random Forests for Survival, Regression, and Classification (RF-SRC) R package version 3.2.0 (Ishwaran and Kogalur 2023). The tune () function was used to select the optimal values of mtry (the number of features randomly selected at each split) and nodesize (minimum size of terminal nodes). The number of trees was set to 500. Models were validated with external cross-validation, splitting data into 90% training and 10% test sets, repeated 1000 times (100 external folds, 10 iterations each). The training set identified discriminatory patterns, while the test set assessed the Receiver Operating Characteristic area under the curve (ROC AUC), out of bag (OOB) error, accuracy, sensitivity, and specificity during each loop. Significant separation was determined by comparing results to a null distribution with randomised class labels. Variables were ranked by the average Variable Importance (VIMP) score across the iterations, which measures the decrease in predictive performance when each feature is randomly permuted. The top variables (according to a point of inflection) were then selected and further characterised by univariate analysis.

3.6.3. Univariate Analysis

Univariate comparisons were performed using Two-tailed T tests or ANOVAs were performed as appropriate, with significance thresholds set to * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Pearson correlation analyses were performed, with false discovery rate correction

set at 0.05 using the Benjamini-Hochberg method. All statistical tests were carried out in R version 4.3.0 (R_Core_Team 2024).

3.6.4. Symptom clustering analysis

Symptom data were collected from participants reporting persistent symptoms following acute COVID-19 infection. As the symptom responses were binary (i.e., “yes” or “no” to each symptom), pairwise dissimilarity between participants was calculated using Gower’s distance via the *daisy* function from the *cluster* package (Maechler M 2025). This distance matrix was then used as the input for unsupervised clustering using the Partitioning Around Medoids (PAM) algorithm. To visualise the clustering structure in two dimensions, classical multidimensional scaling (MDS) was applied to the Gower distance matrix. Symptom profiles within each cluster were further characterised by generating a heatmap using the *pheatmap* package (Kolde 2019). Symptom frequencies were expressed as proportions of each cluster to account for differences in group size, and a blue gradient was used to indicate the relative prevalence of symptoms across clusters.

3.6.5. Narrative analysis

Qualitative narrative data were analysed independently by two researchers in order to gain an understanding of each participant’s interpretation of their illness narrative according to Frank’s definitions, which are outlined below. Any disagreements were discussed, and if there was still discordance, an external perspective was sought. We then explored whether these narrative types aligned with any symptom improvement over the duration of the study.

Restitution: Restitution narratives centre around experiences of recovery, improvement, or positive transformation. Participants may discuss how they have overcome challenges, regained health, or found ways to manage their symptoms. Examples of typical phrases given by Arthur Frank include: “Yesterday I was healthy, today I’m sick, but tomorrow I’ll be healthy again.” and “as good as new”.

Tragedy: Tragic narratives focus on the negative experiences of the patient journey. It may be that despite the doctor’s best effort, or indeed the patient’s willingness to improve, their symptoms remain or worsen and they are not representative of their pre-pandemic selves. Participants may describe the anger or deep sadness they have experienced throughout their illness, and this may also be conveyed by participants crying as they provide their narrative. Tragic narratives often evoke empathy and sympathy from the reader.

Humour: This narrative involves light-hearted or comical elements, where the participant may laugh while describing the challenges they face. This humour may reflect the absurd nature of their illness/patient journey.

Chaos: Chaos narratives lack a clear structure or coherence, often characterised by disjointed or fragmented storytelling. Participants may struggle to convey their experiences in a linear or organised manner, leading to confusion or ambiguity in their narratives. Chaos narratives may involve overlapping events, contradictory statements, or abrupt shifts in focus (Frank. 1995).

3.7. Results/Discussion

3.7.1. Cohort characteristics at baseline

Participants were matched for age, sex, BMI, and ethnicity (Table 3.1 and Figure 3.2). The number of comorbidities was higher in the long COVID group, although this could reflect the onset of new conditions as a result of COVID-19 infection. Interestingly, the number of symptoms experienced in the first 6 weeks of COVID-19 infection was also greater in the long COVID group, which may indicate that the severity of the acute disease was greater in this cohort compared to the controls. This is consistent with reports showing severity of infection is a risk factor for long COVID (Greenhalgh et al. 2024); however, there were no hospitalisations as a result of SARS-CoV-2 infection in either group. Further details on the type of symptoms participants had in the acute and long phase of infection are detailed in Appendix B, Table S3.1. As expected, the fatigue severity was greater in the long COVID group (Fatigue Severity Score, Figure 2E), and this also appears to be the driving factor for the high Patient Health Questionnaire-9 (PHQ-9) scores in this cohort (Appendix B, Figure S3.1, question 4). The short form-12 (SF-12) survey, which measures the impact of health on quality of life, was used to generate physical and mental composite scores (P/MCS). A score above 50 indicates better than average health-related quality of life in both the PCS and MCS (Ware, Kosinski, and Keller 1996). As shown in Figures 2G and 2H, the long COVID cohort had significantly lower SF-12 scores than controls, particularly in the PCS, indicating that their physical health is especially impacting their everyday life and well-being. These outcome measures were repeated in the follow up questionnaire, allowing for longitudinal changes to be measured and associated with alterations in self-reported symptoms and biomarkers.

	Control (n=64)	Long COVID (n=65)	P value
Age (years), mean (SD) †	45.97 (16.37)	49.68 (14.87)	
Female (%) ‡	44 (68.8%)	48 (73.8%)	
Ethnicity ‡			
White – British, Irish, other	54 (84.4%)	60 (92.3%)	
Other	9 (14.1%)	3 (4.6%)	
Did not respond	1 (1.6%)	2 (3.1%)	
BMI, mean (SD) †	24.89 (5.097)	26.71 (5.627)	
Smoking status ‡			
No	41 (64.1%)	48 (73.8%)	
Yes	1 (1.6%)	1 (1.5%)	
Previous smoking	14 (21.9%)	14 (21.2%)	
Did not respond	8 (12.5%)	2 (3.1%)	
Work situation ‡			0.0002 ***
Employed full-time	48 (75%)	31 (47.7%)	
Reduced hours	0	11 (16.9%)	
Unemployed	2 (3.1%)	9 (13.8%)	
Retired	10 (15.6%)	14 (21.5%)	
Did not respond	4 (6.3%)	0	
Comorbidities ‡			
Heart disease	2 (3.1%)	5 (7.7%)	
Diabetes	2 (3.1%)	1 (1.5%)	
Hayfever (seasonal allergies)	11 (17.2%)	21 (32.3%)	
Eczema	9 (14.1%)	7 (10.8%)	
Asthma	5 (7.8%)	12 (18.5%)	
Any joint disease such as rheumatoid arthritis	2 (3.1%)	4 (6.2%)	
Other	11 (17.2%)	26 (40%)	0.0061 **
Did not respond	7 (10.9%)	3 (4.6%)	
Medication ‡			
NSAID	4 (6.3%)	9 (13.8%)	
Aspirin	3 (4.7%)	8 (12.3%)	
Asthma medication	3 (4.7%)	11 (16.9%)	0.0445 *
Blood pressure medication	6 (9.4%)	13 (20.0%)	
Immunosuppressants	0	1 (1.5%)	
Other	15 (23.4%)	37 (56.9%)	0.0001 ***
Did not respond	7 (10.9%)	3 (4.6%)	
Vitamins/supplements ‡			
Vitamin C	6 (9.4%)	17 (26.2%)	0.0201 *
Vitamin D	20 (31.3%)	36 (55.4%)	0.0076 **
Omega-3 (fish oil)	3 (4.7%)	15 (23.1%)	0.0041 **
Zinc	2 (3.1%)	12 (18.5%)	0.0087 **
Garlic	0	2 (3.1%)	

Probiotics	3 (4.7%)	19 (29.2%)	0.0003 ***
Multi-vitamins	12 (18.8%)	18 (27.7%)	
Quercetin	0	4 (6.2%)	
Coenzyme Q10	0	4 (6.2%)	
Other	13 (20.3%)	19 (29.2%)	
Did not respond	7 (10.9%)	3 (4.6%)	

Table 3.1: Participant characteristics. † Continuous data analysed by a two-tailed T test, ‡ Categorical data analysed using Pearson's Chi Squared test or Fisher's Exact Test for 2x2 contingency tables. Where no p values are shown, there was no significant difference between groups. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

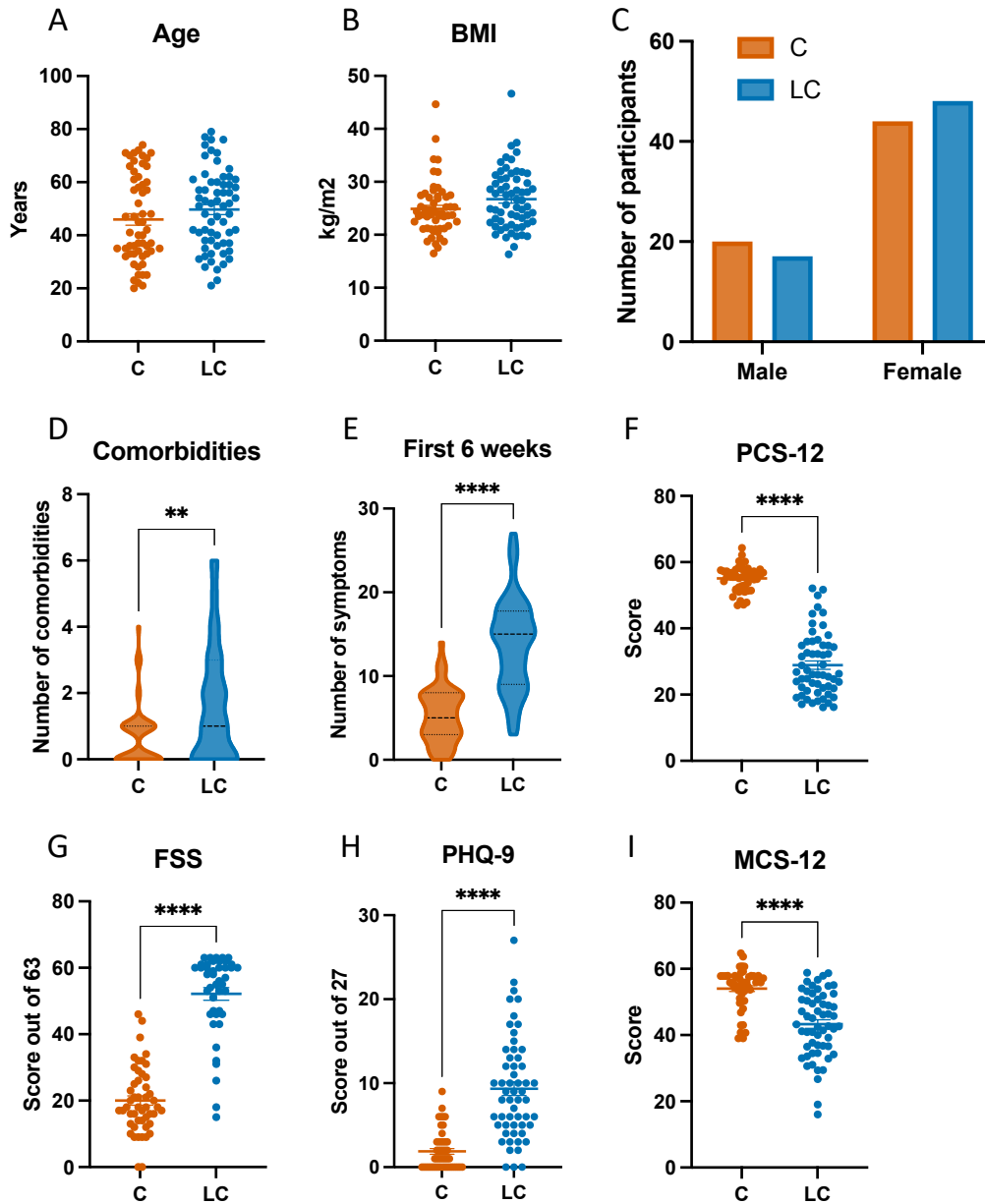


Figure 3.2: Baseline participant characteristics from questionnaire data. Graphs compare individuals with Long COVID (LC, blue) and controls (C, orange) across multiple domains: (A) Age (years) and (B) Body Mass Index (BMI, kg/m²) show no significant group differences. (C) Sex distribution reveals more females than males in both groups. (D) Number of comorbidities is significantly higher in the LC group ($p < 0.01$). (E) Number of symptoms reported during the first 6 weeks of COVID-19 infection is markedly elevated in LC participants ($**p < 0.0001$). (F) Physical Component Score (PCS-12) and (I) Mental Component Score (MCS-12) from the SF-12 health survey are both significantly lower in the LC group ($**p < 0.0001$), indicating poorer health-related quality of life. (G) Fatigue Severity Scale (FSS) and (H) Patient Health Questionnaire-9 (PHQ-9) scores are significantly elevated in LC participants, consistent with higher levels of fatigue and depressive symptoms ($**p < 0.0001$). Data are presented as mean SEM Statistical significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

3.7.2. Long COVID symptom clusters reveal distinct neurological and systemic profiles

Unsupervised clustering of binary symptom data using Partitioning Around Medoids (PAM) revealed two distinct participant subgroups based on symptom co-occurrence patterns. Classical multidimensional scaling (MDS) applied to the Gower distance matrix demonstrated clear separation between these two clusters in two-dimensional space (Figure 3.3, Panel A). To further characterise the symptom burden associated with each cluster, we visualised symptom frequencies as a heatmap, scaled within each symptom and expressed as a proportion of participants within each cluster (Panel B). Cluster 1 (light blue) was characterised predominantly by neurological symptoms, with a particularly high prevalence of neurocognitive and fatigue-related symptoms such as memory problems, severe fatigue, difficulty with attention and word-finding. However, most other symptoms within this cluster had a relatively low prevalence, indicating that these individuals may primarily experience neurological sequelae. In contrast, Cluster 2 (dark blue) displayed a higher overall symptom burden, featuring a more systemic and acute-like symptom profile. Symptoms such as cough, fever, abdominal pain, loss of smell or taste, and gastrointestinal disturbances were notably more frequent in this group, suggesting a broader spectrum of post-viral symptoms. These findings suggest that this long COVID cohort is heterogeneous, comprising two distinct clinical subtypes: one predominantly neurological and fatigue-related, and another with more widespread, systemic symptoms resembling a lingering acute inflammatory response. This differentiation may have important implications for diagnosis, prognosis, and the development of tailored management strategies. Future work should assess whether these

symptom clusters correlate with underlying biological differences or differential recovery trajectories. This work will be presented in the next chapter.

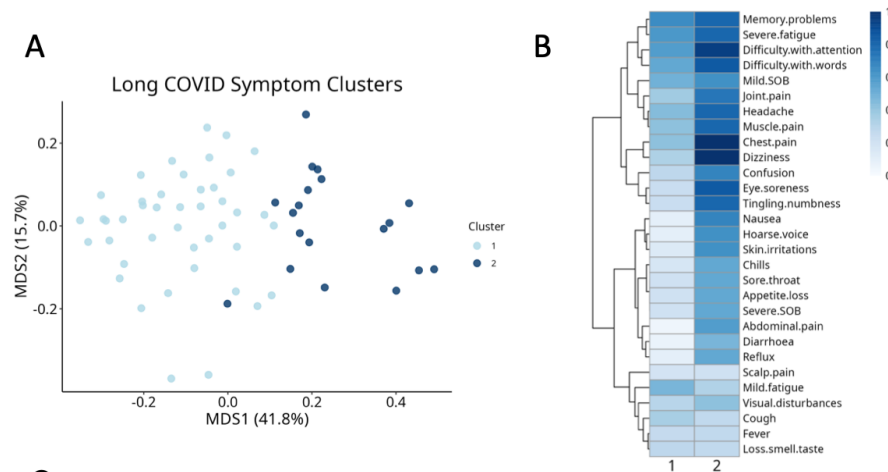


Figure 3.3: Symptom-based clustering in individuals with Long COVID. (A) *Multidimensional scaling (MDS) plot showing two distinct clusters based on binary symptom data using Gower distance and Partitioning Around Medoids (PAM).* (B) *Heatmap of symptom prevalence within each cluster, scaled by row and expressed as a proportion of individuals in each group. Cluster 1 (light blue) is enriched for neurocognitive and fatigue-related symptoms, while Cluster 2 (dark blue) shows a multi-system symptom profile with higher prevalence of acute-phase features.*

3.7.3. Cohort characteristics at follow up

Demographic and clinical characteristics of the study cohort at follow-up (9-12 months) are presented in Figure 3.4A–G. Age (Figure 3.4A) and body mass index (BMI) (Figure 3.4B) did not significantly differ between control participants and individuals with long COVID, regardless of symptom improvement status. In contrast, significant differences were observed in fatigue severity and depressive symptoms. The Fatigue Severity Scale (FSS) scores (Figure 3.4C) were significantly elevated in participants who did not report symptom improvement compared to both improved participants ($p < 0.001$) and controls ($p < 0.0001$).

Similarly, scores on the Patient Health Questionnaire (PHQ-9), assessing depressive symptoms (Figure 3.4D), were markedly higher in the non-improved group compared to improved participants ($p < 0.001$) and controls ($p < 0.0001$), indicating persistent psychological distress and fatigue in this subgroup.

Health-related quality of life was assessed using the Mental Component Summary (MCS-12) and Physical Component Summary (PCS-12) scores (Figures 3.4E and F). Both MCS-12 and PCS-12 scores were significantly lower in non-improved participants compared to improved participants ($p < 0.01$ and $p < 0.0001$, respectively) and controls ($p < 0.0001$ for both), reflecting poorer mental and physical well-being in those who reported no symptom improvement at follow up.

The number of ongoing symptoms reported at follow-up (Figure 3.4G) was significantly higher in the non-improved group compared to those who reported improvement ($p < 0.05$), demonstrating a continued burden of symptoms in a subset of individuals. Together, these findings highlight distinct subgroups within the long COVID cohort at follow-up, with a clear separation between those who have recovered or improved and those who continue to experience a significant symptom burden, fatigue, depressive symptoms, and reduced quality of life.

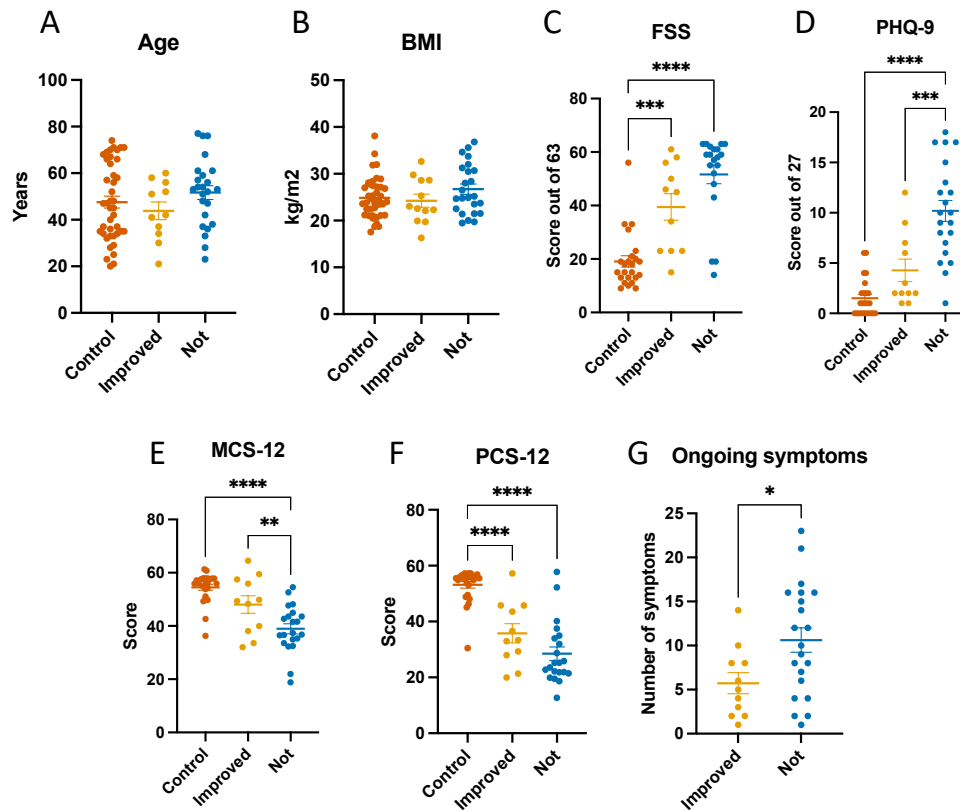


Figure 3.4: Cohort characteristics at follow-up. (A–B) Age and BMI across control, improved, and non-improved groups. (C–D) Fatigue Severity Scale (FSS) and Patient Health Questionnaire (PHQ-9) scores comparing groups. (E–F) Mental Component Summary (MCS-12) and Physical Component Summary (PCS-12) scores. (G) Number of ongoing symptoms in each group. Data presented as mean $\pm$ SEM. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.7.4. Associations with symptom improvement

To explore potential factors associated with recovery in long COVID, we compared demographic, psychological, and symptom cluster characteristics between participants who reported improvement and those who did not. Analysis of demographic and symptom cluster characteristics between participants who reported improvement and those who did not revealed no statistically significant differences ($p > 0.05$, chi-squared or Fisher's exact test as

appropriate). However, several trends were observed. A higher proportion of participants who did not report improvement were classified as tragedy (T) responders compared to the rest of the responders (R). This latter group, which comprised participants assigned humour (H) and chaos (C) narratives given the small sample size in these cohorts, was predominantly made up of those assigned with restitution (R) ($n = 7$). Specifically, among non-improved participants, 10 out of 16 (62.5%) were assigned tragedy narratives, while those who reported improvement were more evenly distributed across the restitution, tragedy, humour, and chaos categories. These findings suggest that individuals who view their illness experience through a tragic lens may be less likely to report recovery, although the small sample size and lack of statistical significance warrant cautious interpretation. Additionally, there was a trend towards more females reporting persistent symptoms compared to males. A greater proportion of participants in the depressed group also fell within the non-improved category compared to those without depression. Furthermore, Cluster 1, which predominantly featured neurological symptoms, contained a higher proportion of non-improved participants compared to Cluster 2, which was characterised by more systemic symptoms. Despite these observable trends, none reached statistical significance.

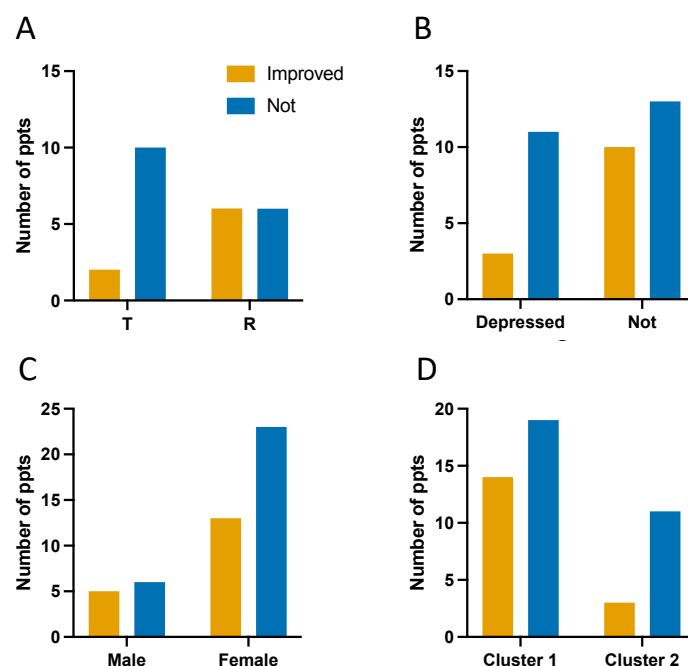


Figure 3.5: Trends in demographic, psychological, and symptom cluster characteristics associated with symptom improvement in long COVID (not statistically significant). Bar plots show the number of participants (ppts) who reported improvement (orange) or no improvement (blue) according to four variables: responder narrative (T = tragedy, R = restitution/humour/chaos), depression status (depressed or not), sex (male or female), and symptom cluster (Cluster 1 = predominantly neurological symptoms, Cluster 2 = predominantly systemic symptoms). Although no statistically significant differences were found ($p > 0.05$, chi-squared or Fisher's exact test), several trends were observed. Non-improved participants were more frequently classified as tragedy responders compared to those who reported improvement, who were more evenly distributed across the narrative categories. A greater proportion of females reported persistent symptoms compared to males. Additionally, participants with depression and those in Cluster 1 were more likely to report no improvement compared to those without depression and those in Cluster 2, respectively. The lack of statistical significance warrants cautious interpretation of these trends.

3.8. Conclusion

A key strength of the study is that the cohorts were matched for age, BMI, and sex. This is crucial when interpreting results, particularly in the context of metabolomics, where these factors can significantly influence the biological signature. Moreover, the demographic and

clinical characteristics of the cohort align with the wider long COVID literature, with a predominance of female participants, a high prevalence of depressive symptoms, and a diverse symptom profile reflecting both neurological and systemic manifestations. Clustering analysis further revealed that long COVID presentations are heterogeneous, with at least two distinct clinical subtypes identified: one predominantly characterized by neurological symptoms and fatigue, and another with more systemic, acute-like symptoms. Despite these clear differences in symptomatology, there were no significant differences in recovery between these clusters. Likewise, narrative analysis highlighted that individuals with tragedy-based illness narratives were more likely to fall into the non-improved category, although this trend did not reach statistical significance. Overall, these findings suggest that the experience of recovery from Long COVID may be highly individual and not easily predicted based on demographic profiles, symptom clusters, or narrative frameworks alone. These insights highlight the need for a more nuanced understanding of recovery trajectories, potentially involving biological or contextual factors not captured in this analysis. Moving forward, we will explore biological differences underpinning recovery and persistent symptoms to gain a deeper understanding of the factors that may influence long-term outcomes in long COVID.

Chapter 4: Multi-omics analysis identifies distinct metabolic subtypes in long COVID and their links to symptom severity

Chapter Summary

Building on the cohort characterisation and methodology established in the previous chapter, Chapter 4 presents the longitudinal multi-omics investigation aimed at identifying diagnostic and mechanistic metabolic signatures of long COVID. Using ^1H NMR-based metabolomics and lipidomics, alongside validated patient-reported outcome measures, this study analyses serum and urine samples from the matched cohort of individuals with long COVID and fully recovered controls. By incorporating data from two time points and combining metabolomic with symptom-based data, this chapter work sought not only to improve diagnostic accuracy, but also to gain insight into the biochemical processes associated with persistent symptomatology.

The integration of metabolomic and lipidomic data enabled the development of multivariate classification models that achieved robust discriminatory power when both serum and urine profiles were analysed in combination. Notable metabolic features distinguishing long COVID from recovered controls included elevated GlycA – an established marker of systemic inflammation – as well as perturbations in steroid hormones and amino acid metabolism. Importantly, symptom-specific correlations revealed biologically meaningful associations between metabolite levels and fatigue, depression, and physical function scores, thereby linking molecular alterations to clinical outcomes.

Through unsupervised clustering, two distinct metabolic subtypes of long COVID were identified: one characterised by elevated inflammatory markers and the other by neuroendocrine and amino acid dysregulation. These subtypes corresponded to different symptom profiles, suggesting heterogeneity not only in clinical presentation but also in underlying pathophysiology. Longitudinal analysis further demonstrated that metabolic recovery tracked with symptomatic improvement, whereas individuals who failed to recover showed sustained deficits in energy and amino acid metabolism, particularly in relation to glutamine, acetate, and branched-chain amino acids.

Together, these findings support the concept of long COVID as a biologically distinct condition with reversible features in a subset of individuals. They also underscore the value of metabolomics for patient stratification and as a potential tool for monitoring disease progression and therapeutic response. This chapter thus advances the central thesis aim of identifying biomarkers for post-viral syndromes and supports further exploration of metabolically targeted interventions.

4.1. Introduction

The emergence of Long COVID, also known as Post-Acute Sequelae of SARS-CoV-2 Infection (PASC), has introduced a significant global health challenge, affecting millions of individuals long after the acute phase of COVID-19 has resolved. Estimates suggest that 10–30% of individuals who contract SARS-CoV-2 develop persistent symptoms lasting beyond three months, with some cases extending over two years (Peter et al. 2025; Wynberg et al. 2023). The symptoms of Long COVID are highly heterogeneous, encompassing fatigue, cognitive impairment, shortness of breath, autonomic dysfunction, and persistent inflammatory

responses, among others (Al-Aly, Xie, and Bowe 2021; Greenhalgh et al. 2022). Risk factors for long COVID include female sex, older age, and experiencing multiple symptoms during acute infection (Sudre et al. 2021). These lingering effects pose a profound burden not only on individual health but also on healthcare systems and economies worldwide, with productivity losses estimated in the billions due to disability, reduced work capacity, and healthcare utilisation (Datta, Fazlul, and Khan 2024).

Despite the substantial impact of Long COVID, diagnosing and understanding its underlying mechanisms remain challenging. The condition is primarily identified through self-reported symptoms, which introduces issues related to subjectivity and variability in diagnosis (Nalbandian et al. 2021), and complicates patient stratification and biomarker validation (Reese et al. 2023). Thus, the absence of definitive diagnostic biomarkers further complicates efforts to standardise clinical management and therapeutic interventions. Emerging evidence from longitudinal studies suggests that persistent inflammation, immune dysregulation, and metabolic alterations may underlie the pathophysiology of Long COVID (Talla et al. 2023). Elevated levels of pro-inflammatory cytokines, including interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF- α), and interleukin-1 beta (IL-1 β), have been reported in numerous patient cohorts (Maciel et al. 2024), but the results have often been equivocal and the measurement of highly labile cytokine and chemokines can be problematic. However, the majority of these findings suggest a sustained inflammatory response that extends well beyond the acute infection phase, potentially contributing to ongoing tissue damage and symptom persistence. In addition to cytokine dysregulation, increased activation of inflammatory pathways such as the JAK-STAT and NF- κ B signalling cascades has been observed, further reinforcing the notion of chronic immune activation (Talla et al. 2022).

Some long COVID studies have demonstrated that inflammatory markers remain elevated for up to two years post-infection (Redondo-Calvo et al. 2024), suggesting that Long COVID may share pathophysiological features with other chronic inflammatory conditions. The presence of neutrophil activation markers, such as elevated IL-8 and circulating nucleosomes, implies a prolonged state of immune activation, potentially leading to tissue-specific damage (Talla et al. 2022). However, despite the growing body of evidence linking immune dysregulation to Long COVID, inconsistencies in biomarker profiles across studies highlight the need for standardised methodologies and longitudinal analyses to validate these findings.

Neurological symptoms, including cognitive impairment, headaches, dizziness, and neuropathic pain, are frequently reported in Long COVID patients. Biomarker studies have begun to shed light on potential mechanisms underlying these symptoms. Neurofilament Light Chain (NFL) and Glial Fibrillary Acidic Protein (GFAP), markers of neuronal injury and astrocyte activation, respectively, were detected at elevated levels in COVID-19 patients with neurological manifestations (Zingaropoli et al. 2023; Spanos et al. 2022), but the data reported from Long COVID sufferers has rarely been compared to age- and BMI matched controls (Ruchisrisarod 2024) and it is unclear whether there is ongoing enhanced neuronal injury in the Long COVID population who report on-going 'brain fog'. Oxidative stress markers, such as Cytochrome c Oxidase Subunit 7A1 (COX7A1), have been associated with cognitive dysfunction and fatigue in Long COVID (Hanson et al. 2023). Given that mitochondrial dysfunction and energy metabolism abnormalities have been implicated in neurodegenerative diseases, it is plausible that similar mechanisms contribute to the neurological symptoms observed in Long COVID. However, few studies have integrated omics

studies to delineate the precise relationship between metabolic dysregulation and neuroinflammation in this condition.

Metabolomic and lipidomic analyses have emerged as powerful tools for uncovering biochemical alterations in disease states. In the context of Long COVID, several studies have highlighted disruptions in lipid metabolism, amino acid metabolism, and oxidative stress pathways (Hanson et al. 2023). Lipidomic analyses have revealed abnormalities in phospholipid and sphingolipid composition, suggesting alterations in membrane integrity and signalling processes that may impact cellular function. NMR-based metabolomics, a technique capable of detecting small-molecule metabolites with high precision, has shown promise in identifying metabolic signatures associated with Long COVID. In previously hospitalised patients, preliminary findings suggest that dysregulation in energy metabolism, including alterations in glucose and fatty acid utilisation, may play a role in the persistence of symptoms (Ansone et al. 2024). Additionally, markers of impaired mitochondrial function and oxidative stress have been identified, supporting the hypothesis that metabolic dysfunction contributes to the pathogenesis of Long COVID (Molnar et al. 2024).

While these findings are compelling, metabolomic studies face challenges related to interindividual variability, confounding factors such as preexisting conditions, and the need for larger, well-characterised cohorts. The integration of multi-omics approaches, combining metabolomics, lipidomics, and proteomics, in conjunction with symptom questionnaire data is required for a more comprehensive understanding of the molecular alterations underlying Long COVID and facilitate the identification of clinically relevant biomarkers. Indeed, cluster analysis has revealed distinct symptom patterns, suggesting varied underlying mechanisms

(Kenny et al. 2023). The heterogeneous nature of Long COVID suggests that multiple subtypes may exist, each with distinct pathophysiological mechanisms. Identifying these subgroups through biomarker-based classification will be crucial for developing targeted therapeutic strategies.

Longitudinal studies with standardised methodologies are essential to track biomarker evolution over time and distinguish persistent abnormalities from transient post-viral changes. Here, we sought to address these gaps by utilising a multi-modal approach, incorporating NMR-based metabolomics (Xiong et al. 2024), lipidomics (Thomas et al. 2024), neurofilament light chain (NfL) levels, Glial Fibrillary Acid Protein (GFAP) (Yeo et al. 2021), and patient-reported questionnaires to characterise metabolic and inflammatory changes in individuals with Long COVID compared to recovered individuals. By integrating biochemical and clinical data, we have uncovered objective biomarkers that can aid in diagnosis, risk stratification, and potential therapeutic targeting.

4.2. Materials and Methods

4.2.1. Participants

The primary objective was to develop a diagnostic test using metabolomics, while secondary objectives included stratifying Long COVID patients based on metabolic and immune profiles and identifying potential pathophysiological mechanisms underlying persistent symptoms. Chapter 3 presents a full description of the study protocol, participant recruitment, eligibility criteria and characteristics.

4.2.2. Clinical questionnaires

Demographic and symptom data were collected as described in Chapter 3, at baseline and at 9-12 months follow up.

4.2.3. GFAP and NfL

The concentrations of sNfL and sGFAP were measured using the Simoa® Neurology 2-Plex B Kit on a Simoa® HD-X analyzer (Quanterix, Billerica, MA, USA). This ultrasensitive immunoassay kit is designed for the quantification of NfL and GFAP in human serum. Analyses were conducted at the University of Basel in accordance with the manufacturer's guidelines. All samples were measured in duplicate using the same kit lot to ensure consistency. Measurements were performed on the same machine by laboratory personnel blinded to the sample identities and clinical data. The intra-assay coefficient of variation (CV) for all samples, including the quality control samples, was maintained below 20%.

4.2.4. Mass spectrometry

The analytical method was developed for serum samples and all solvents were LC-grade (Sigma-Aldrich Inc., St. Louis, MO). Initially, the internal standards were added to 200 µL of serum in silylated 2 mL glass chromatography vials. The proteins were then precipitated using acetonitrile (400 µL) containing 0.1% v/v formic acid (LC-grade, Sigma-Aldrich). This was done in metal and glass syringes to ensure no contamination from plastic. The samples were then briefly vortexed and placed at -20 °C for 30 min. The samples were then centrifuged ($10,000 \times g$, 4 °C) for 10 min. The supernatant was then removed and placed in fresh silylated vials and stored for no more than 24 h before solid-phase extraction (SPE) of the lipids.

4.2.5. NMR Sample preparation

Biological samples were collected at baseline and follow-up visits (9-12 months), either at participants' homes or at a designated study site. Participants provided a first-void urine sample, and blood samples (~35 mL) were drawn into serum and plasma tubes. Serum and plasma samples were stored upright at room temperature for 30 minutes after drawing and then processed by centrifugation at $3,000 \times g$ at room temperature. Aliquoted samples were then immediately placed on dry ice and transferred to a -80°C freezer. Urine samples were rendered acellular prior to storage at -80°C for downstream metabolomic analysis.

4.2.6. NMR-based metabolomics

Metabolomic profiling was performed using ^1H -NMR spectroscopy, following standardised protocols (Dunstan et al. 2024; K Dunstan, C Anthony, and Ladds 2025). Spectra were acquired and processed in TopSpin 4.1.4 (Bruker), phased, referenced, and baseline-corrected before being manually binned with ACD/Labs Spectrus Processor Academic Edition 12.01. Metabolite integrals were sum-normalised prior to statistical analysis.

4.2.7. Statistical analysis

All statistical analyses are outlined in Chapter 3. A visualisation of the random forest approach is also presented in Figure 4.1A.

4.2.8. Data management

All data were managed in compliance with UK GDPR and the Data Protection Act 2018. Samples were anonymised immediately after collection and securely stored at the Department of Pharmacology, University of Oxford. Participants provided consent for long-

term sample storage and potential future analyses, including autoantibody and cytokine profiling.

This study successfully applied advanced NMR-based metabolomics, lipidomics, and patient-reported outcomes to characterise biochemical alterations in Long COVID. A random forest classification model distinguished Long COVID from fully recovered controls, revealing distinct metabolic signatures associated with persistent inflammation, altered energy metabolism, and disease subtypes. These findings contribute to the development of biomarker-based diagnostics and provide novel insights into the pathophysiological mechanisms underlying Long COVID.

4.3. Results

4.3.1. Metabolomic and Lipidomic Signatures Distinguish Long COVID from Recovered COVID-19 Controls

To evaluate the robustness and accuracy of the metabolomic model in distinguishing Long COVID from recovered controls, we employed a nested cross-validation framework with random forest classification (Figure 4.1A). The outer loop consisted of 100 iterations, each involving a 90% training and 10% test set split, while the inner loop performed 10-fold cross-validation within the training set. To account for potential bias, we also performed classification using randomised group assignments to generate a “random model” for comparison. The true model consistently outperformed the random model across all key performance metrics (Figure 4.1B). The average ROC AUC for the true model was 0.70, significantly higher than the random model (**** $p < 0.0001$), indicating robust discriminatory ability. Accuracy (0.62), sensitivity (0.63), and specificity (0.64) also significantly exceeded those of the random model (**** $p < 0.0001$ for all comparisons), demonstrating that the

classification was not driven by random associations. To further understand the key drivers of classification performance, we calculated the variable importance (VIMP) scores for all metabolites (Figure 4.1C). The most discriminatory variables included metabolites from both serum and urine, with urine glutamine, urine L-alanine, urine 4-deoxythreonic acid, serum proline, and serum cortisone being the most impactful. These findings underscore the potential of metabolomic profiling to reveal distinct biochemical alterations associated with Long COVID, offering valuable insights into the underlying pathophysiology.

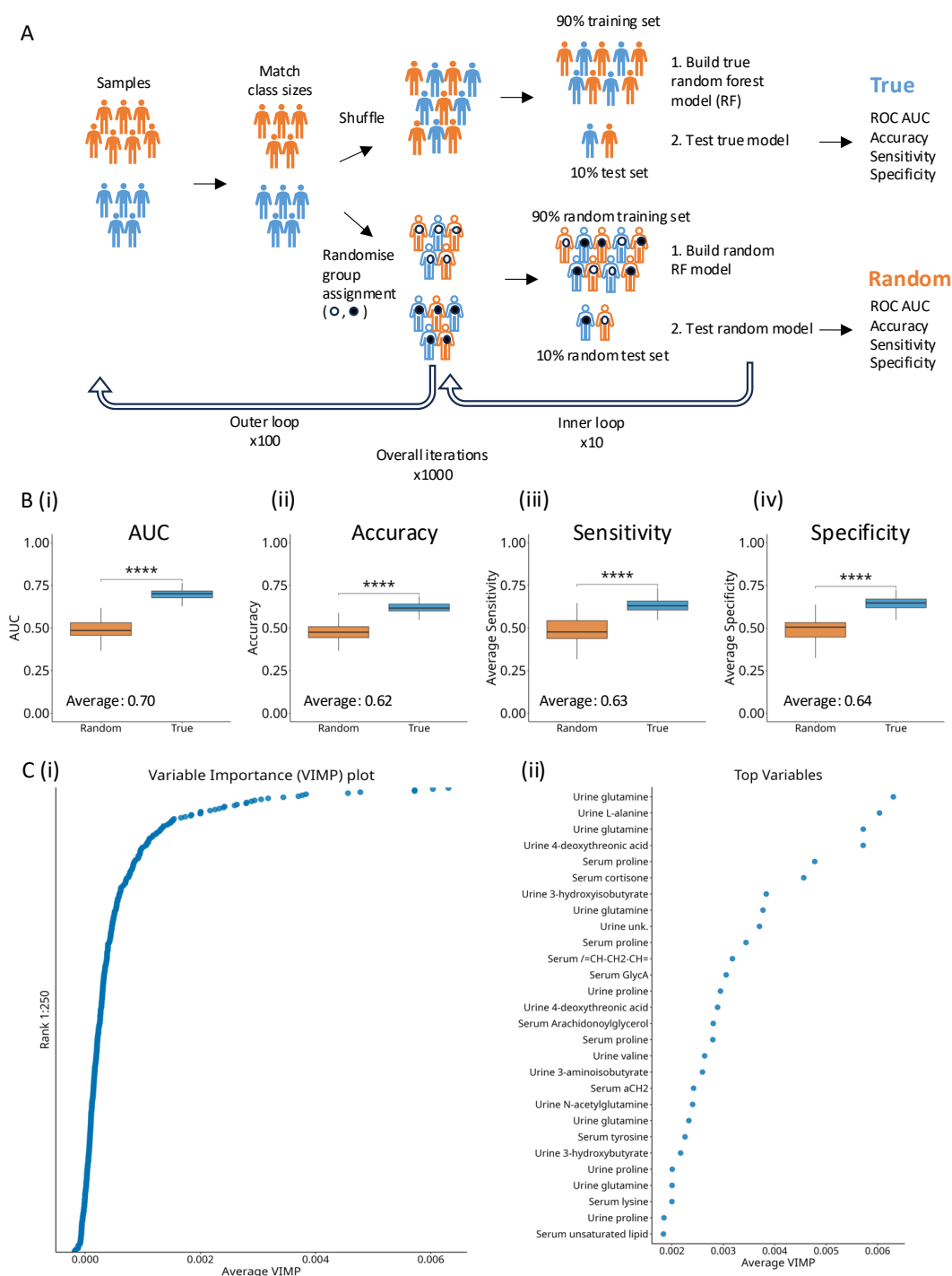


Figure 4.1: Metabolomic and lipidomic signatures distinguish Long-COVID from age-, sex-, and BMI-matched recovered COVID-19 controls. (A) Schematic of the nested cross-validation approach used to assess model robustness and classification performance. The dataset was divided into a training set (90%) and a test set (10%) over 100 iterations (outer loop), with 10-fold cross-validation within each iteration (inner loop), resulting in a total of 1,000 overall iterations. To evaluate the robustness of the model, the process was repeated with randomised class labels (random model), and performance metrics including ROC AUC,

accuracy, sensitivity, and specificity were compared between true and random models. (B) Performance metrics comparing true and random models: (i) ROC AUC, (ii) Accuracy, (iii) Sensitivity, (iv) Specificity. The true model (blue) significantly outperformed the random model (orange) across all metrics (**** $p < 0.0001$). (C) Variable importance analysis for distinguishing Long COVID from recovered controls: (i) Variable Importance (VIMP) plot showing ranked metabolites according to their contribution to model accuracy. (ii) Top variables contributing to classification performance, highlighting key metabolites from both serum and urine, including urine glutamine, urine L-alanine, urine 4-deoxythreonic acid, serum proline, and serum cortisone.

4.3.2. Serum Metabolic Alterations in Long COVID

To identify specific serum metabolites that distinguish Long COVID from recovered COVID-19 controls, we performed univariate analysis on the top variables identified by the random forest classification model (Figure 4.2). Several serum metabolites showed significant differences between the groups. Long COVID participants exhibited significantly lower levels of serum proline ($p < 0.001$) and serum cortisone ($p < 0.01$) compared to controls (Figure 4.2A, 2B). In contrast, GlycA levels, a marker of systemic inflammation, were significantly elevated in the Long COVID group ($p < 0.01$) (Figure 4.2D). Other notable differences included increased levels of serum 2-arachidonoylglycerol ($p < 0.001$, Figure 4.2E), lysine ($p < 0.05$), and unsaturated lipid content ($p < 0.05$) (Figure 4.2G-H), alongside reduced levels of serum tyrosine ($p < 0.01$, Figure 4.2F) in Long COVID. Additionally, serum cortisol ($p < 0.05$) and 11-deoxycortisol ($p < 0.05$) were also reduced, indicating potential dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 4.2I, 2J). In contrast, markers of neuronal injury and glial activation, including serum neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP), did not differ significantly between groups (NS), suggesting the absence of overt neurodegeneration or glial reactivity in Long COVID patients (Figure 4.2K, 2L). These findings indicate that the metabolic alterations in Long COVID primarily involve

systemic inflammation and dysregulation of amino acid metabolism, rather than ongoing neuroinflammation or neuronal injury. The reduction in cortisol and related metabolites further suggests impaired stress response regulation, which may contribute to the persistent symptoms observed in Long COVID.

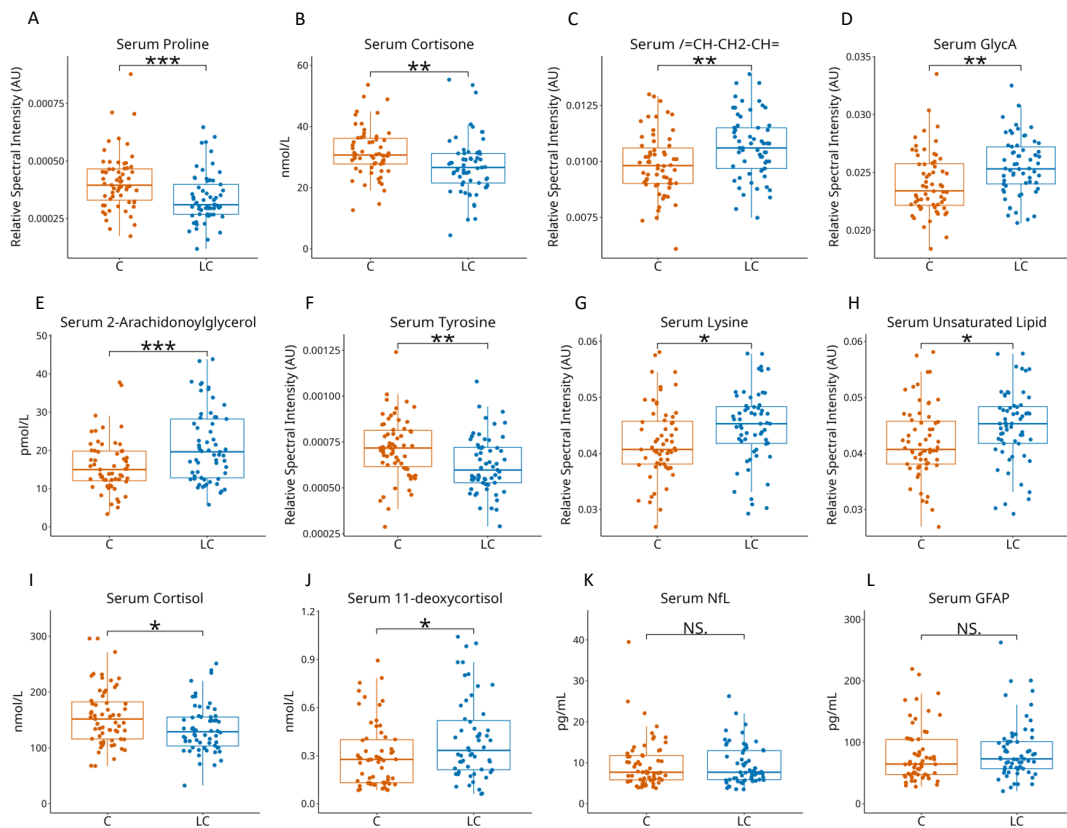


Figure 4.2: Univariate analysis of top serum metabolites distinguishing Long COVID from post-COVID age-, sex-, and BMI-matched controls. (A–H) Box plots representing the relative spectral intensity (AU), as determined by NMR, or concentration (nmol/L or pmol/L), as determined by mass spectrometry, of key serum metabolites identified as significant discriminators between Long COVID (LC) and post-COVID recovered controls (C). Metabolites shown include serum proline (A), serum cortisone (B), serum $\text{-CH-CH}_2\text{-CH=}$ (C), serum GlycA (D), serum 2-arachidonoylglycerol (E), serum tyrosine (F), serum lysine (G), and serum unsaturated lipid (H). (I–J) Serum cortisol (I) and serum 11-deoxycortisol (J), related to HPA axis dysregulation, are shown due to their relevance to metabolic pathways implicated by the variable importance plots (VIPs). (K–L) Serum neurofilament light chain (NfL) (K) and glial fibrillary acidic protein (GFAP) (L) levels, assessed to investigate potential ongoing neuroinflammation or neuronal injury, show no significant difference between Long COVID and recovered controls (NS = not significant). Each box plot displays the median, interquartile range, and standard deviation, highlighting the variation within and between groups.

*Statistically significant differences between groups are denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

4.3.3. Urine Metabolites Distinguish Long COVID from Recovered Controls

A parallel analysis of the urine metabolites that were most important for discriminating Long COVID from control samples in the random forest model revealed further metabolic differences between the groups (Figure 4.3A-H). Several key metabolites exhibited significant differences between the groups, primarily involving glutamine and valine metabolism. Urine glutamine levels were significantly reduced in Long COVID compared to controls ($p < 0.01$) (Figure 4.3A), as were levels of urine 4-deoxythreonic acid ($p < 0.01$) (Figure 4.3B), N-acetylglutamine ($p < 0.001$) (Figure 4.3G), and proline ($p < 0.05$) (Figure 4.3D). These changes indicate altered amino acid metabolism, potentially linked to increased protein turnover or metabolic stress. Additionally, metabolites related to valine catabolism were notably decreased in Long COVID urine samples. Urine valine levels were significantly lower ($p < 0.05$) (Figure 4.3E), as were urine 3-hydroxyisobutyrate ($p < 0.01$) (Figure 4.3C) and urine 3-aminoisobutyrate ($p < 0.05$) (Figure 4.3F). Urine 3-hydroxybutyrate levels were also significantly reduced ($p < 0.01$) (Figure 4.3H), suggesting alterations in branched-chain amino acid breakdown and energy metabolism. Given valine's role in muscle function and protein synthesis, its reduction in Long COVID may reflect muscle catabolism or impaired amino acid utilisation, consistent with reported symptoms of muscle fatigue and post-exertional malaise in Long COVID patients. These findings underscore the metabolic dysfunction characteristic of Long COVID and support the hypothesis that disrupted amino acid and energy metabolism contribute to symptom persistence.

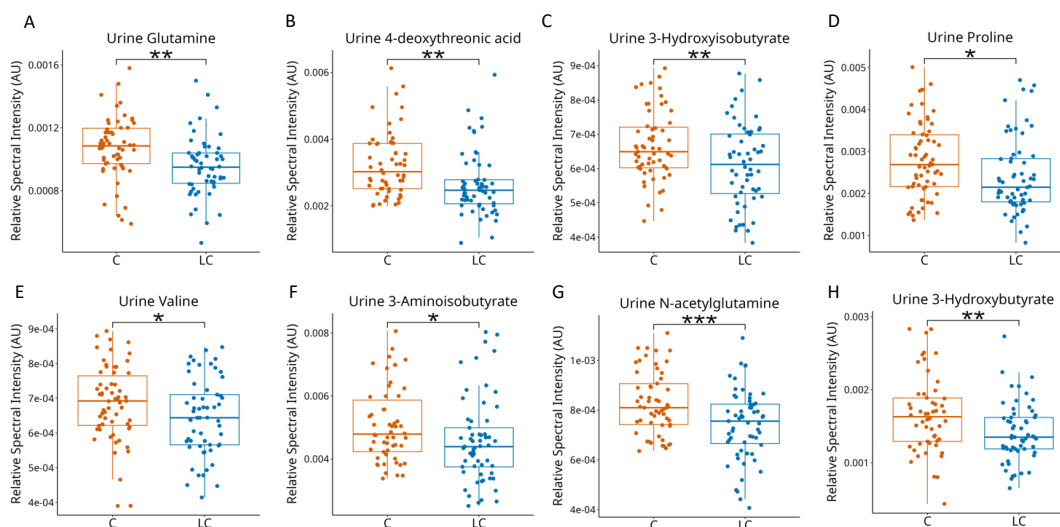


Figure 4.3: Univariate analysis of top urine metabolites distinguishing Long COVID from post-COVID matched controls. (A–H) Box plots showing the relative spectral intensity (AU) of key urine metabolites identified as significant discriminators between Long COVID (LC) and recovered COVID-19 controls (C). The metabolites shown include urine glutamine (A), urine 4-deoxythreonic acid (B), urine 3-hydroxyisobutyrate (C), urine proline (D), urine valine (E), urine 3-aminoisobutyrate (F), urine N-acetylglutamine (G), and urine 3-hydroxybutyrate (H). Each box plot displays the median, interquartile range, and standard deviation, highlighting intergroup variability. Statistically significant differences between groups are denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.3.4. Combining Serum and Urine Metabolites Enhances Classification Accuracy

Given that both serum and urine metabolites independently contributed to distinguishing Long COVID from recovered controls, we investigated whether integrating both datasets could improve classification performance. Random forest models built separately on serum and urine metabolites each demonstrated moderate discriminatory ability, with AUC values of 0.65 ± 0.16 and 0.67 ± 0.16 , respectively (Figure 4.4A-B). Both models significantly outperformed their respective randomly permuted null models (serum random AUC = 0.50 ± 0.17 , $p < 0.0001$; urine random AUC = 0.49 ± 0.18 , $p < 0.0001$, Figure 4.4A-B), indicating that the observed differences were not due to random chance. To enhance classification accuracy,

we combined the top variable importance metabolites (VIPs) from both serum and urine datasets. This integrated approach significantly improved model performance, achieving an AUC of 0.81 ± 0.13 (compared to the random model AUC of 0.50 ± 0.18), with a sensitivity of 0.70 ± 0.20 and specificity of 0.71 ± 0.22 (both significantly better than random models, $p < 0.0001$, Figure 4.4C). This finding highlights the complementary nature of serum and urine metabolomic profiles and underscores the advantage of multi-biofluid analyses in identifying robust metabolic signatures of Long COVID.

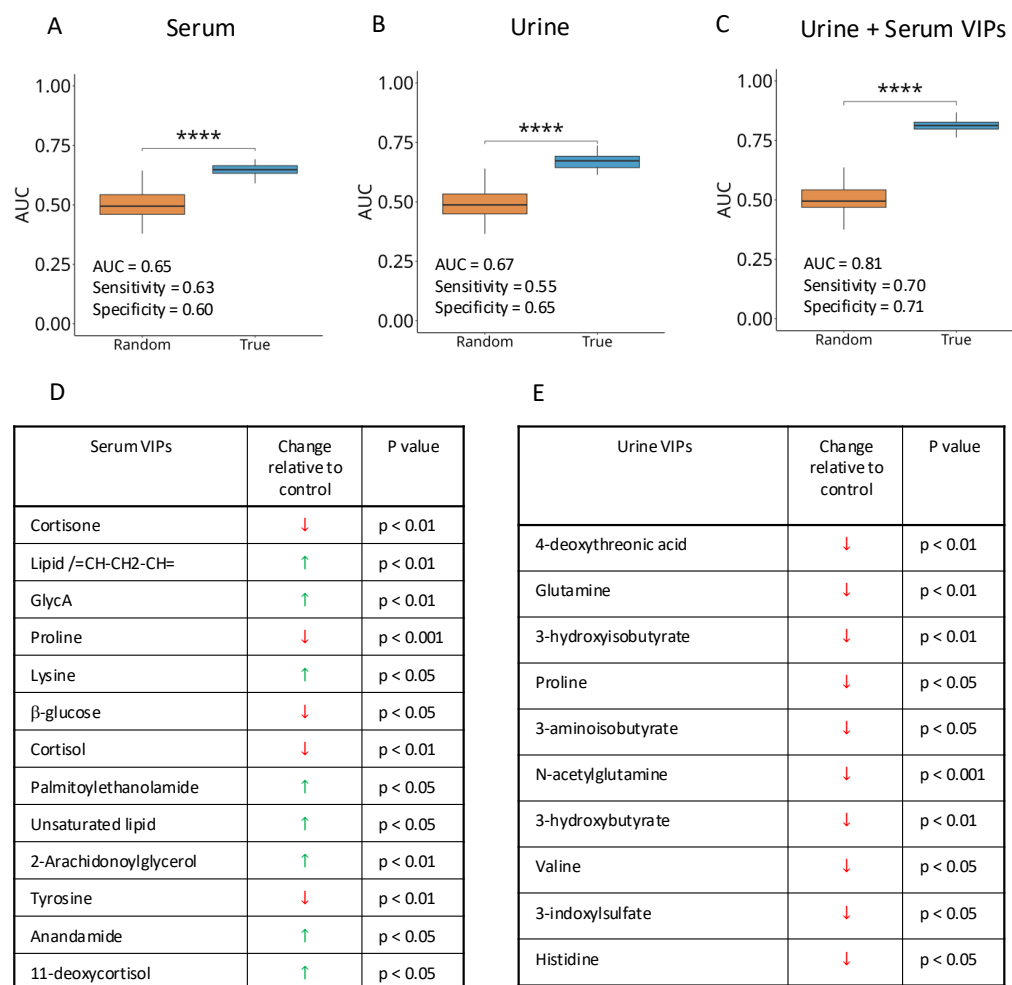


Figure 4.4: Combining serum and urine metabolites improves classification accuracy for Long-COVID. (A–C) Random forest models built separately using serum metabolites (A, AUC = 0.65) and urine metabolites (B, AUC = 0.67) show moderate discriminatory power. Combining the top variables of importance (VIPs) from both models (C: Urine + Serum VIPs) significantly improves classification performance, achieving an AUC of 0.81 with a sensitivity of 0.70 and specificity of 0.71. The true model (blue) significantly outperforms the random model (orange) across all comparisons (****p < 0.0001). (D–E) Tables summarizing the top variable importance (VIP) metabolites identified from serum (D) and urine (E) datasets. Arrows indicate the direction of change in Long COVID compared to controls (↑ = increased, ↓ = decreased). P values indicate statistical significance of differences between groups. Notably, serum metabolites such as cortisone, GlycA, proline, and cortisol, and urine metabolites such as 4-deoxythreonic acid, glutamine, and 3-hydroxyisobutyrate, are among the most discriminative.

4.3.5. Metabolomic Profiles Correlate with Symptom Severity and Functional Impairment

To investigate the relationship between metabolic alterations and symptom severity in Long COVID, we conducted a correlation analysis between the top serum VIP metabolites identified by the serum random forest model and questionnaire data assessing symptom burden and functional impairment including the Patient Health Questionnaire (PHQ-9), Fatigue Severity Scale (FSS), and the 12-Item Short Form Health Survey (SF-12 PCS/MCS scores) (Figure 4.5). The correlation plot demonstrates that the number of symptoms reported during the first 6 weeks and after 6 weeks, as well as scores from the PHQ-9 (depression), FSS (fatigue), PCS-12 (physical function), and MCS-12 (mental function), are closely interrelated. However, the PCS-12 scores, reflecting physical function impairment, showed the most consistent correlations with key metabolic markers. Among the metabolites, increased GlycA levels exhibited a strong positive correlation with symptom severity, particularly with the number of symptoms experienced during both the acute and chronic phase of COVID-19, indicating that heightened systemic inflammation is associated with greater symptom burden. Reduced levels of cortisone and cortisol correlated positively with PCS-12 scores, suggesting that impaired HPA axis function is linked to poorer physical function. Other significant correlations included decreased serum proline and increased unsaturated lipids, both of which were associated with greater physical impairment. Interestingly, while several symptom scores were highly correlated with each other, the metabolic markers displayed more specific associations, particularly with physical function as measured by PCS-12. This indicates that distinct metabolic disruptions may underlie the physical limitations observed in Long COVID, rather than merely reflecting a generalised symptom burden. These findings highlight the complex interplay between persistent metabolic disturbances and functional impairment in

Long COVID, with specific biomarkers potentially serving as indicators of physical recovery. Further studies integrating multi-omics data with longitudinal symptom tracking may provide deeper insights into the metabolic basis of symptom persistence.

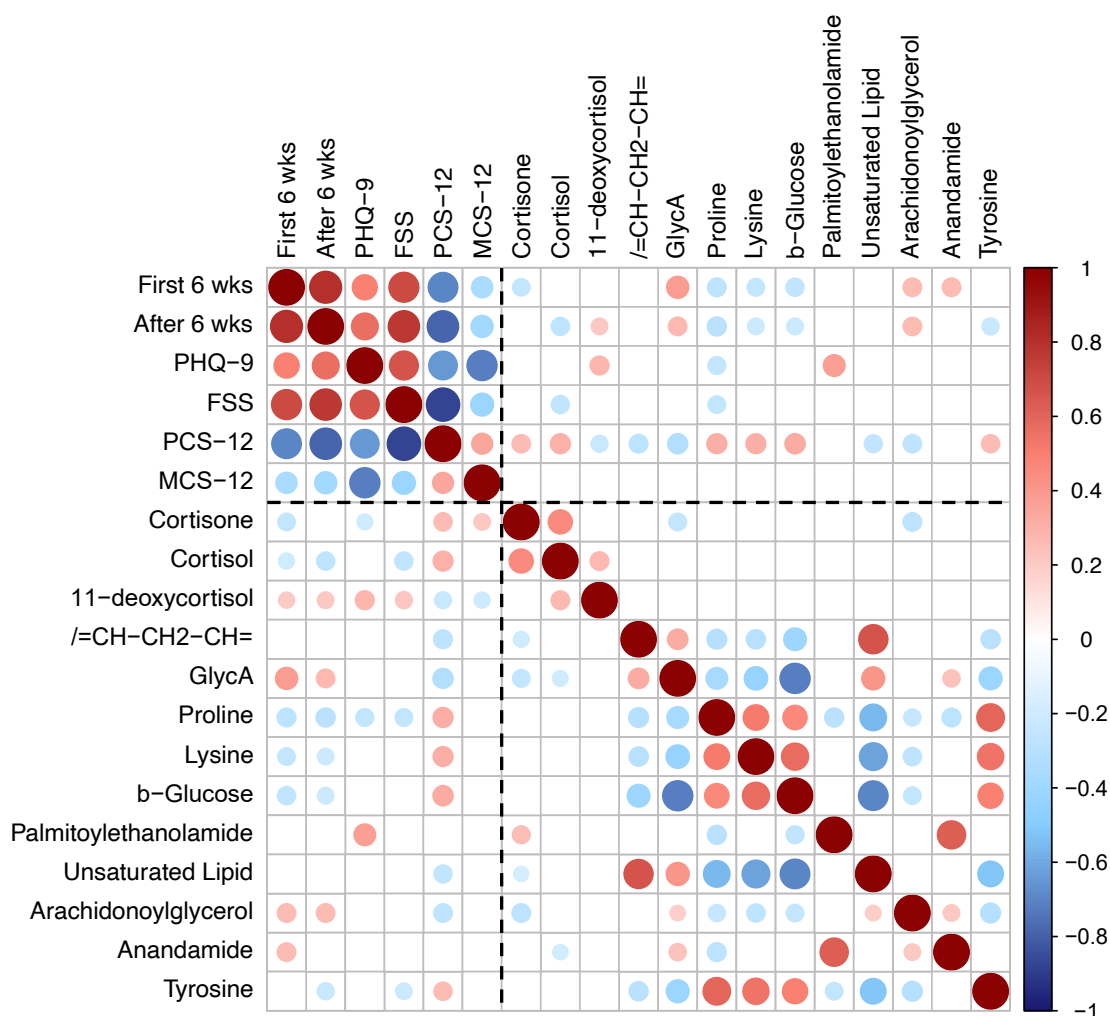


Figure 4.5: Correlation plot of the serum metabolites and questionnaire data with the top VIP scores (most responsible for the separation between Long COVID and control in the model). The colour intensity of the circles reflects the strength of the Pearson correlation coefficient (R value), as indicated by the colour scale on the right (red = positive correlation, blue = negative correlation). The size of the circles corresponds to the magnitude of the correlation coefficient ($|R|$), with larger circles indicating stronger correlations (closer to -1 or 1). Dotted lines separate VIP metabolites from questionnaire scores. Symptom variables include the number of symptoms reported during the first 6 weeks (First 6 wks) and after 6 weeks (After 6 wks), as well as scores from the Patient Health Questionnaire (PHQ-9), Fatigue Severity Scale (FSS), Physical Component Summary (PCS-12), and Mental Component

Summary (MCS-12) from the 12-Item Short Form Health Survey (SF-12). Notably, the PCS-12 scores showed the strongest correlations with the VIP metabolites, while other symptom scores were more interrelated with each other. This pattern highlights the relationship between altered metabolic profiles and physical function impairment in Long COVID.

4.3.6. Long COVID Subtypes Revealed by Cluster Analysis

Given the heterogeneity of Long COVID symptoms, we conducted unsupervised cluster analysis to identify potential metabolic subtypes. Two distinct clusters emerged, corresponding to different symptom profiles, as presented in Chapter 3 (Figure 4.6A-B shows a repeat of the cluster characteristics in the same figure for easy comparison). Most participants fell into cluster 1, which was characterised predominantly by neurological symptoms such as memory problems, severe fatigue and difficulty with attention. On the other hand, these participants had far fewer systemic symptoms. A smaller subset of Long COVID participants fell into cluster 2, with proportionally more multi-system symptoms, including chest pain, dizziness, and GI symptoms that were absent in cluster 1. A targeted analysis of the top discriminatory serum metabolites similarly revealed distinct metabolic profiles associated with each cluster. One way ANOVA revealed a significant effect of group on serum cortisone ($p < 0.01$), 11-deoxycortisol ($p < 0.01$), and cortisol ($p < 0.05$) as well as GlycA ($p < 0.01$), a lipid resonance (2.8 ppm broad signal arising from $=CH-CH_2-CH=$ in macromolecules, $p < 0.05$) and serum proline ($p < 0.01$). Post-hoc Tukey tests revealed cluster 1, associated with greater neurological symptoms, was characterised by lower cortisone ($p < 0.01$) (Figure 4.6C-i), and proline levels ($p < 0.001$) (Figure 4.6C-vi), alongside increased 11-deoxycortisol ($p < 0.05$) (Figure 4.6C-iii) and specific lipid species ($p < 0.001$) (Figure 4.6C-vi) indicative of altered steroid metabolism.

In contrast, Cluster 2, which was marked by systemic inflammatory symptoms, showed significantly elevated GlycA levels, consistent with persistent immune activation (Figure 4.6C-iv). Interestingly, cortisol levels were significantly lower in Cluster 2 compared to controls (Figure 4.6C-ii), suggesting a possible impairment in stress response regulation. These findings reinforce the concept that Long COVID is not a single homogeneous condition but instead consists of distinct subtypes with specific metabolic and symptomatic profiles. Identifying these clusters could have implications for developing targeted therapeutic strategies and personalised interventions, addressing the underlying pathophysiological mechanisms in each subgroup.

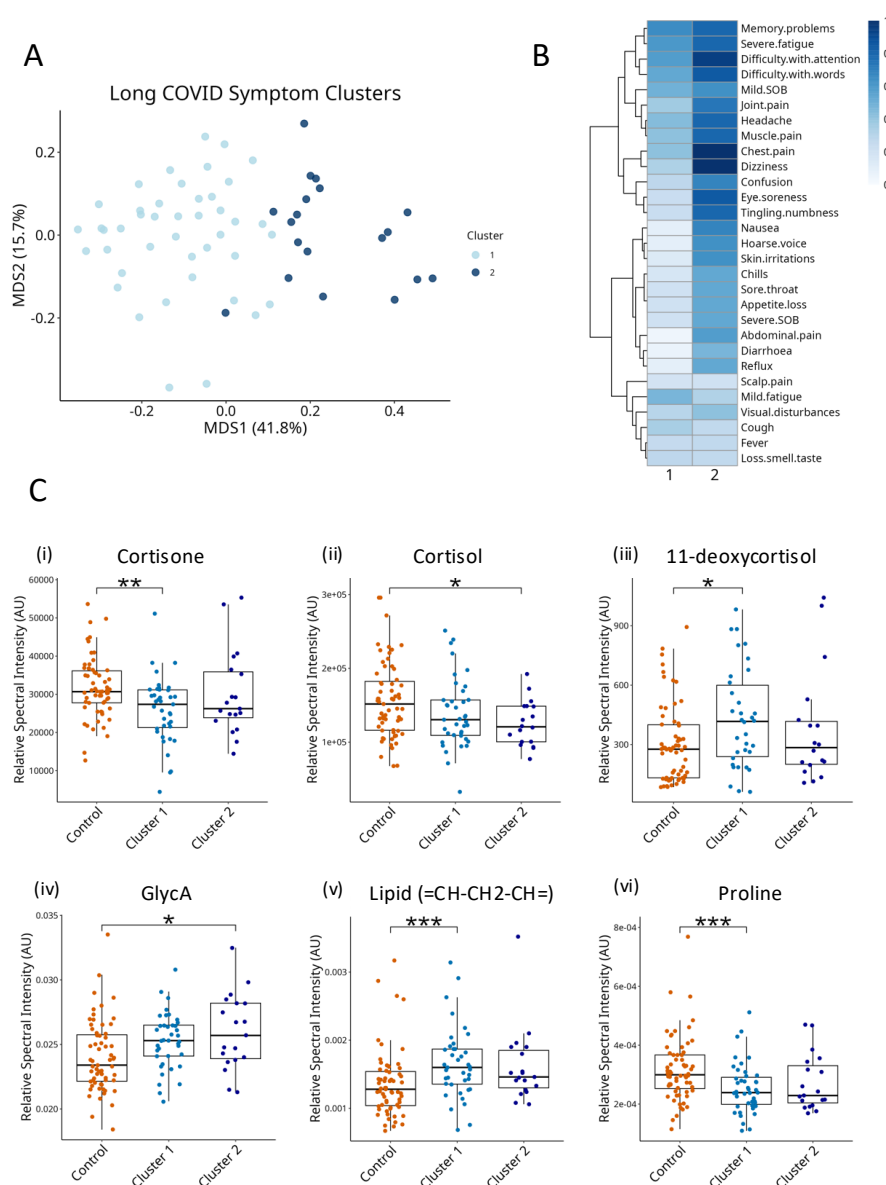


Figure 4.6: Cluster analysis at baseline reveals two prominent clusters with distinct metabolite profiles. (A) Multidimensional scaling (MDS) plot displaying two distinct symptom clusters identified using Gower distance and Partitioning Around Medoids (PAM) clustering. Cluster 1 (light blue) predominantly consists of individuals with neurocognitive and fatigue-related symptoms, while Cluster 2 (dark blue) includes individuals with a more multi-system symptom profile. (B) Heatmap showing the prevalence of specific symptoms within each cluster, scaled by row and expressed as the proportion of individuals in each group. Cluster 1 is enriched for neurological symptoms such as memory problems, severe fatigue, and difficulty with attention, while Cluster 2 shows a higher prevalence of multi-system symptoms, including chest pain, dizziness, and gastrointestinal issues. (C) Box plots illustrating differences in key serum metabolites between the two clusters and the control group. (i) Cortisone levels were significantly lower in Cluster 1 compared to controls ($p < 0.01$). (ii) Cortisol levels were significantly reduced in Cluster 2 compared to controls ($p < 0.05$). (iii) 11-

deoxycortisol levels were elevated in Cluster 1 compared to controls ($p < 0.05$). (iv) GlycA levels were significantly elevated in Cluster 2 compared to controls ($p < 0.05$). (v) Lipid resonance ($=CH-CH_2-CH=$ in macromolecule) was significantly higher in Cluster 1 compared to controls ($p < 0.001$). (vi) Proline levels were significantly lower in Cluster 1 compared to controls ($p < 0.001$). Each plot shows the median, interquartile range, and standard deviation. One-way ANOVA followed by Tukey post-hoc tests were used to determine statistical significance.

4.3.7. Follow-Up metabolome identifies recovery from long COVID

To explore the evolving metabolic landscape of Long COVID, we conducted a follow-up analysis comparing individuals who reported improvement with those who did not, as well as healthy controls (Figure 4.7A). Demographic variables, including age (Figure 4.7A-i) and BMI (Figure 4.7A-ii), showed no significant differences between the groups, ensuring comparability. All Long COVID participants were significantly more fatigued, as measured by the fatigue severity scale (FSS, Figure 4.7Aiii) and had lower PCS-12 scores (Figure 4.7Avi); however, those who reported either no symptom improvement or a worsening of symptoms had significantly elevated PHQ-9 scores (Figure 4.7iv) and lower MCS-12 scores (Figure 4.7v), indicating a greater impact of symptoms on mental health. The total number of ongoing symptoms was also significantly higher in those reporting no symptom improvement, indicating a severer burden of persistent disease (Figure 4.7vii).

Using a random forest classification model, it was possible to effectively distinguish non-recovered Long COVID patients from controls, achieving an average AUC of 0.71 and an accuracy of 0.65 (**** $p < 0.0001$) (Figure 4.7B). This finding suggests that metabolic disturbances persist in non-recovered individuals, allowing for robust discrimination from healthy controls. In contrast, the model failed to distinguish improved Long COVID patients

from controls (NS) (Figure 4.7C), indicating that individuals who report symptom improvement tend to have metabolic profiles closer to those of the control group. The generation of a Variable Importance Plot (VIMP) (Figure 4.7D) highlights the top metabolites associated with non-recovery status. Serum acetate, glutamine, leucine, and other essential amino acids were among the most discriminatory variables. These findings indicate that persistent disruptions in amino acid and energy metabolism may underlie ongoing symptoms in non-recovered individuals, reflecting metabolic stress and impaired protein turnover. By contrast, the normalisation of metabolic profiles in those who report improvement highlights the potential reversibility of these disturbances, emphasising the importance of metabolic monitoring in Long COVID management.

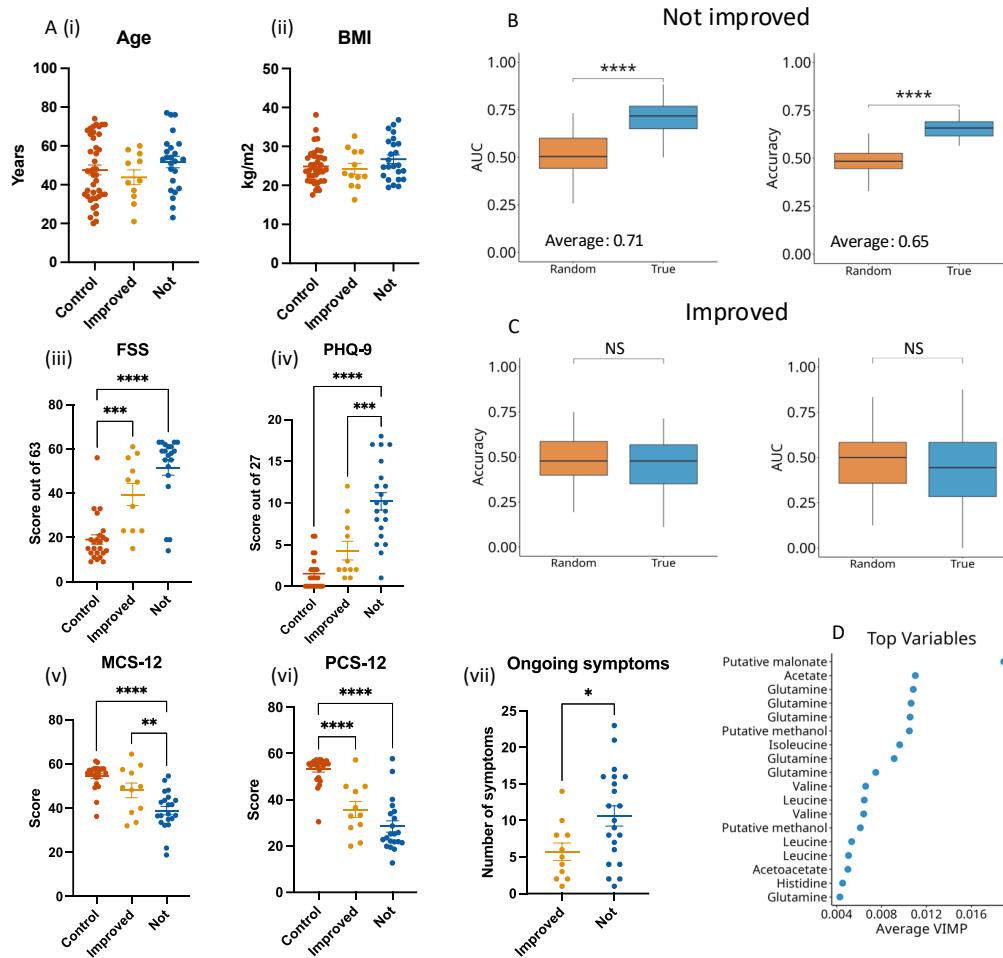


Figure 4.7: Characteristics of long COVID patients and controls at follow-up and serum random forest classification results. (Ai) Age and (Aii) BMI showing no significant differences between groups. (Aiii) Fatigue Severity Scale (FSS) scores, (Aiv) Patient Health Questionnaire-9 (PHQ-9) scores, (Av) Mental Component Score (MCS-12) and (Avi) Physical Component Score (PCS-12) from the SF-12 survey, (Avii) Ongoing symptoms experienced in the long COVID cohorts. (B) Random forest classification results for non-recovered vs. control groups. The model shows significant discrimination with an average AUC of 0.71 and accuracy of 0.65 (**** $p < 0.0001$). (C) Random forest classification results for improved vs. control groups showing no significant discrimination (NS). (D) Variable Importance Plot (VIMP) from the significant random forest model, highlighting key serum metabolic features associated with non-recovered status, including acetate, glutamine, and various essential amino acids. Error bars indicate mean $\pm$ SEM. Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, and NS (not significant).

4.3.8. Low amino acid availability defines non-recovered Long COVID at follow up

To further characterise the metabolic differences at follow up, the metabolites with highest VIMP scores (Figure 4.7D) were compared across all three groups, showing their levels relative to both controls and the improved Long COVID group (Figure 4.8A-F). One-way ANOVAs revealed a significant main effect of group on serum acetate ($F_{(2, 77)} = 7.667$, $p = 0.0009$) and glutamine ($F_{(2, 77)} = 5.349$, $p=0.0067$), as well as on several essential amino acids (isoleucine ($F_{(2, 77)} = 4.833$, $p=0.0105$), valine ($F_{(2, 77)} = 7.296$, $p=0.0013$), leucine ($F_{(2, 77)} = 7.647$, $p=0.0009$), and histidine ($F_{(2, 77)} = 5.323$, $p=0.0068$)). In all cases, post-hoc tukey tests revealed these were only significantly altered between controls and those who did not report symptom improvement. The depletion of these metabolites in the non-recovered group also correlated with measures of symptom severity, such as fatigue, depression, and the PCS-12 measure (Figure 4.8G). These results suggest a link between altered serum metabolite levels and the persistence of symptoms in Long COVID patients, particularly highlighting the role of acetate and branched chain amino acids (BCAAs) in symptomatology.

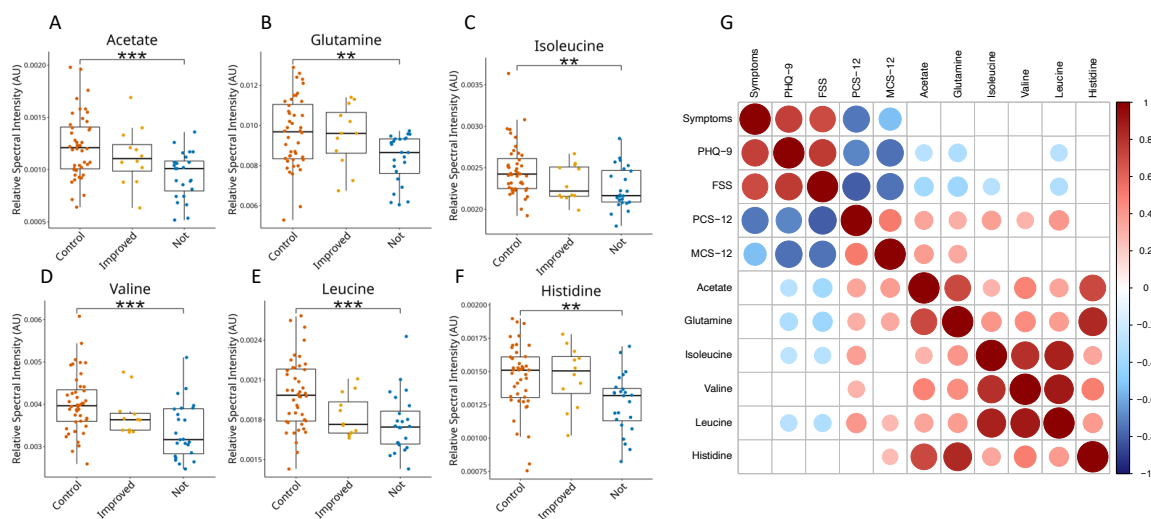


Figure 4.8: Altered metabolite levels in serum from long COVID patients stratified by symptom improvement. (A–F) Box plots displaying relative spectral intensity (AU) of key serum metabolites that discriminate between control individuals, Long COVID patients with symptom improvement at follow-up (Improved), and Long COVID patients with no improvement or worsening (Not). Metabolites shown include acetate (A), glutamine (B), isoleucine (C), valine (D), leucine (E), and histidine (F). One-way ANOVA was used to assess differences across the three groups. Controls are shown in orange, improved Long COVID patients in yellow, and non-improved Long COVID patients in blue. Statistical significance between groups is denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars represent the median, interquartile range, and standard deviation. (G) Correlation matrix depicting relationships between clinical parameters (number of ongoing symptoms, PHQ-9, FSS, PCS-12, MCS-12) and metabolite levels. Correlations are visualized as coloured circles, with the size of the circle indicating the strength of the correlation and the colour representing the direction (red: positive, blue: negative). Strong positive correlations are observed between PCS-12 and MCS-12 scores with metabolites such as acetate and glutamine, while PHQ-9 and FSS scores, while show inverse correlations with the latter metabolites, as well as leucine and isoleucine.

4.4. Discussion

This study reveals that metabolomic characterisation of Long COVID can identify distinct metabolic alterations that differentiate affected individuals from recovered COVID-19 controls. Few studies to date have successfully developed a model capable of distinguishing Long COVID from fully recovered individuals with such a high level of discriminatory accuracy

(AUC = 0.81), underscoring the robustness of a multi-biofluid approach, which we have employed successfully elsewhere (Probert et al. 2021). Moreover, our findings demonstrate that the metabolic profile of individuals with Long COVID evolves over time. In some cases, as symptoms resolve, the metabolome increasingly resembles that of the control group. However, those who do not report recovery exhibit a distinct and persistent biomarker profile, suggesting ongoing metabolic disruption.

Our baseline metabolomic analysis revealed distinct alterations in both serum and urine metabolites that differentiate Long COVID from recovered controls. Notably, urine metabolites alone provided a reasonable discriminatory ability (AUC = 0.67), indicating that non-invasive urine profiling can serve as a practical tool for initial screening, despite not being as discriminative as a multi-fluid approach. The reduction of urinary glutamine, valine, and related metabolites in Long COVID is consistent with reports of altered energy metabolism and metabolic stress in chronic fatigue conditions, where impaired amino acid availability has been associated with mitochondrial dysfunction (Armstrong et al. 2012). Although urine metabolite profiles in ME/CFS vary across studies, a notable consistency is the generalised reduction of both urinary and circulating amino acids, which may reflect pathological alterations in nitrogen and energy metabolism (Armstrong et al. 2012; Niblett et al. 2007; Jones et al. 2005). Serum metabolites in this study also exhibited significant differences between Long COVID patients and recovered controls. A key strength of our study is the integration of NMR-based serum metabolomics with mass spectrometry analysis of steroids and endocannabinoids, providing a comprehensive and multidimensional characterisation of metabolic alterations. Notably, elevated levels of endocannabinoids, including 2-arachidonoylglycerol and anandamide, were observed in the Long COVID group. These

changes may represent an adaptive response aimed at counteracting systemic inflammation or maintaining metabolic homeostasis (Turcotte et al. 2015). Endocannabinoids are known to play a crucial role in modulating stress responses and immune function, and dysregulation within this pathway has been implicated in chronic inflammatory conditions (Turcotte et al. 2015). However, their specific role in the context of Long COVID remains insufficiently explored and warrants further investigation.

Our unsupervised clustering analysis revealed two distinct subtypes of Long COVID: one characterised predominantly by neurological dysfunction and reduced steroid metabolism, and another marked by systemic inflammation with elevated GlycA. The neurological cluster, associated with symptoms such as cognitive impairment and fatigue, demonstrated lower cortisone and proline levels and higher 11-deoxycortisol and specific lipid resonances, suggesting a disruption in the hypothalamic-pituitary-adrenal (HPA) axis. Several studies have identified clusters based on symptom patterns, severity, and affected organ systems (Mfouth Kemajou et al. 2024; Blankestijn et al. 2024; Basharat, Chao, and McGill 2022; Yong and Liu 2021; Mahase 2020). Other proposed subtypes have included post-intensive care syndrome, post-viral fatigue syndrome, and syndromes affecting specific organ systems (Yong and Liu 2021; Mahase 2020). More closely related to our findings, other cluster analyses studies have revealed groups characterised by myalgia-like pain, neurocognitive disorders, and multisystem involvement (Blankestijn et al. 2024; Kemajou et al. 2023), and pathophysiological mechanisms underlying these subtypes have been argued to include immune dysregulation, cerebral hypoperfusion, impaired gas transfer, endothelial damage, and autoimmunity (Diar Bakerly et al. 2024; Altmann et al. 2023). The utility of identifying distinct subtypes, beyond understanding the pathogenesis of this condition, would also be to

be able to direct the individual to the most appropriate specialist and treatment approaches (Diar Bakerly et al. 2024; Altmann et al. 2023).

Disruptions in cortisol, cortisone, and 11-deoxycortisol levels observed in the neurological cluster suggest a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, which has been a long-standing area of interest in post-viral syndromes (Yavropoulou, Tsokos, et al. 2022; Yavropoulou, Filippa, et al. 2022). Cortisol, a glucocorticoid produced by the adrenal cortex, plays a pivotal role in regulating the stress response. Reduced cortisol levels may result in an inadequate anti-inflammatory response, contributing to symptoms such as fatigue, mood disturbances, and cognitive impairment – features commonly reported in chronic fatigue syndrome and other post-infectious states (Triffaux et al. 1994). Some studies have revealed disruptions in the hypothalamic-pituitary-adrenal (HPA) axis, including pituitary hormone dysregulation and central adrenal insufficiency is present in a subset of long COVID patients (Sims et al. 2024; Sahoo et al. 2024). The observed reductions in both cortisol and cortisone may reflect impaired HPA axis function, possibly linked to hypothalamic dysfunction or adrenal insufficiency following viral infection. It has been reported that dysregulation of cortisol secretion, particularly the shift towards increased evening and nocturnal levels, suggests a potential disruption of the HPA axis in long COVID-19 (Dalhuisen et al. 2024). IL-6 also appears to play a role in this altered regulation, highlighting the inflammatory modulation of adrenal function, which might persist and contribute to ongoing symptoms in Long COVID (Dalhuisen et al. 2024).

GlycA, which was prominent in the multi-system cluster, is a composite marker associated with acute-phase proteins such as α 1-acid glycoprotein and reflects ongoing systemic

immune activation (Otvos et al. 2015). This aligns with existing evidence indicating that prolonged immune dysregulation, including elevated cytokines and altered immune cell profiles, may underpin the pathophysiology of Long COVID (Yin et al. 2024; Low, Low, and Akrami 2023). Importantly, our findings provide a metabolic perspective on chronic inflammation, highlighting GlycA as a potential biomarker for clinical assessment and monitoring. GlycA has been associated with cardiovascular disease risk, subclinical atherosclerosis, and all-cause mortality (Ritchie et al. 2015; Ballout and Remaley 2020). It demonstrates low intra-individual variability and correlates with other inflammatory markers including C-reactive protein and interleukin-6 (Otvos et al. 2015). GlycA levels are elevated in chronic inflammatory conditions such as rheumatoid arthritis, psoriasis, and lupus, and decrease with anti-inflammatory treatments (Mehta et al. 2020). It has shown predictive value for future severe infections, cardiovascular events, and cancer (Duprez et al. 2016).

A key aspect of our study is the inclusion of longitudinal follow-up data, which revealed that while metabolic distinctions between Long COVID and recovered controls are evident early on, these differences become less pronounced over time. After 9-12 months, the metabolomic profiles of Long COVID individuals who reported symptom improvement were indistinguishable from those of fully recovered controls. This suggests that metabolic recovery is achievable in some individuals, highlighting the potential for spontaneous resolution of metabolic dysregulation in Long COVID. However, a subset of participants with persistent symptoms exhibited sustained metabolic alterations. Non-recovered patients continued to exhibit metabolic abnormalities, particularly involving amino acid metabolism and energy-related pathways, such as persistent disruptions in branched-chain amino acids (BCAAs) like valine, histidine, leucine, and isoleucine. These findings align with prior reports

that have identified ongoing disturbances in amino acid metabolism in Long COVID (Martínez et al. 2024; López-Hernández et al. 2023). The observed depletion of BCAAs, which are critical for muscle protein synthesis and energy indicate ongoing muscle catabolism or altered energy demands in non-recovered patients (Calder 2006). This pattern supports previous observations that persistent immune activation in Long COVID could increase amino acid requirements to support protein synthesis and lymphocyte proliferation (Kelly and Pearce 2020). Additionally, the correlation between disrupted amino acid levels and symptom severity, including fatigue and reduced physical health scores, is consistent with the idea that metabolic disturbances underlie the prolonged symptom burden observed in some patients. These findings add nuance to the evolving understanding of Long COVID by suggesting that recovery may involve a re-establishment of metabolic homeostasis, while persistent symptoms could reflect unresolved metabolic strain. Therefore, targeting metabolic pathways related to amino acid metabolism might represent a therapeutic opportunity for patients with prolonged Long COVID symptoms.

While our study provides valuable insights into the metabolic alterations associated with Long COVID and identifies potential diagnostic biomarkers, it has several limitations that should be considered. The primary limitation is the absence of a fully independent test set from a separate cohort or trial, which could validate our findings in a different population. Although we employed rigorous cross-validation methods within our study cohort to assess model robustness, the lack of external validation may limit the generalisability of our results. Furthermore, while our multi-omics approach enhances the comprehensiveness of metabolic profiling, the heterogeneity inherent to Long COVID presents challenges in identifying universally applicable biomarkers. Future studies that include larger, more diverse

populations and independent replication cohorts will be essential to corroborate our findings and strengthen the utility of these biomarkers in clinical practice. Despite these limitations, our study offers a robust foundation for further investigation into the metabolic basis of Long COVID and the development of diagnostic tools.

In conclusion, this study significantly advances our understanding of the metabolic underpinnings of Long COVID. By identifying key biomarkers and metabolic subtypes, we have laid the groundwork for future research aimed at elucidating the mechanistic drivers of symptom persistence. Integrating metabolomic data with proteomics, transcriptomics, and immune profiling in longitudinal studies will be essential to refine multi-omic signatures and develop targeted therapeutic strategies.

Chapter 5: The EXPLAIN trial for long COVID

Chapter summary

Following the identification of distinct metabolic phenotypes in long COVID using community-based samples (Chapter 4), this chapter presents a hospital-based sub-study conducted as part of the EXPLAIN trial. The primary aim of this investigation was to explore whether metabolic and coagulation profiling could complement advanced imaging data obtained via hyperpolarised xenon MRI (XeMRI) in individuals experiencing persistent breathlessness despite normal lung scans. While XeMRI results did not reveal significant functional impairments in the studied cohort, this chapter shifts focus to the secondary aim: identifying biochemical and metabolic subgroups within long COVID using blood- and urine-based analyses. My role in this study involved processing all biological samples on clinic days and performing ^1H NMR metabolomics, forming the basis of the biochemical analyses presented here.

Despite limited overall discrimination between long COVID participants and controls in random forest models, unsupervised clustering of coagulation data revealed two distinct subgroups within the long COVID cohort. One subgroup (Cluster 1) displayed a hypercoagulable and inflammatory phenotype, characterised by elevated levels of CRP, D-dimer, serum amyloid A, and prolonged clot lysis time. In contrast, Cluster 2 exhibited profiles indistinguishable from controls. Integration of metabolomics with coagulation data further supported this distinction, with Cluster 1 showing unique metabolic alterations – including reductions in serum glucose, histidine, and its derivative – associated with greater fatigue and

poorer quality of life. These findings suggest that metabolic dysfunction in this subgroup may be linked to underlying immune and platelet activation processes.

Incorporating functional outcomes and symptom burden, this chapter proposes that at least one biologically distinct long COVID phenotype may be driven by thromboinflammatory mechanisms. The observed depletion of histidine in this subgroup raises the possibility of altered histamine synthesis contributing to platelet activation, although whether this is causal or consequential remains to be established. The absence of significant findings in the broader cohort underscores the heterogeneity of long COVID and the limitations of applying group-level analyses to such a diverse condition, particularly with low sample sizes. Moreover, the differing context of this study – conducted in a hospital setting under time pressure – may have introduced stress-related confounds not present in earlier chapters.

Nonetheless, this work highlights the importance of resolving patient heterogeneity through integrative biochemical and clinical analyses. The identification of a metabolically distinct, hypercoagulable subgroup offers a potential avenue for therapeutic stratification and reinforces the value of clustering approaches in the study of long COVID. These findings support the development of further validation studies and suggest that targeted intervention strategies may be needed for individuals with specific thromboinflammatory signatures.

5.1. Introduction

The EXPLAIN trial aimed to investigate the underlying causes of persistent lung dysfunction in long COVID patients, focusing on individuals who experience breathlessness despite normal or near-normal conventional imaging, such as CT scans. Preliminary findings from hyperpolarised xenon 129 MRI (XeMRI) demonstrated impaired alveolar gas transfer in some

patients, indicating potential alveolar-capillary diffusion limitation even in the absence of structural lung abnormalities (Grist et al. 2021). However, the limitations of XeMRI, such as cost and limited availability, make its use as a routine diagnostic tool difficult. To complement these imaging findings, we undertook a blood sub-study to investigate whether metabolic profiling could detect similar functional impairments and identify distinct biological subgroups within long COVID. This blood-based approach aimed to map metabolic signatures to lung function metrics obtained from XeMRI and explore whether specific metabolic patterns could predict disease severity. In parallel, a colleague measured clotting parameters to investigate whether hypercoagulability might be associated with persistent symptoms. Unlike the previous long COVID study (Chapter 3-4), I was not responsible for participant recruitment, consent, or collection of questionnaire data, though presented in this chapter. My role on the trial days involved processing all blood and urine samples, as well as downstream NMR experiments and analysis. We collected 106 blood and urine samples from long COVID patients as well as a small subset of healthy controls (n=27). Unlike the pilot study, the XeMRI findings from the EXPLAIN trial did not demonstrate any impairment of lung function or structure and therefore, this chapter will focus on the second aim: exploring metabolic and biochemical heterogeneity within the long COVID cohort.

5.2. Methods

5.2.1. Study Design

This prospective observational cohort study aimed to assess lung function and potential pathophysiological mechanisms underlying breathlessness in patients with Long COVID using hyperpolarised xenon magnetic resonance imaging (HPX-MRI). The study included three

cohorts: post-hospitalised Long COVID patients (Cohort A), non-hospitalised Long-COVID patients (Cohort B), and COVID-19 positive controls without ongoing symptoms (Cohort C). A blood and urine sub-study (presented here) aimed to assess clotting abnormalities, markers of inflammation, and metabolic differences across groups.

5.2.2. Participants

Participants in Cohorts A and B were recruited from Long COVID clinics at participating NHS sites. Cohort C participants were recruited through public advertising throughout the hospital sites. Cohort A included post-hospitalised Long COVID patients with previous COVID-19 pneumonia. Cohort B consisted of non-hospitalised Long COVID patients, with subgroups based on the presence or absence of breathlessness on exertion. Cohort C included individuals with previous confirmed COVID-19 infection but without ongoing symptoms. All participants underwent demographic and medical history collection, lung function tests, exercise capacity tests, low-dose CT, and HPX-MRI scans. Blood and urine samples were collected for coagulation and metabolomics analysis before scans but after lung function and exercise tests. Structured Light Plethysmography (SLP) was performed optionally at some sites. The following lists describe participant inclusion/exclusion criteria; however, Table 5.1 details the final number of participants recruited in each group. Notably, only five participants were hospitalised in the final cohorts.

Common Inclusion Criteria (for all cohorts):

1. Aged 18 years or over
2. Willing and able to give informed consent

Cohort A – Post-hospitalised Additional Inclusion Criteria: 3. Previously hospitalised with COVID-19 pneumonia 4. Group 1 ONLY: a. More than 3 months post-infection b. Diagnosis of Long-COVID or ‘post-COVID syndrome’ (NICE 2020) via specialised assessment at a long-COVID clinic 5. Group 2 ONLY: a. 1 - 12 months post-infection b. Microbiological evidence of COVID-19 infection c. Evidence of architectural distortion on CT

Cohort B – Non-Hospitalised Additional Inclusion Criteria: 3. Group 3 ONLY: a. More than 12 months since infection b. Clinical diagnosis of Long-COVID or ‘post-COVID syndrome’ (NICE 2020) via specialised assessment at a long-COVID clinic c. Significantly breathless upon exertion 4. Group 4 ONLY: a. More than 3 months since infection b. Microbiological evidence of COVID-19 infection c. Clinical diagnosis of Long-COVID or ‘post-COVID syndrome’ (NICE 2020) via specialised assessment at a long-COVID clinic d. Significantly breathless upon exertion 5. Group 5 ONLY: a. More than 3 months since infection b. Microbiological evidence of COVID-19 infection c. Clinical diagnosis of Long-COVID or ‘post-COVID syndrome’ (NICE 2020) via specialised assessment at a long-COVID clinic d. No respiratory symptoms

Cohort C – Controls Additional Inclusion Criteria: 3. Microbiological evidence of COVID-19 infection at any time since March 2020, confirmed verbally by the participant 4. No ongoing symptoms post-COVID infection

Common Exclusion Criteria (for all cohorts):

1. Pregnant, lactating or planning pregnancy during the course of the study
2. Known chronic renal impairment (EGFR below 60 mL/min) as measured by local policy
3. Known prior reaction to the contrast media used in this study, as locally determined
4. Inability to lie flat for imaging

5. Contraindications to MRI examinations, as locally determined
6. COVID infection in the last 3 months
7. Known significant current or prior cardiorespiratory disease, including significant smoking history (>10 pack years)
8. History of bleeding and clotting disorders
9. Currently on antiplatelets and/or anticoagulants (excluding Aspirin)
10. Any other reason, as determined by the study investigators, that renders the participant ineligible

Cohort-Specific Exclusion Criteria:

- Cohort A (Group 1 ONLY): Participant received mechanical ventilation
- Cohort B and C: Admission to hospital with COVID-19 pneumonia
- Cohort C: Symptoms fitting the definition of Long-COVID or 'Post-COVID syndrome' (NICE 2020)

Group	n	Cohort	Hospitalised	Date of infection	Breathless	LC/C
1	4	A	Yes	2020	NA	LC
2	1	A	Yes	2022	NA	LC
3	11	B	No	2020	Yes	LC
4	57	B	No	2021+	Yes	LC
5	33	B	No	2021+	No	LC
6	27	C	No	2021+	No	C

Table 5.1: Final participant groups included in the study. Cohort A consists of post-hospitalised Long COVID (LC) patients, divided into those infected in 2020 (Group 1) and 2022 (Group 2). Cohort B consists of non-hospitalised LC patients, subdivided into those with breathlessness (Group 3: infected in 2020, Group 4: infected in 2021+) and without breathlessness (Group 5: infected in 2021+). Cohort C includes control participants (Group 6) with previous COVID-19 infection but no ongoing symptoms.

5.2.3. Questionnaires

Participants completed a series of validated questionnaires to assess symptoms, quality of life, anxiety, and fatigue. These included the Dyspnoea-12 Questionnaire, Nijmegen Questionnaire, Functional Assessment of Chronic Illness Therapy - Fatigue (FACIT-F), Generalized Anxiety Disorder - 7 (GAD-7), Patient Health Questionnaire - 9 (PHQ-9), Visual Analogue Fatigue Rating, Work and Social Adjustment Scale (WSAS), and EuroQol 5-Dimension 5-Level (EQ-5D-5L). The questionnaires were administered at baseline and follow-up visits to capture changes in symptoms and functional status over time.

5.2.4. Sample collection

Blood samples for coagulation and metabolomics analyses were collected prior to scanning patients, and prior to receiving contrast if applicable. Coagulation samples were collected into blue-top vacutainer tubes (citrate) and centrifuged at 3000xg for 15 minutes at room temperature immediately after collection. Plasma was then aliquoted and placed immediately on dry ice before storing at -80°C. For metabolomics analysis, blood was collected into red-top (silica coated, for serum) and green-top (lithium heparin, for plasma) vacutainer tubes. These tubes were stored upright for 30 minutes at room temperature prior to centrifugation at 2000xg for 10 minutes at room temperature. Urine samples for coagulation and metabolomics studies were collected and immediately aliquoted into Eppendorf tubes and placed on dry ice. All aliquots were transferred to a -80°C freezer within 4 hours of collection and stored upright.

5.2.5. ELISAs and Clinical Biochemistry

Coagulation analyses were performed on plasma samples at the Oxford Haemophilia and Thrombosis Centre. The following assays were used:

Assay	Method	Sample Type
Prothrombin fragment F1.2	96 well ELISA	Plasma
Myeloperoxidase (MPO)	Human Myeloperoxidase ELISA Kit	Plasma
Soluble CD40 ligand (sCD40L)	Human sCD40L Platinum ELISA Kit	Plasma
Soluble P-selectin	96 well ELISA	Plasma
11-dehydro thromboxane B2 (and Creatinine)	96 well ELISA (paired with urine Creatinine)	Spot urine
Soluble thrombomodulin	96 well ELISA	Plasma
Plasminogen activator inhibitor-1	Antigen ELISA, 96 well ELISA	Plasma
Serum amyloid A1/2 (SAA1/2)	Human SAA ELISA kit	Plasma

All assays were performed according to manufacturer instructions, and the results were recorded and analysed in batches. These analyses were performed by a colleague, and the results were uploaded into the EXPLAIN database, where they could be combined with the metabolomics results for an integrated downstream analysis.

5.2.6. ¹H NMR Metabolomics

Serum and urine ¹H NMR spectra were obtained in an identical manner to the methods outlined in Chapter 3.8.

5.2.7. Statistical Analysis

All statistical analyses, multivariate and univariate, were carried out using the same methods outlined in Chapter 3, with the exception of the clustering analysis, as the variables were all continuous unlike the prior analyses on binary and mixed data. K-means clustering was

performed using the kmeans function, specifying two clusters (centres = 2) and setting the number of random starts to 25 (nstart = 25) to ensure stable clustering. Euclidean distance was used as the distance metric. The pheatmap package was used to visualise the clustering results. The MatchIt package in R was used to generate a 1:1 matching between long COVID patients and controls based on age, sex, ethnicity, and BMI, using optimal matching with Mahalanobis distance to improve balance. Statistical comparisons between groups were performed using one-way ANOVA or unpaired t-tests, as appropriate. Significance levels were set at $p < 0.05$, with additional thresholds for increased significance ($p < 0.01$ and $p < 0.001$) indicated where applicable. Post-hoc analyses were conducted where necessary to identify differences between clusters. All data visualisation and statistical analyses were performed using R and GraphPad Prism 10.

5.2.8. Ethics Statement

This study was approved by the South Central - Oxford C Research Ethics Committee (REC) (Ethics Ref: 21/SC/0398) and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice (GCP) guidelines. Written informed consent was obtained from all participants prior to any study-related procedures.

5.3. Results

5.3.1. Participant characteristics

The demographic and clinical characteristics of the study participants are presented in Table 5.2. The study included 27 control participants and 106 individuals with Long COVID. There were no significant differences between the control and Long COVID groups in terms of age, sex distribution, BMI, smoking history, asthma, diabetes, or vaccination status. Ethnicity was

slightly different between the groups, with a higher proportion of White British participants in the Long COVID group (80.2%) compared to the control group (51.9%); however, this difference was not statistically significant. Physiological measurements, including heart rate, systolic and diastolic blood pressure, respiratory rate, and oxygen saturation (SpO₂), were comparable between the two groups. Pulmonary function tests, as measured by FEV Z-score and FVC Z-score, also showed no significant differences. Importantly, although not presented here, imaging studies and scans revealed no significant abnormalities distinguishing the control group from the Long COVID group. These results suggest that the Long COVID cohort did not exhibit significant differences in baseline physiological measurements compared to controls. However, it is important to address the class size imbalance between the groups. When building a classification model, it is necessary to balance class sizes to ensure accurate evaluation of model performance. If one class substantially outweighs the other, the model may appear to perform well simply because it is more likely to detect the larger class, rather than genuinely distinguishing between groups. Therefore, when matching for class size in each iteration, it is essential to consistently create balanced subsets. To achieve this, the next step involved selecting a matched subset of controls and Long COVID participants, thereby minimising potential bias and enhancing the robustness of the classification analysis.

	Control (n=27)	Long COVID (n=106)
Age (years), mean (SD) †	47.63 (14.00)	50.62 (12.40)
Female (%) ‡	20 (74%)	69 (65%)
Ethnicity §		
White – British	14 (51.9%)	85 (80.2%)
White - Any other background	6 (22.2%)	14 (13.2%)
Asian - Chinese	2 (7.4%)	0
Asian - Indian	3 (11.1%)	1 (0.9%)
Asian - Any other background	2 (7.4%)	3 (2.8%)

Mixed - White & Asian	0	1 (0.9%)
Black - British	0	1 (0.9%)
Prefer not to say	0	1 (0.9%)
BMI, mean (SD) †	30.40 (6.44)	29.54 (6.45)
Smoking history ‡		
No	21 (77.8%)	76 (71.7%)
Yes, former	6 (22.2%)	28 (26.4%)
Yes, current	0	2 (1.9%)
Asthma ‡		
No	24	97
Yes	3	9
Diabetes ‡		
No	26	100
Yes	1	6
Vaccination ‡		
No	0	4
Yes	27	102
COVID-19 Test ‡		
PCR	15	48
Lateral flow	12	42
Heart rate (bpm) †	76.74 (14.01)	78.60 (12.41)
Systolic blood pressure (mmHg) †	135.1 (21.22)	128.5 (18.26)
Diastolic blood pressure (mmHg) †	82.44 (15.25)	83.25 (12.02)
Respiratory rate (breaths/min) †	16.33 (3.00)	16.92 (3.28)
SpO2 (%) †	97.56 (1.31)	97.84 (1.44)
FEV Z-score †	0.102 (0.89)	-0.027 (1.04)
FVC Z-score †	0.1211 (1.08)	0.0622 (0.97)

Table 5.2: Participant characteristics. † Continuous data analysed by a two-tailed T test and presented as mean (SD), ‡ Categorical data analysed using Chi-square or Fisher's Exact Test for 2x2 contingency tables. There were no significant differences between the two groups.

5.3.2. Random forest modelling demonstrates metabolic differences between long COVID and age- and BMI-matched controls with weak discriminatory power

Due to the substantial class imbalance, we employed the R Matchit package to generate a subset matched for age, sex, and BMI (Figure 5.1). To assess whether random forest classification modelling could differentiate long COVID from control, we utilised the serum and urine metabolome, alongside routine biochemistry tests and clotting factors listed in

Section 5.2.4. While the true model yielded an AUC of 0.58, significantly different from the random model (AUC = 0.49, Kolmogorov-Smirnov test, $p < 0.0001$), the average accuracy was only 0.51 ($p < 0.05$ better than random). Despite both metrics being statistically superior to the random model, they lack substantial discriminatory power, indicating only subtle differences in the metabolome between long COVID and control, contrasting with the previous results in Chapter 4. The average VIMP scores across iterations revealed that, even among the top metabolites, many differences were statistically non-significant between long COVID and control. Notably, these top features did not include previously identified biomarkers, such as serum GlycA or BCAAs (Figure 5.1). Among the significant variables, urine proline, urinary 11-dehydro-thromboxane B2, and prothrombin time were all significantly decreased in long COVID compared to controls. While these findings may hint at specific metabolic or coagulation alterations in long COVID, they should be interpreted with caution given the subtlety of the differences observed.

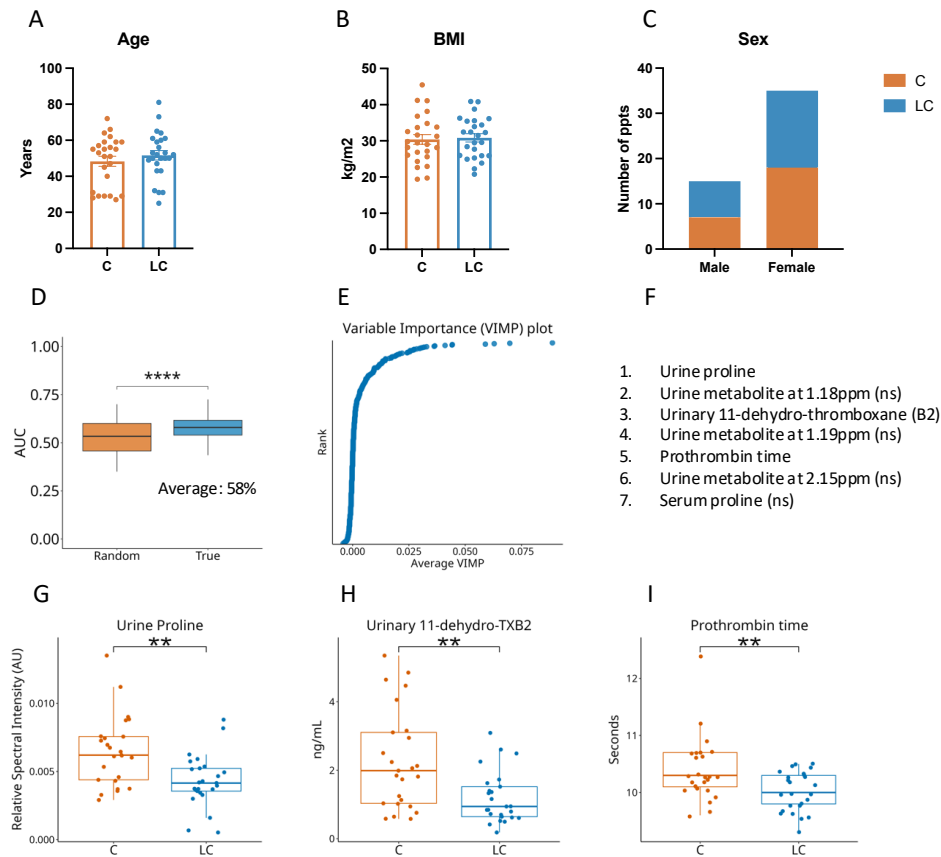


Figure 5.1: Demographics, model performance, and key metabolites distinguishing Long COVID from controls. (A) Age distribution of control (C) and Long COVID (LC) participants, showing no significant difference between groups. (B) Body Mass Index (BMI) distribution between control and Long COVID groups, also showing no significant difference. (C) Sex distribution between control and Long COVID participants, showing a higher proportion of females in both groups, with no significant difference between groups. (D) Performance of the random forest classification model distinguishing Long COVID from controls using the serum and urine metabolome, as well as routine blood biochemistry and clotting-related measures. The true model achieved an AUC of 0.58, significantly better than the random model (Kolmogorov-Smirnov test, **** $p < 0.0001$), indicating a modest discriminatory ability. (E) Variable Importance (VIMP) plot from the random forest model, showing ranked importance of metabolites in distinguishing Long COVID from controls. (F) Ranked list of the top seven variables identified by the model, including urine proline, urinary 11-dehydro-thromboxane B2 (TXB2), and prothrombin time, all of which were significantly lower in Long COVID compared to controls, as well as four other metabolites that did not reach significance. (G-I) Box plots showing significantly lower levels of urine proline (** $p < 0.01$), urinary 11-dehydro-TXB2 (** $p < 0.01$), and prothrombin time (** $p < 0.01$) in the Long COVID group compared to controls. Data are presented as mean $\pm$ SEM. Statistical comparisons between groups were performed using one-way ANOVA with post hoc Tukey tests where appropriate.

5.3.3. Identification of clotting subgroups within long COVID

Given the small sample size and the heterogeneous nature of Long COVID, it is possible that differences between controls and Long COVID are being masked by the presence of distinct subgroups within the Long COVID cohort. Previous analyses (Chapter 4) identified metabolically distinct subgroups within a small sample, suggesting that a similar phenomenon might exist within the clotting data. Therefore, we conducted an unsupervised clustering analysis to investigate potential clotting subgroups within the Long COVID cohort, focusing on the hypothesis that the heterogeneity of Long COVID may arise from a mixture of pathophysiological processes. Using the clotting data alone, unsupervised clustering identified two distinct clusters with unique clotting profiles (Figure 5.2A). These clusters were compared to controls to determine whether their clotting profiles align with or diverge from the healthy baseline (Figure 5.2B and Appendix D, Figure S5.1 for all). Cluster 1 was characterised by significantly elevated levels of C-reactive protein (CRP) ($p < 0.01$), Serum Amyloid A (SAA) ($p < 0.01$), D-Dimer ($p < 0.05$), and prolonged clot lysis time (CLT50) ($p < 0.05$) compared to controls (Figure 5.2B, i-iv). These findings indicate a pro-inflammatory and hypercoagulable state relative to controls. Cluster 2, on the other hand, exhibited levels of these markers comparable to those of the control group, suggesting of distinct clotting phenotypes, which may reflect underlying biological differences in disease mechanisms.

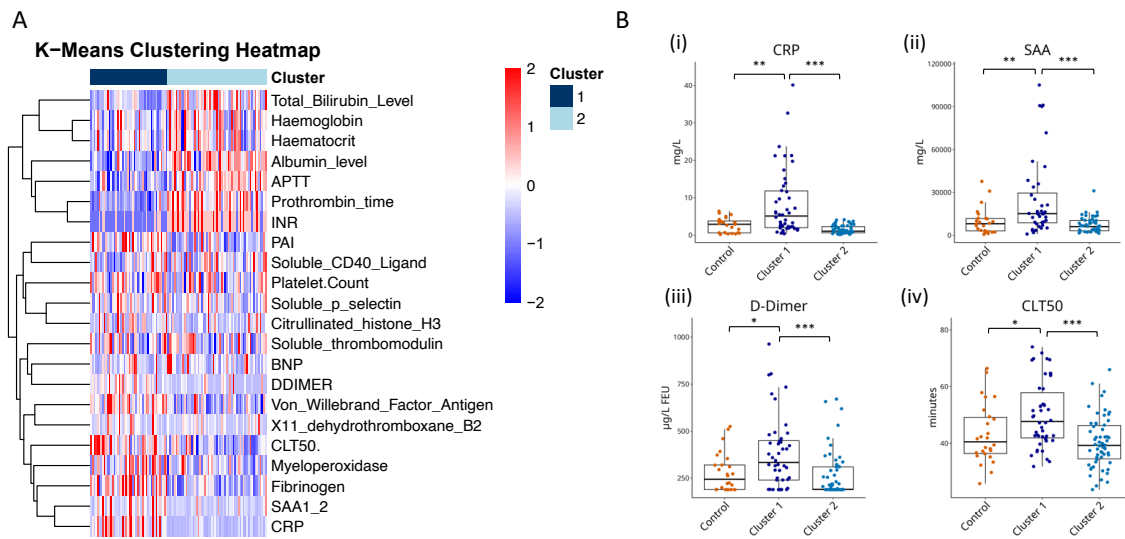


Figure 5.2: Identification of Clotting Subgroups within Long COVID. (A) *K-Means Clustering Heatmap*: Unsupervised *k*-means clustering analysis of clotting markers identified two distinct clusters (Cluster 1 and Cluster 2) within the Long COVID cohort. The heatmap displays scaled expression levels (z-scores) of key clotting and inflammatory markers across the two clusters. Cluster 1 (dark blue) is characterised by elevated levels of markers associated with inflammation and hypercoagulability, including CRP, Serum Amyloid A (SAA), D-Dimer, and increased clot lysis time (CLT50). In contrast, Cluster 2 (light blue) demonstrates a profile more comparable to controls, with lower levels of these markers. (B) *Key Markers Differentiating Clusters and Controls*: Box plots illustrating the distribution of four key markers across controls, Cluster 1, and Cluster 2: (i) CRP: Significantly elevated in Cluster 1 compared to controls ($p < 0.01$) and Cluster 2 ($p < 0.001$). (ii) SAA: Significantly higher in Cluster 1 compared to both controls ($p < 0.01$) and Cluster 2 ($p < 0.001$). (iii) D-Dimer: Significantly increased in Cluster 1 compared to controls ($p < 0.05$), and Cluster 2 ($p < 0.001$) indicating a hypercoagulable state. (iv) CLT50: Significantly prolonged in Cluster 1 compared to controls ($p < 0.05$) and Cluster 2 ($p < 0.001$), indicating impaired clot lysis. Data are presented as box plots with individual data points. Statistical comparisons between groups were performed using one-way ANOVA with post hoc Tukey tests. The clustering results indicate that Cluster 1 is characterised by a more inflammatory and hypercoagulable profile compared to both controls and Cluster 2, while Cluster 2 exhibits a clotting and inflammatory profile more similar to that of the control group.

5.3.4. Metabolomic characterisation of clotting subgroups within Long COVID

Following the identification of distinct clotting subgroups within the Long COVID cohort, we aimed to investigate whether the observed differences in clotting profiles correspond to differences in the metabolome. Specifically, we hypothesised that Cluster 1, which exhibited a pro-inflammatory and hypercoagulable state, would also demonstrate a distinct metabolic signature compared to controls, whereas Cluster 2, which presented with clotting and inflammatory features similar to controls, would not. This approach aligns with the notion that the heterogeneity observed within Long COVID may result from differing pathophysiological processes. To test this, we employed random forest classification modelling to determine whether the serum and urine metabolomes could distinguish between the identified clusters and controls. When comparing Cluster 2 to controls, random forest modelling did not achieve significant discrimination, consistent with the similarity of their clotting and inflammatory profiles. However, when comparing Cluster 1 to controls, random forest classification of the serum and urine metabolome yielded a cross-validated AUC of 0.60, significantly better than the random permuted model ($p < 0.001$, Kolmogorov-Smirnov test). These findings suggest that the metabolic profile of Cluster 1 is distinct from that of controls, mirroring the differences observed in clotting and inflammatory markers. When the model included both metabolomic and clotting data, the discrimination between Cluster 1 and controls improved further, achieving an AUC of 0.65 (Supplementary Figure 5.3). In contrast, the combined model still failed to distinguish Cluster 2 from controls, reinforcing the conclusion that Cluster 2 remains metabolically and clinically similar to controls. Additionally, Cluster 1 was associated with a significantly higher EQ-5D score (indicating worse health-related quality of life) and a significantly lower FACIT score

(indicating greater fatigue) compared to both Cluster 2 and controls, suggesting that Cluster 1 represents a subgroup with more severe and persistent symptoms.

To gain insights into the metabolites contributing most to the classification, we analysed the variable importance (VIMP) scores averaged across model iterations. Among the top VIMPs, glucose was notably lower in Cluster 1 compared to both controls ($p < 0.01$) and Cluster 2 ($p < 0.05$), as determined by one-way ANOVA with post hoc Tukey tests. This decrease in glucose may reflect ongoing immune activation and an inflammatory state, consistent with the pro-inflammatory profile of Cluster 1. Other significant VIMPs included histidine and a histidine derivative, both of which were significantly reduced in Cluster 1 compared to controls (histidine: $p < 0.001$, histidine derivative: $p < 0.001$) and also compared to Cluster 2 (histidine derivative: $p < 0.0001$). Histidine has been implicated in platelet function and clotting, as it serves as a precursor to histamine and influences platelet aggregation (Saxena et al. 1989). The reduction of histidine and its derivative in Cluster 1 may therefore reflect alterations in platelet activation or function, further linking the metabolic profile of this cluster to its pro-thrombotic state. These findings highlight the metabolic distinctions that underpin the more severe clinical phenotype observed in Cluster 1. Identifying these differences not only enhances our understanding of the pathophysiology of long COVID but also provides potential metabolic targets for therapeutic intervention. Furthermore, the integration of clotting, inflammatory, and metabolomic data allows for a more nuanced classification of long COVID subgroups, which may be crucial for tailoring treatments to specific patient profiles.

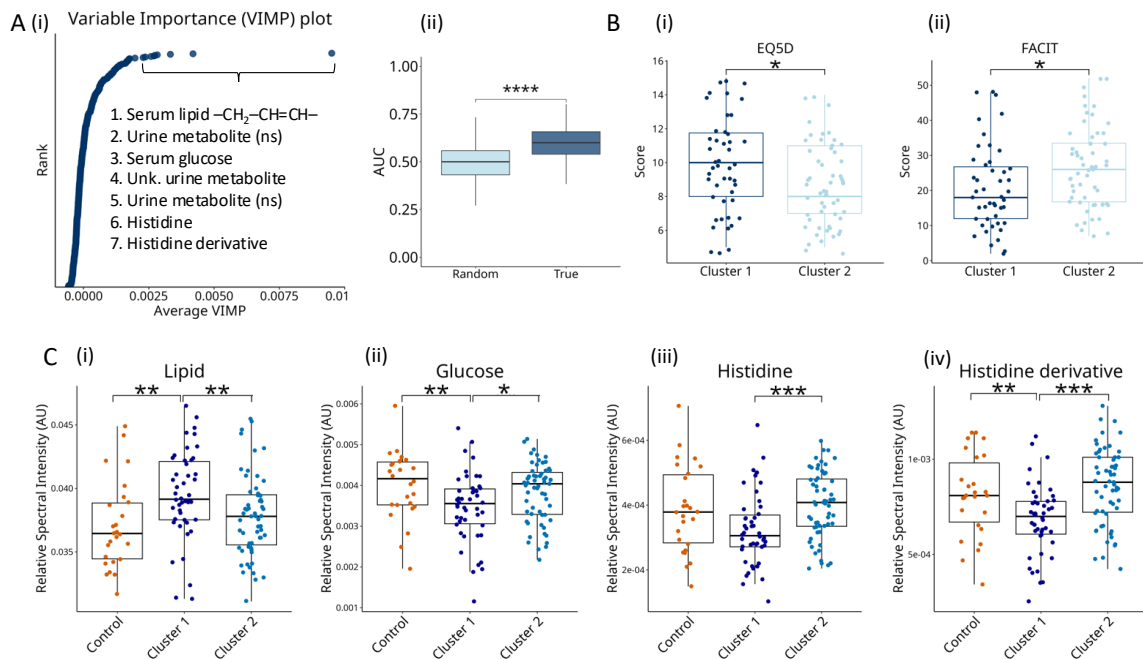


Figure 5.3: Metabolomic Characterisation of Clotting Subgroups within Long COVID. (A) Random Forest Classification and Variable Importance: (i) Variable Importance (VIMP) plot showing the most important variables for distinguishing Cluster 1 from controls based on the serum and urine metabolome. Top-ranked variables include a serum lipid ($-\text{CH}_2-\text{CH}=\text{CH}-$), serum glucose, histidine, and a histidine derivative. Two urine metabolites and an unknown urine metabolite were also identified as important but did not reach statistical significance (ns). (ii) Random forest model performance distinguishing Cluster 1 from controls, with a cross-validated AUC of 0.60, significantly better than the random permuted model (Kolmogorov-Smirnov test, $****p < 0.0001$). (B) Clinical Characterisation of Clusters: (i) EQ-5D Scores: Cluster 1 had significantly higher EQ-5D scores compared to Cluster 2 ($p < 0.05$), indicating worse health-related quality of life. (ii) FACIT Scores: Cluster 1 exhibited significantly lower FACIT scores compared to Cluster 2 ($p < 0.05$), indicating greater fatigue. (C) Key Metabolites Distinguishing Clusters and Controls: (i) Lipid: Significantly lower in Cluster 1 compared to both controls and Cluster 2 ($p < 0.01$), indicating potential alterations in lipid metabolism related to inflammatory states. (ii) Glucose: Significantly decreased in Cluster 1 compared to controls ($p < 0.01$) and compared to Cluster 2 ($p < 0.05$), possibly reflecting increased immune activation and metabolic stress. (iii) Histidine: Significantly reduced in Cluster 1 compared to controls ($p < 0.001$), suggesting altered histidine metabolism that may relate to platelet activation, as histidine can influence platelet aggregation through its role in histamine biosynthesis. (iv) Histidine Derivative: Lower in Cluster 1 compared to both controls ($p < 0.01$) and Cluster 2 ($p < 0.001$), supporting the potential link between altered histidine metabolism and pro-thrombotic states. Data are presented as box plots with individual data points. Statistical comparisons were performed using one-way ANOVA with post hoc Tukey tests. These findings

indicate that Cluster 1 exhibits a distinct metabolomic profile, correlating with increased inflammation, fatigue, and poorer health-related quality of life compared to both controls and Cluster 2.

5.4. Discussion

5.4.1. Limited evidence for long COVID vs controls

The findings from our initial analysis indicate limited evidence for distinguishing Long COVID from controls based on the serum and urine metabolome, as well as routine biochemistry and clotting markers. One potential reason for this limitation is the relatively small sample size, which may reduce the statistical power to detect nuanced differences. Additionally, Long COVID is inherently heterogeneous, likely encompassing multiple pathophysiological pathways and clinical phenotypes. As a result, pooling all Long COVID cases together may obscure subgroup-specific metabolic changes, highlighting the need for a more nuanced approach to classification and analysis. Indeed, due to the large sample size of the long COVID cohort, we were able to apply unsupervised clustering analysis to investigate this hypothesis.

5.4.2. Clustering analysis reveals a distinct hypercoagulable subgroup within long COVID

Our results demonstrate that within the Long COVID cohort, a distinct subset of individuals (Cluster 1) exhibits elevated levels of markers associated with coagulation and inflammation. While these findings indicate a hypercoagulable phenotype within Cluster 1, it is important to note that not all individuals within this cluster necessarily display clinically significant abnormalities. However, the overall pattern of elevated markers within Cluster 1 is biologically meaningful and warrants further investigation. In contrast, Cluster 2 did not exhibit significant differences compared to controls for the markers assessed, indicating that

this subgroup may represent a different clinical phenotype not captured by the markers measured in this study.

Existing literature supports the notion of a hypercoagulable state in Long COVID, marked by persistent vascular damage, endothelial dysfunction, and coagulation abnormalities. For instance, studies have reported increased levels of inflammatory markers and endothelial biomarkers, such as von Willebrand factor and platelet factor 4, indicative of thrombotic endothelialitis (Turner et al. 2024). Additionally, fibrinolysis-resistant microclots containing inflammatory molecules have been observed in long COVID plasma samples (Pretorius et al. 2021). These microclots, along with platelet hyperactivation, may impair microcirculation and organ function, contributing to ongoing symptoms (Wang, Yu, et al. 2022). The thrombotic state observed in Long COVID is hypothesised to result from persistent endothelial damage, residual viral reservoirs, and immunopathology (Nicolai, Kaiser, and Stark 2023). Indeed, promising therapeutic approaches include early anticoagulation, which has been associated with improved outcomes (Wang, Yu, et al. 2022; Violi et al. 2024), as well as novel small molecules targeting vascular damage and clotting abnormalities (Samarelli et al. 2024). Our findings, therefore, align with these reports by identifying a subgroup of Long COVID patients with a potentially hypercoagulable and inflammatory profile, warranting further exploration into targeted treatments for this specific phenotype.

5.4.3. Low blood glucose is associated with an inflammatory long COVID subtype

Our study uniquely integrates metabolomic profiling with coagulation and inflammatory markers to better understand the hypercoagulable phenotype within a subset of Long COVID

patients. Notably, we identified significantly lower glucose levels in Cluster 1 compared to both controls and Cluster 2, suggesting a link between altered glucose metabolism and the inflammatory state in this subgroup. Glucose plays a crucial role in immune activation and function, as immune cells require glucose for energy and biosynthesis. During activation, immune cells increase glucose uptake to meet heightened metabolic demands (Maciver et al. 2008; Calder, Dimitriadis, and Newsholme 2007). This relationship is particularly relevant in inflammatory states, where immune activation can deplete circulating glucose, as seen in acute responses like endotoxin challenge (Kvidera et al. 2017). Furthermore, both hypo- and hyperglycaemia can impair immune function and promote inflammation (Calder, Dimitriadis, and Newsholme 2007; Shomali et al. 2021), highlighting the complex regulation of glucose in chronic inflammatory conditions. Interestingly, Cluster 1 was also associated with significantly worse fatigue and poorer health-related quality of life (QOL), as indicated by lower FACIT and higher EQ-5D scores, respectively. This link between metabolic disruption and symptom severity suggests that the inflammatory subgroup may experience a more severe clinical phenotype, warranting tailored therapeutic strategies rather than a one-size-fits-all approach.

5.4.4. Low systemic histidine levels may contribute to a hypercoagulable state in long COVID

The observed reduction in histidine levels within Cluster 1, characterised by a hyperinflammatory and hypercoagulable profile, may reflect the increased utilisation of histidine as a precursor for histamine production, consistent with histamine's role in platelet aggregation. Previous evidence demonstrates that histamine functions as an intracellular messenger mediating platelet aggregation, with histidine decarboxylase (HDC) catalysing the

conversion of histidine to histamine within activated platelets (Saxena et al. 1989). This histamine synthesis is rapid and parallels platelet activation, highlighting the potential for increased histidine consumption in hypercoagulable states. The increased production of histamine within platelets during aggregation may thus explain the lower circulating levels of histidine observed in this cluster. Histidine depletion has been previously linked to inflammatory states, as demonstrated by studies in chronic kidney disease patients, where low plasma histidine concentrations are associated with heightened inflammation, oxidative stress, and increased mortality (Watanabe et al. 2008). Furthermore, histidine supplementation has been shown to improve metabolic outcomes, including reductions in BMI, adiposity, and markers of glucose homeostasis (Thalacker-Mercer and Gheller 2020). In animal models, histidine has been shown to alleviate inflammation in adipose tissue via the NF- κ B and PPAR γ pathways, indicating potential anti-inflammatory effects (Sun et al., 2014). However, it remains unclear whether the depletion of histidine itself directly contributes to the hyperinflammatory and hypercoagulable state observed in Cluster 1 or whether it is a downstream consequence. This raises the intriguing possibility that histidine supplementation could modulate the hypercoagulable phenotype, but it is equally plausible that replenishing histidine might inadvertently exacerbate histamine-driven inflammation and coagulation. Future studies are warranted to investigate the potential therapeutic implications of histidine modulation in the context of thromboinflammatory states observed in Long COVID.

5.4.5. Limitations of the study

This study had several limitations that may have influenced the findings. Firstly, we did not collect clinical questionnaire data from control participants, and the small number of controls reduced the power to detect differences. Additionally, we did not collect symptom data comparable to that obtained in Chapter 4, which limits our ability to compare the metabolic clusters identified in that study. Furthermore, we did not perform lipidomics, which played a key role in separating groups in the previous analysis.

The hospital-based setting of the EXPLAIN trial also differed significantly from the more relaxed environments in Chapter 4, where participants were seen either at home or in a department setting, typically by a single, known researcher. In contrast, the hospital setting was busy, with multiple staff members present, creating a potentially stressful environment, particularly as participants were preparing for their scans. Due to the fast expiry of xenon, there was also often a rush to ensure participants completed all tests prior to scanning on time. This difference may have induced stress-related metabolic changes, commonly known as "white coat syndrome," which could confound results. Moreover, blood sampling in the EXPLAIN trial often followed lung function tests or the sit-to-stand test, but the order was inconsistent due to the practicalities of clinical workflow. Since physical activity and stress can significantly impact the metabolome, this variation could contribute to metabolic heterogeneity within the dataset.

Unlike Chapter 4, we did not measure cortisol, which was a primary driver of group separation in the previous long COVID versus control analysis, limiting our ability to validate findings.

Additionally, the low number of hospitalised participants made it difficult to explore the effects of hospitalisation within the long COVID cohort.

5.4.6. Conclusions

This study identified distinct metabolic and coagulation profiles within long COVID patients, revealing heterogeneity that may underlie persistent symptoms. Despite the absence of significant findings from XeMRI, which did not demonstrate impairments in lung function or structure in the long COVID cohort, the blood sub-study highlighted potential biological differences between clusters, particularly in relation to coagulation markers and metabolic changes. These findings advance our understanding of the diverse presentations of long COVID, but independent validation cohorts are still needed to confirm these results and assess their generalisability.

Chapter 6: Metabolomic and Neuroinflammatory Signatures of Fibromyalgia Reveal Distinct Pain Subtypes

Chapter summary

Expanding the thesis beyond post-viral syndromes, this chapter investigates fibromyalgia (FM), a prevalent yet poorly understood chronic pain disorder, to explore whether similar metabolic and inflammatory signatures underlie its symptomatology. Following the identification of metabolically and immunologically distinct subgroups in long COVID (Chapters 3–5), this chapter applies a comparable multi-modal approach to FM. By integrating ^1H NMR metabolomics with inflammatory and neurodegenerative protein profiling in both serum and cerebrospinal fluid (CSF), and linking these molecular signatures to pain phenotyping through quantitative sensory testing (QST), this chapter aims to uncover the biological heterogeneity that may underpin fibromyalgia and its varied clinical presentations.

The study draws on a large cohort of 143 FM patients and 70 healthy, pain-free controls from the Danish Pain Research Biobank (collected and supplied by Morten Blichfeldt-Eckhardt). Analyses revealed systemic alterations in amino acid metabolism in FM, with consistent reductions in serum and CSF levels of glutamine and proline, and additional depletions of histidine and lysine in serum. These findings point towards dysregulation of glutamatergic signalling and inflammatory pathways. Notably, elevated CSF CRP levels in FM participants suggest the presence of neuroinflammation, which may contribute to central pain amplification mechanisms.

Pain threshold assessments via QST confirmed widespread sensory hypersensitivity in FM patients. Hierarchical clustering based on QST metrics identified two distinct FM subgroups: one exhibiting pain thresholds similar to healthy controls and another demonstrating marked hyperalgesia. These subgroups also displayed differing metabolic signatures, suggesting a biological basis for the observed heterogeneity in pain processing. Together, these results support the notion that fibromyalgia is not a uniform condition but rather comprises biologically distinct subtypes that may respond differently to treatment.

By linking metabolic and inflammatory dysregulation to central and peripheral pain processing, this chapter supports the argument for a precision medicine approach in chronic pain conditions. The findings highlight the potential of metabolic profiling not only as a tool for patient stratification but also as a gateway to targeted therapeutic development.

6.1. Introduction

Fibromyalgia is a complex pain syndrome that affects approximately 2-8% of the global population. It is the third most common musculoskeletal condition and the most common cause of chronic widespread pain (Sarzi-Puttini et al. 2020). In addition to diffuse musculoskeletal pain, fibromyalgia is characterised by fatigue, sleep disturbances, cognitive dysfunction, and mood alterations, contributing to significant morbidity and a high socioeconomic burden. Despite widespread impact, the causes and underlying pathophysiology remain elusive, but are thought to be multifactorial (Bellato et al. 2012). Indeed, there is an emerging consensus that fibromyalgia encompasses multiple subtypes rather than being one clinical entity, which further complicates diagnostic and treatment pathways (Sarzi-Puttini et al. 2020). A major challenge in research and clinical management is

the lack of objective biomarkers, which hinders early diagnosis, patient stratification, and the development of targeted therapeutics. The identification of validated biological markers alongside an improved understanding of the underlying mechanisms is critical for addressing this gap and improving patient outcomes (Siracusa et al. 2021).

The classification and diagnosis of fibromyalgia have evolved over time, reflecting an increasing recognition of its complex symptomatology (Sarzi-Puttini et al. 2020). The 1990 American College of Rheumatology (ACR) criteria established fibromyalgia as a condition characterised by widespread pain across all four quadrants of the body and the axial skeleton, along with tenderness at 11 or more tender points (Wolfe et al. 1990). However, due to impracticalities and variability in tender point examination, the 2016 ACR criteria (among others) introduced a broader assessment approach, requiring generalised pain in at least four of five body regions and symptom persistence for at least three months. The updated criteria also incorporated the Symptom Severity Score (SSS) alongside the Widespread Pain Index (WPI) to capture the multidimensional nature of fibromyalgia, including fatigue, cognitive dysfunction, and sleep disturbances (Wolfe et al. 2016). In parallel, the International Classification of Diseases, 11th Revision (ICD-11) recognised fibromyalgia as a distinct subset of chronic widespread pain within the broader category of chronic primary pain, reinforcing its status as a discrete clinical entity (Treede et al. 2015). In the current study, the FM cohort satisfies both the 2016 revised criteria and the FM subset of CWP in the ICD-11 classification system. These inconsistencies in clinical practice as well as research contribute to the challenges in understanding disease pathology and developing targeted therapeutics. This underscores the need for objective biomarkers of pain to enhance classification and patient stratification, ultimately improving diagnosis and treatment approaches.

While not incorporated into formal diagnostic criteria, Quantitative Sensory Testing (QST) has consistently demonstrated altered pain processing in patients with fibromyalgia (Petersen et al. 2025). QST measures pain thresholds across multiple sensory modalities and provides insight into central and peripheral pain mechanisms. For example, studies using pressure pain threshold (PPT) testing have shown that fibromyalgia patients experience pain at significantly lower levels of applied pressure compared to healthy controls, reflecting widespread mechanical hyperalgesia. Similarly, thermal QST studies have found that FM patients exhibit lower heat pain thresholds (HPT) and exaggerated responses to repeated noxious thermal stimuli, supporting the role of central sensitisation in FM pathophysiology (Hurtig et al. 2001). By assessing how a painful stimulus alters the perception of a subsequent painful stimulus, QST has demonstrated impaired conditioned pain modulation (CPM) in FM patients, indicating deficits in descending pain inhibition (Petersen et al. 2025). Additionally, fibromyalgia patients often show enhanced temporal summation of pain (TSP), where repeated stimuli of the same intensity lead to a progressively stronger pain response, indicating heightened central excitability and chronic pain amplification (Petersen et al. 2025). While these reports are consistent at the group level, there is substantial inter-individual variability, and it remains unclear whether such variation reflects biological differences or is a result of technical variability (Petersen et al. 2025). At present, we lack validated biomarkers or biological correlates with these QST results, making their clinical use or validity uncertain.

The mechanisms driving pain amplification in fibromyalgia remain poorly understood. Neuroimaging studies have demonstrated heightened activity in pain processing areas of the brain, including the insula, anterior cingulate cortex, and somatosensory cortices, which may contribute to central sensitisation (Gracely et al. 2002; Cook et al. 2004). Neurochemical

imbalances have also been implicated, including elevated cerebrospinal fluid (CSF) levels of substance P (Russell et al. 1994) and decreased serotonin, noradrenaline, and dopamine (Russell et al. 1992). Additionally, reduced μ -opioid receptor availability in pain-modulating brain regions suggests dysfunction in endogenous pain inhibition, and may be responsible for the CPM deficits seen in patients (Harris et al. 2007). In addition to central mechanisms, peripheral immune and metabolic dysfunction may contribute to the persistent pain state. Increased levels of circulating pro-inflammatory cytokines, particularly IL-6 and IL-8, have been reported in fibromyalgia patients (Mendieta et al. 2016) alongside an increased prevalence of rheumatic disease and small-fiber neuropathy. Inflammatory markers are known to activate nociceptors and elevation of these mediators may contribute to sensitisation and sustained sensory dysfunction (Ji, Xu, and Gao 2014). Increased oxidative stress and mitochondrial dysfunction has also been evidenced in patients with fibromyalgia, and these may contribute to the exercise intolerance, fatigue, and cognitive deficits present (Cordero et al. 2010). While these biomarkers offer insights into the underlying pathology, their presence is inconsistent across patients and they lack diagnostic specificity (Favretti, Iannuccelli, and Di Franco 2023). Given the multifactorial nature of fibromyalgia, it is unlikely that a single biomarker will define the disease. Further research is needed to better characterise these biological perturbations, ideally by simultaneously investigating both central and peripheral mechanisms within the same individuals. A more integrated approach is essential to unravel the complex interactions driving the disease, rather than focusing on pathways in isolation.

Metabolomic studies show promising potential for both biomarker discovery and uncovering the pathophysiological mechanisms of fibromyalgia. Metabolites, defined as molecules <1.5

kDa, represent the end products of cellular processes, integrating influences from both genetic and environmental factors. As a result, they provide a valuable snapshot of both physiological and pathological states. By measuring metabolites simultaneously, metabolomics offers a more comprehensive view of disease processes than individual biomarkers, reflecting the complex interplay of systemic dysfunction (Smolinska et al. 2012). The metabolome is particularly sensitive to inflammatory processes, as any changes in immune function are reliant on changes in metabolism, and has been used to detect subtle differences in immune dysfunction in patients with inflammatory diseases of the central nervous system (Yeo et al. 2020; Yeo et al. 2019; Jurynczyk et al. 2017). Indeed, prior analyses of serum metabolomics in fibromyalgia have identified alterations in amino acid metabolism, energy production pathways, and oxidative stress markers (Menzies et al. 2020). Notably, changes in glutamate, tryptophan, and lipid metabolism have been linked to symptom severity, suggesting a metabolic underpinning to central sensitisation (Zetterman et al. 2024). However, CSF metabolomics remains underexplored and may provide more direct insights into central nervous system alterations in chronic pain.

This chapter integrates ^1H Nuclear Magnetic Resonance (NMR) metabolomics with inflammatory and neurodegenerative protein profiling in both serum and cerebrospinal fluid (CSF) to investigate the biological underpinnings of fibromyalgia (FM). Given the lack of objective biomarkers and the complex, multifactorial nature of FM, we aim to identify metabolic and inflammatory signatures that distinguish FM from pain-free controls and provide insight into disease mechanisms. We aim to use such multimodal screening of CSF and serum biomarkers to understand the contribution and interaction of peripheral and central mechanisms, as well as the complex interplay with other factors by exploring clinical

pain scores, mood, cognitive, and fatigue assessments. Lastly, we aim to explore differences within FM patients, with a particular focus on distinguishing those with different pain thresholds.

6.2. Materials and Methods

6.2.1. Participants

Participants aged 18 to 80 years, capable of understanding and completing the questionnaires, were recruited from The Danish Pain Research Biobank (DANPAIN Biobank), as previously described (Blichfeldt-Eckhardt et al. 2023). For this study, samples were obtained from two groups: individuals with fibromyalgia, according to the ICD-11 classification system (Nicholas et al. 2019) (n=143) and pain-free controls (n=70). Further details on the recruitment process and cohort demographics have been described previously (Blichfeldt-Eckhardt et al. 2023).

6.2.2. Clinical questionnaires

Several questionnaires were used to obtain quantitative measures of functional impact of pain, including the Revised Fibromyalgia Impact Questionnaire (FIQR), Pain Disability Index (PDI), Major Depression Inventory (MDI), Generalised Anxiety Disorder 7 (GAD7), and Insomnia Severity Index (ISI). A numerical rating scale (NRS) ranging from 0 – 10 was used to measure self-assessed pain intensity during exercise, during rest, and on average. A total NRS score was taken as the sum of these measures, in which a higher score indicates higher self-assessed pain.

6.2.3. Quantitative Sensory Testing (QST)

Quantitative sensory tests were carried out on all participants within a week of sample collection. The QST protocol (16,57 in DANPAIN paper) was used to assess sensitivity to heat, pinprick, and pressure in this order, with detailed procedures described previously (Blichfeldt-Eckhardt et al. 2023). Table 6.1 provides definitions of the key QST terminology used in this thesis.

Heat pain threshold: Briefly, heat detection (HDT) and heat pain thresholds (HPT) were measured using an MSA Thermotester (Somedic AB, Hörby, Sweden) over the most painful area (or lower back in pain-free participants) and the thenar eminence of the left hand. Ramp stimuli (1°C/s) were applied from a 32°C baseline until participants pressed a stop button, with a 50°C cut-off. HDT was defined as the first noticeable temperature change, while HPT was the point at which heat was perceived as painful. The final thresholds were averaged from three trials.

Pinprick pain threshold: A single or repeated pinprick (512 mN force) was applied to participants over the most painful area and the thenar eminence of the left hand. TSP was assessed by comparing pain ratings after a single stimulus to those after a train of 10 pinpricks ($1/\text{s}$) within a 1 cm^2 area. Ratings were given on a 0–10 NRS scale, with TSP calculated as the ratio of pain ratings after repeated vs. single pinpricks.

Pressure pain threshold: Increasing pressure (1 kPa/s) was applied via a cuff pressure algometer (CPAR) to both lower legs until participants indicated pain onset (cPPT) or their pain tolerance threshold (cPTT), either by pressing a stop button or reaching a score of 10 using a visual analogue scale (VAS). Repeated cuff pressure stimuli (10 cycles) at cPTT

intensity were applied to one leg, with pain continuously rated using VAS. Temporal Summation of Pressure Pain (cTSP) scores were calculated by comparing pain ratings from early vs. late stimuli, with values >1 indicating increased pain sensitivity over time.

Conditioned Pain Modulation (CPM): CPM was assessed using a conditioning stimulus cuff on the right lower leg, inflated to the pressure corresponding to VAS = 5 (moderately painful) for up to 100 seconds. Five seconds after applying the conditioning cuff, the test stimulus cuff on the left leg was inflated, and cPPT and cPTT were reassessed while the participant was instructed to focus on the left leg. The CPM effect was calculated as the difference in thresholds between baseline and conditioning, with positive values indicating hypoalgesia.

HDT	Heat Detection Threshold	Temperature at which participant detected heat
HPT	Heat Pain Threshold	Temperature at which participant perceived pain
Pinprick	NA	Pain intensity rating in response to a single pinprick stimulus
TSP pinprick	Temporal summation of pain – pinprick test	Pain intensity after repeated pinprick stimulation compared to a single pinprick
cPPT	Cuff pressure pain threshold	Pressure at which participant first perceives pain (VAS = 1)
cPTT	Cuff pressure pain tolerance	Maximum pressure participant is willing to tolerate before stopping the test (pressure at termination or VAS = 10)
cTSP	Temporal summation of pain – pressure test	The ratio of pain ratings between late and early stimuli during repeated pressure stimulation, indicating pain amplification over time
CPM response	Conditioned pain modulation	The change in pain perception in response to a conditioning stimulus, reflecting endogenous pain modulation efficiency (positive=hypoalgesia)

Table 6.1: Quantitative Sensory Testing (QST) definitions.

6.2.4. Sample collection

Blood (12mL) was collected in plain tubes and left to stand for 30 minutes at room temperature prior to centrifugation at 2,000 XG for 10 minutes at 4°C. The resulting serum was then aliquoted and stored at -80°C. CSF was collected via lumbar puncture, with the first 10 drops discarded to avoid contamination with blood, or until the fluid ran clear. All samples were centrifuged at 2,000 XG for 10 minutes at 4°C, aliquoted on ice, and transferred to -80°C for storage.

6.2.5. Neuroimmune protein panels

The following experiments were performed in The Department of Biochemistry and Immunology, Lillebaelt Hospital, University Hospital of Southern Denmark, Vejle, Denmark. A panel of cytokines and receptors was quantified in CSF and plasma/serum using multiplex assays (Mesoscale Discovery), including TNF, LT α , IL-8, IL-6, IL-1 α , IL-1 β , IL-1Ra (CSF/plasma) and IL-6R, gp130, TNFR1, TNFR2 (CSF/serum) (Martin et al. 2016). hsCRP (mg/L) was measured using a high-sensitivity ELISA (TECAN, IBL International GmbH) with a 0.02 mg/L detection limit and intra-/inter-assay CVs of 4.1–6.9% and 5.8–6.3%, respectively. Serum suPAR (ng/mL) was assessed via suPARnostic® AUTO Flex ELISA (ViroGates A/S), with a detection limit of 0.4 μ g/L and CVs of 2.3–6.0%.

Neurofilament light (NfL) and glial fibrillary acidic protein (GFAP) were measured in CSF and serum using a Simoa® HD-X Analyzer (Quanterix), blinded to clinical data (. Detection limits were 0.065 ng/L (NfL) and 0.475 ng/L (GFAP), with quantification limits of 0.8 ng/L (serum NfL), 8 ng/L (CSF NfL), 16.6 ng/L (serum GFAP), and 166 ng/L (CSF GFAP). Assay performance was monitored using four quality controls (two manufacturer-provided, two in-house). Total analytical variation ranged from 9–16% (NfL) and 9–18% (GFAP).

To account for the known age-dependent increase serum/CSF NfL levels, exponential models were applied for age adjustment. Raw NfL concentrations in serum and CSF were first log-transformed to achieve normality and exponential regression model was fitted to the data. Residuals were then used for subsequent analyses, representing age-independent deviations in NfL levels.

6.2.6. ¹H NMR metabolomic sample processing

Serum (150 µL) and CSF (100 µL) were mixed with NMR buffer (75 mM sodium phosphate in D₂O, pH 7.4; 400 µL for serum, 450 µL for CSF) and transferred to 5 mm NMR tubes. Spectra were acquired using a 700-MHz Bruker AVII spectrometer (16.4 T, ¹H [¹³C/¹⁵N] TCI cryoprobe, University of Oxford). Serum was analysed with a Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence (with a τ interval of 400 µs, 80 loops, 32 scans, 1.5 s acquisition time, 2 s relaxation delay), while CSF was processed using a 1D ¹H NOESY presaturation scheme (2 s pre-saturation) for water suppression. A fixed receiver gain was used throughout. All samples were analysed within 9 hours of thawing and with pooled plasma samples spread throughout the run to monitor technical variation for quality control purposes.

6.2.7. ¹H NMR data processing

¹H NMR spectra were phased, baseline corrected and referenced to the lactate methyl resonance at $\delta = 1.33$ ppm using Topspin 4.1.4 (Bruker). ACD/Labs Spectrus Processor Academic Edition 12.01 (Advanced Chemistry Development, Inc.) was then used to manually bin each resonance signal, excluding all noise from analysis. Integrals of metabolite bins were sum normalised and exported to R version 4.3.0 (R_Core_Team 2024) for multivariate analysis. Resonances were assigned by reference to literature values (Lenz et al. 2003; Tang

et al. 2004; Snytnikova et al. 2019) and validated through 2D spectral analysis and spiking with known compounds as necessary.

6.2.8. Statistical analysis

Random Forest Classification models were used to compare groups across z-scaled serum and CSF metabolites and proteins using the Random Forests for Survival, Regression, and Classification (RF-SRC) R package version 3.2.0 (Ishwaran and Kogalur 2023), as outlined in Chapter 3.8.2. Univariate analyses of the predictor variables with the highest variable importance (VIMP) scores were performed according to Chapter 3.8.3, with significance thresholds set to $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Pearson correlation analyses were performed, with false discovery rate correction set at 0.05 using the Benjamini-Hochberg method. For cluster analysis, a hierarchical approach was implemented using Ward's minimum variance method with Euclidean distance as the dissimilarity metric (all variables were continuous). The resulting dendrogram was cut at $k = 2$ clusters. All statistical tests were carried out in R version 4.3.0 (R_Core_Team 2024).

6.3. Results

6.3.1. Random Forest modelling of serum and CSF metabolome distinguishes between fibromyalgia and pain-free controls

To assess whether the serum and CSF metabolome alone could detect the presence of fibromyalgia (FM) pain, we used a random forest approach with a 10-fold cross validation scheme and compared the “true” model (control vs FM) against a “random” model (model with random class assignment) (Figure 6.1A). The random forest classification was able to distinguish between control and FM with a cross-validated ROC AUC of 69% (Figure 6.1B-i). This was significantly better than the random model, which achieved a ROC AUC of 50%

($p < 0.001$, Kolmogorov–Smirnov test) (Figure 6.1B-i). Importantly, class sizes were matched during the cross-validation scheme to ensure the model performance was not due to a greater incidence of FM. Further performance metrics are presented in Appendix E, Figure S6.1. All predictor variables (serum and CSF metabolites) were ranked by their average VIMP score across the iterations and the top variables were selected before a point of inflection in the scores (Figure 6.1B-ii). To determine the relationship between these top metabolites and FM, univariate approaches were applied, as described below.

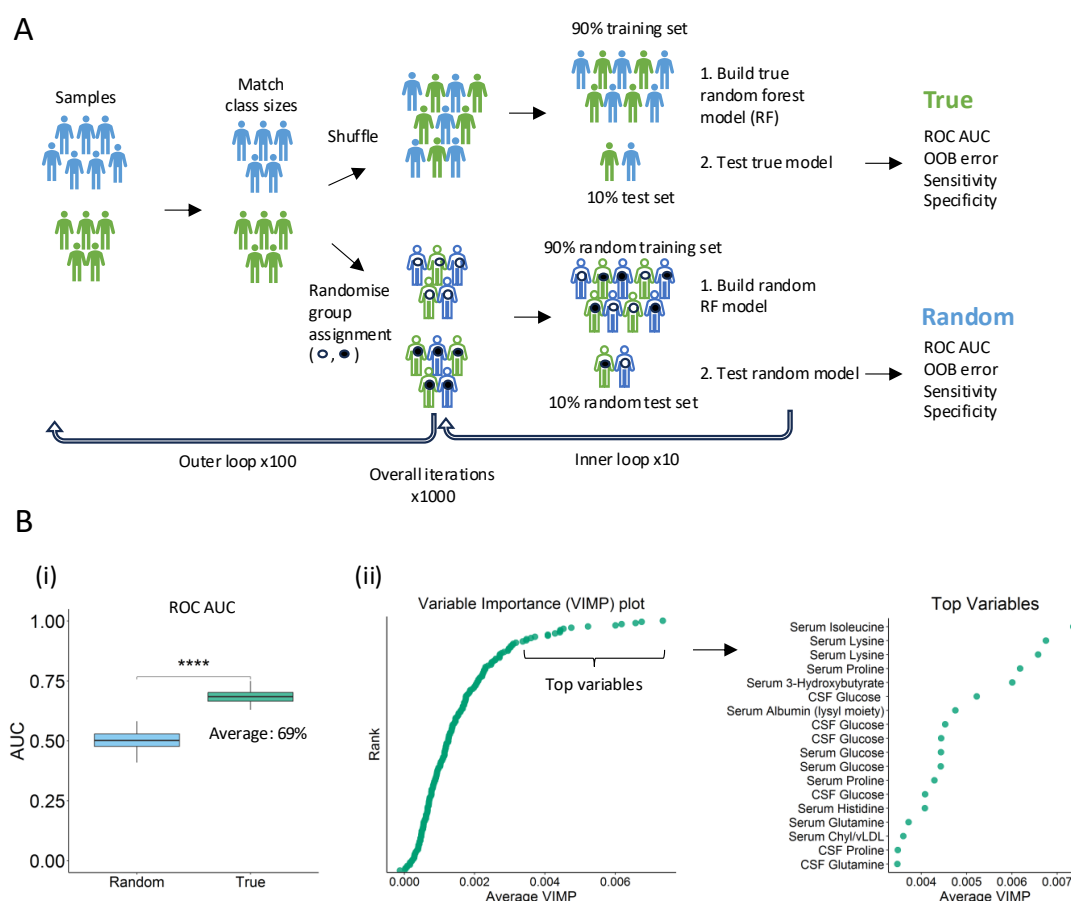


Figure 6.1: Random Forest Classification distinguishes the serum and CSF metabolome of individuals with fibromyalgia (FM) from pain-free controls. A) Schematic of the Random Forest model-building workflow. Sample sizes of FM and pain-free control groups were matched and shuffled. In the true model pipeline, samples were split into 90% training and 10% test sets, and a Random Forest model was trained and tested using 10-fold cross-validation. In the random pipeline, group labels were permuted before model training, and the

same 90% training / 10% test split and 10-fold cross-validation approach was applied to generate a null distribution. Each outer loop was repeated 100 times, with 10 internal cross-validation loops per outer loop, yielding 1000 total iterations. **B)** Outcomes of cross-validated Random Forest classification. **(i)** ROC AUC values for true and random models. The true model significantly outperformed the random control (**** $p < 0.0001$). The average AUC across iterations was 69%. **(ii)** Variable importance (VIMP) plot. Left: ranked VIMP scores across all predictor variables. Right: magnified view of top-ranked features. These include multiple spectral bins corresponding to amino acids and glucose in serum and CSF. Repeated assignments refer to distinct metabolite bins derived from different NMR spectral regions of the same compound.

6.3.2. Top metabolites characterising the metabolic signature of fibromyalgia, which are not influenced by age, sex, or BMI

The metabolites most important for distinguishing between FM and pain-free controls were predominantly from serum. Two tailed T tests revealed decreases in serum lysine ($p < 0.001$), proline ($p < 0.001$), albumin (lysyl moiety, $p < 0.001$), glucose ($p < 0.001$), histidine ($p < 0.001$), and glutamine ($p < 0.001$) in FM compared to controls (Figure 6.2). Conversely, serum 3-hydroxybutyrate and chylomicron/VLDL (resonance signal at 0.87ppm arising from mobile -CH₃) were increased in the FM group relative to controls ($p < 0.001$, Figure 6.2C; $p < 0.001$, Figure 6.2H). Two CSF metabolites, proline and glutamine, were also ranked in the top variables and were significantly decreased relative to controls ($p < 0.01$, Figure 6.2I; $p < 0.01$, Figure 6.2J). To ensure these differences were a result of FM and not of confounds, we assessed the effect of age, sex, and BMI on the metabolome. In a smaller subset matched for age and BMI using the matchit package in R (Ho D 2011), a random forest was still able to predict FM vs controls with a cross-validated AUC of 66% and the top variables remained the same (Appendix E, Figure S6.2-A). To further this, the pain-free control group was divided into two groups according to age to produce equal class sizes (above and below 33 years). Random forest classification had a cross-validated AUC of 91% and was driven by CSF glucose

and CSF NfL (Appendix E, Figure S6.3 (i-ii)). Notably, serum lysine was lower in the older group ($p < 0.05$, Figure S6.3), but also remained lower in the age and BMI-matched FM group relative to those controls, indicating it may be involved in both disease and ageing. No other top metabolites were significantly different in younger vs older control groups (Figure S6.3) or in male vs female controls (Figure S6.4).

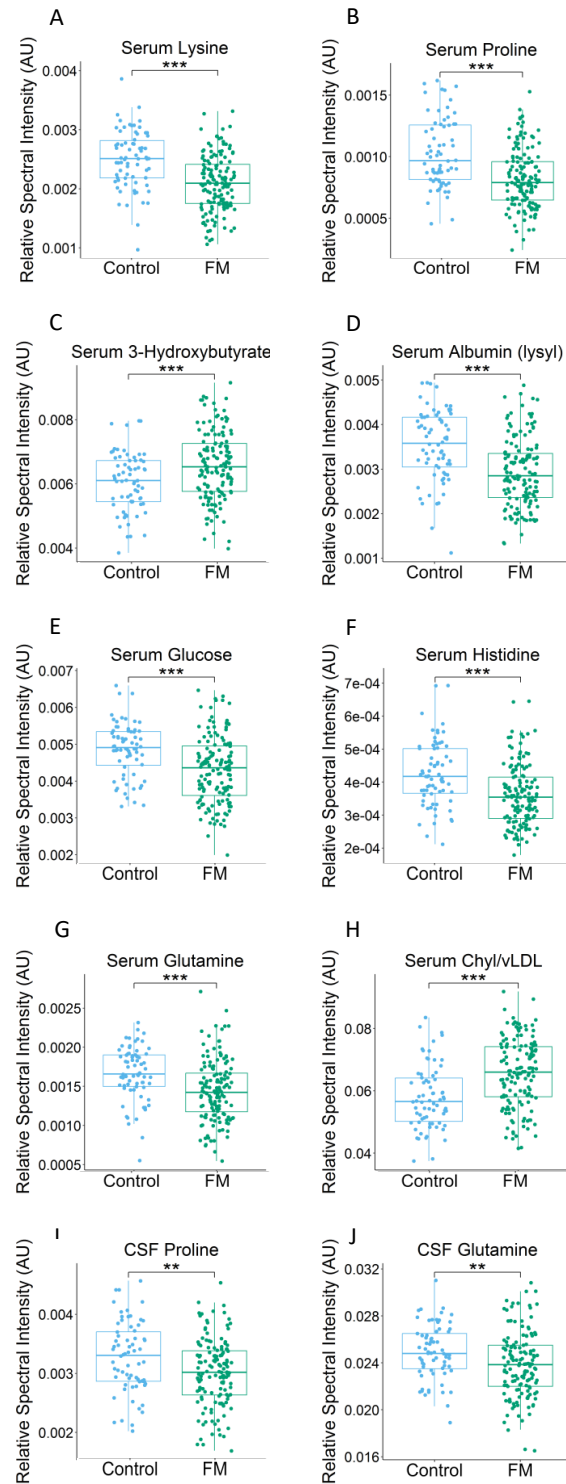


Figure 6.2: Univariate analysis of the top-ranked discriminatory metabolites in serum and CSF of individuals with fibromyalgia (FM) and pain-free controls. *Boxplots show the relative spectral intensity (arbitrary units, AU) for metabolites identified as top contributors to the Random Forest model based on Variable Importance (VIMP) score. Only metabolites that*

were statistically significant after correction for age, BMI, and sex are shown. Data presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

6.3.3. Distinct profiles of inflammation and neurodegeneration protein markers in fibromyalgia

After finding a difference between FM and pain-free metabolomes, we next sought to determine whether there were any differences in markers of inflammation and neuronal damage. First, to account for the known association between neurofilament light chain (NfL) levels and age, we employed exponential curve fitting on control data alone to obtain residuals that reflect age-independent NfL deviations (Figure 3A (i) for CSF and (ii) for serum). Using these age-adjusted NfL levels, no difference was found between NfL concentrations in either CSF (Figure 3A (i)) or serum (Figure 3A (ii)) in FM vs controls. However, a random forest classification model using all protein markers could detect FM over controls with an AUC of 63%. Again, this model performed significantly better than by random chance ($p < 0.0001$, Kolmogorov-Smirnov test), although it was not as robust as the metabolome alone. The top three ranked variables of importance were CSF CRP, plasma IL-1RA, and plasma CRP, all of which were higher in FM relative to the controls (Figure 3B (ii) $p < 0.05$, Figure 3B (iii) $p < 0.01$, Figure 3B (iv) $p < 0.01$). Importantly, CRP in the CSF remained significantly higher in the smaller subset of FM against age- and BMI-matched controls (Appendix 6, Figure S6.2 D-i), suggesting that neuroinflammation may play a pivotal role in pain amplification in FM, independent of peripheral inflammatory contributions. In contrast, both IL-1RA and CRP levels measured in the circulation were no longer significantly different after controlling for age and BMI (Appendix, Figure S6.2 Dii-iii). Notably, plasma CRP levels

were significantly higher in females compared to males within the control group, indicating potential sex-specific variations in baseline inflammatory status (Appendix E, Figure S6.4).

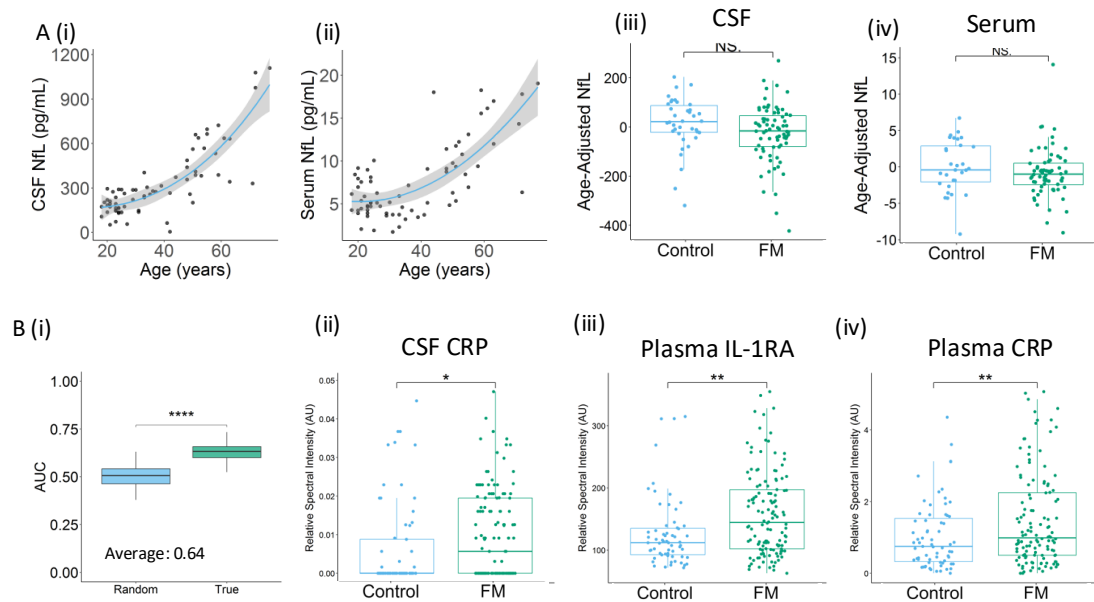


Figure 3: Serum and CSF markers of inflammation and neurodegeneration distinguish individuals with fibromyalgia (FM) from pain-free controls. A) Relationship between age and neurofilament light chain (NfL) concentration in (i) CSF and (ii) serum, modelled using an exponential growth function (blue line, 95% confidence interval shown in grey). (iii–iv) Age-adjusted NfL levels derived from the exponential models show no significant difference between FM and control groups in either (iii) CSF or (iv) serum. B) Random Forest classification model based on serum and CSF inflammatory markers, including age-adjusted NfL levels. (i) ROC AUC values comparing true versus random models. The average AUC of the true model was 0.64, significantly outperforming the random model (**** $p < 0.0001$). (ii–iv) Top variables contributing to model accuracy: (ii) CSF C-reactive protein (CRP), (iii) plasma interleukin-1 receptor antagonist (IL-1RA), and (iv) plasma CRP. p values are indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), NS = not significant.

6.3.4. Hierarchical clustering reveals two distinct FM subtypes with distinct sensory profiles

After identifying metabolic and inflammatory alterations in individuals with FM, we next investigated whether variation in pain processing might reflect biologically meaningful subgroups. Although reduced pain thresholds are commonly associated with FM, our data

revealed substantial inter-individual variability. While some FM participants exhibited the expected hyperalgesic profile, others had pain thresholds comparable to pain-free controls, suggesting the presence of distinct sensory subtypes within the FM population (Figure 6.4). To explore this heterogeneity, we performed hierarchical clustering on z-score normalised Quantitative Sensory Testing (QST) data. This analysis identified two distinct clusters within the FM cohort (Figure 4A–B). Cluster 1 comprised individuals with relatively preserved pain thresholds, similar to those of pain-free controls, whereas Cluster 2 showed marked hypersensitivity across pressure, heat, and mechanical stimuli (Figure 4E–J and Appendix E, Figure S6.5 for all variables). We next examined whether these sensory subtypes were associated with clinical or demographic differences. Cluster 2 contained a significantly higher proportion of individuals who met the 1990 FM diagnostic criteria, which include physical tender point examination (Fisher's exact test, $p = 0.0009$; Figure 4C). Additionally, although females were overrepresented in the FM cohort overall, female participants were disproportionately clustered in Cluster 2, the more pain-sensitive subgroup (Fisher's exact test, $p = 0.0025$; Figure 4D). In contrast, there were no significant differences in clinical pain measures – including the Numerical Rating Scale (NRS), Pain Disability Index (PDI), and Widespread Pain Index (WPI) – between the two clusters (Appendix 6, Figure S6.6). Similarly, while FM participants showed increased anxiety (GAD-7), sleep disturbance (ISI), depression, and functional impairment (FIQR) relative to controls, these psychosocial measures did not differ significantly between Cluster 1 and Cluster 2 (Appendix E, Figure S6.6). These findings highlight a dissociation between objective sensory sensitivity and self-reported clinical pain, emphasising the importance of stratifying FM patients beyond symptom severity alone.

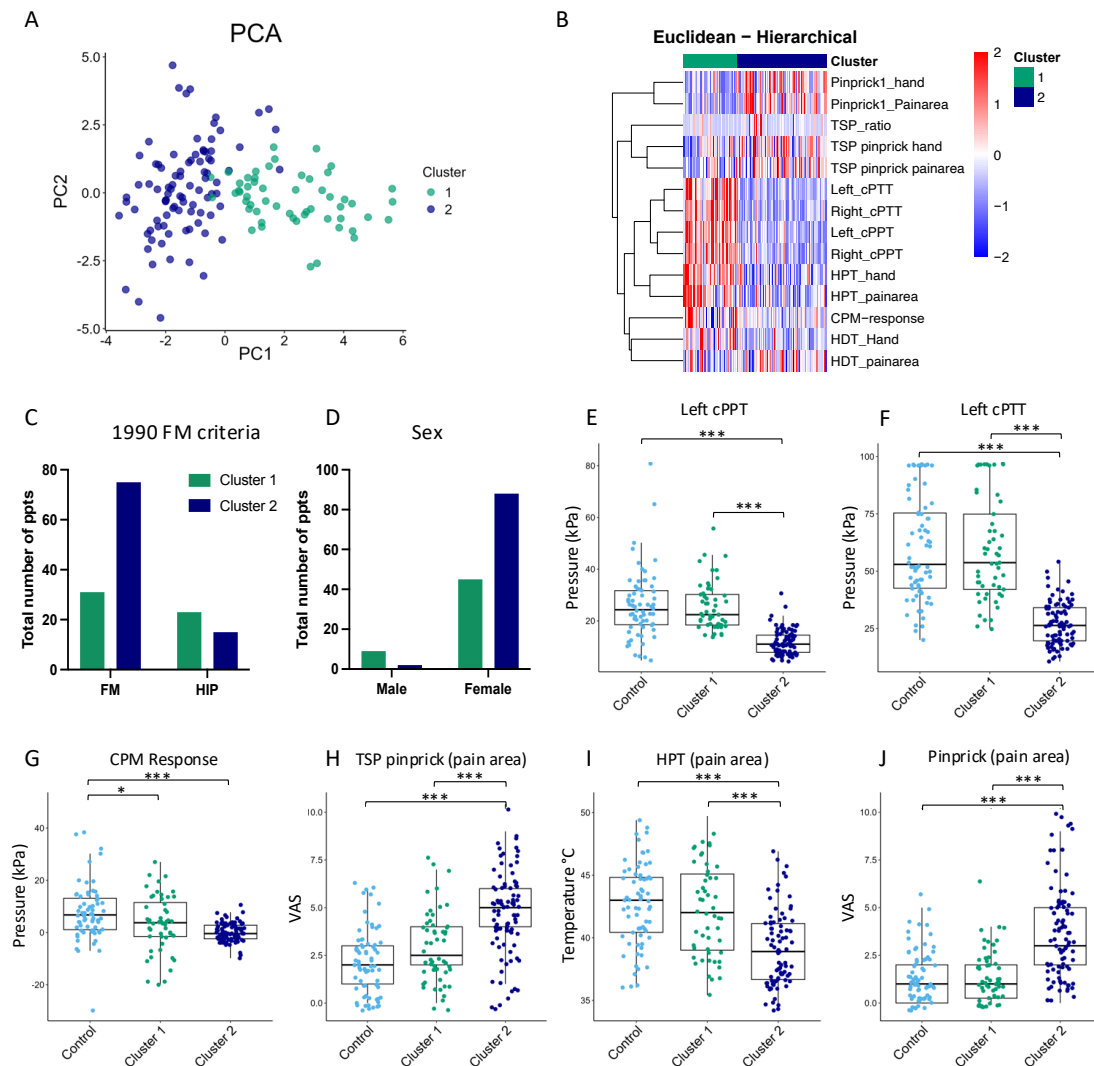


Figure 6.4. Cluster analysis of Quantitative Sensory Testing (QST) scores reveals subgroups within the fibromyalgia (FM) cohort. A) Principal component analysis (PCA) plot visualising participant separation based on QST measures after z-score normalisation. Two clusters were identified by hierarchical clustering (Euclidean distance, $k=2$). B) Heatmap of the standardised QST variables across participants, ordered by hierarchical clustering. C) Distribution of cluster membership according to 1990 FM classification criteria and high-impact pain (HIP). FM cases were overrepresented in Cluster 2 (Fisher's exact test, $p = 0.0009$). D) Distribution of sex across clusters, showing a significant enrichment of females in Cluster 2 (Fisher's exact test, $p = 0.0025$). E–J) One-way ANOVA comparing QST-derived metrics across control participants, Cluster 1, and Cluster 2. VAS: Visual Analogue Scale, cPPT: cuff pressure pain threshold, cPTT: cuff pressure pain tolerance, TSP (pinprick): temporal summation of pain, CPM response: conditioned pain modulation, HPT: heat pain threshold, HDT: heat detection threshold. Data shown as mean $\pm$ SEM, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

6.3.5. Pain sensitivity subtypes associate with distinct metabolic profiles

To determine whether variation in pain sensitivity among individuals with FM was underpinned by biological differences, we assessed whether the top metabolites distinguishing FM from controls were differentially expressed across the sensory subgroups. One-way ANOVAs revealed that participants in Cluster 2 – characterised by lower pain thresholds – exhibited more pronounced metabolic perturbations than those in Cluster 1 (Figure 5). Significant group differences were observed in a range of metabolites, including serum lysine, proline, albumin (lysyl moiety), glucose, histidine, glutamine, and chylomicron/VLDL (resonance at 0.87 ppm), as well as CSF proline. In several cases, notably serum glutamine and histidine, metabolite levels in Cluster 1 did not differ from pain-free controls, whereas Cluster 2 showed significant reductions (glutamine $p < 0.001$; histidine $p < 0.001$ vs. control). Furthermore, post hoc Tukey tests confirmed a significant decrease in histidine in Cluster 2 compared to Cluster 1 ($p < 0.05$), supporting the biological distinction between subgroups. Importantly, these differences were not attributable to age or body mass index (BMI), as there were no significant differences in these variables between the clusters (Appendix E, Figure S6.6). These results suggest that the sensory subtypes of FM are associated with distinct peripheral and central metabolic profiles, further reinforcing the biological validity of the identified subgroups.

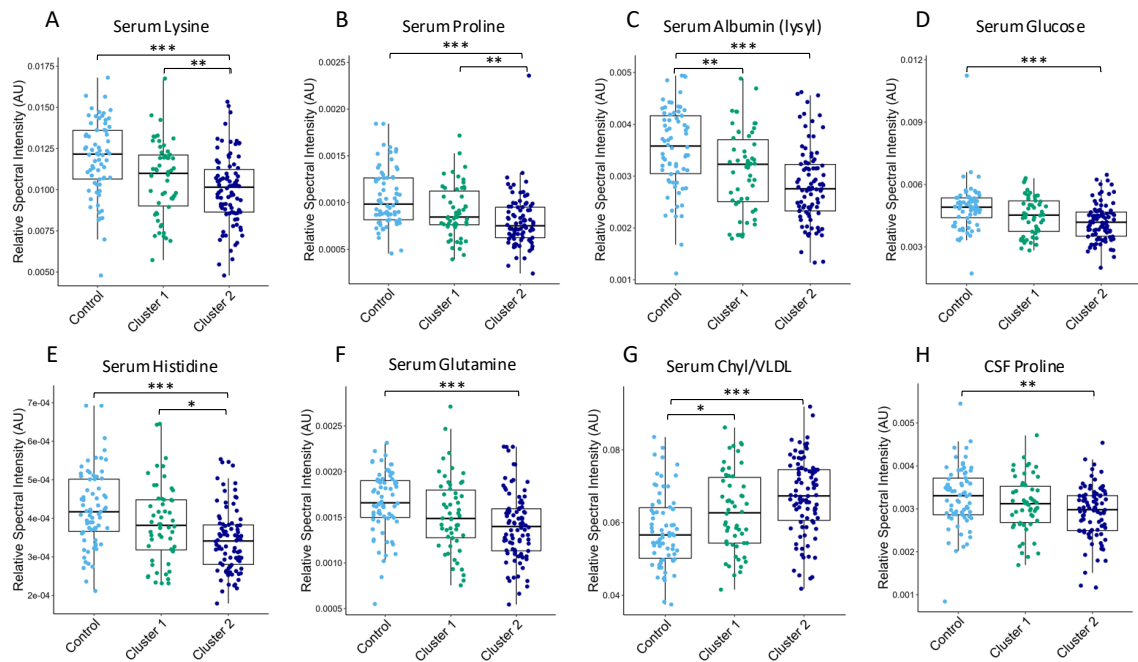


Figure 6.5: Metabolic profiling distinguishes between sensory clusters identified in fibromyalgia (FM). Boxplots show the relative spectral intensity (arbitrary units, AU) for the top discriminatory serum and CSF metabolites identified through Random Forest variable importance analysis, now stratified by QST-derived cluster (Cluster 1 and Cluster 2) alongside pain-free controls. Data presented as mean $\pm$ SEM, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

6.4. Discussion

Our results support previous findings of peripheral metabolic abnormalities in patients with fibromyalgia, including altered amino acid metabolism, energy metabolism, and lipid dysregulation. Further to this, we now show these alterations extend to central metabolism, demonstrating deficits in glutamine and proline metabolites. While previous studies have investigated the serum metabolome, to our knowledge, this is the first metabolomic analysis of CSF in FM. By combining both serum and CSF metabolites with CSF and blood markers of inflammation, we present a more comprehensive picture of FM pathophysiology. Most strikingly, we demonstrate that these metabolic and inflammatory profiles are associated

with differences in pain thresholds. These findings support the concept that FM comprises multiple disease subtypes, identifying distinct biological underpinnings associated with different clinical presentations, and support the use of QST in the clinical setting.

6.4.1. Peripheral and central metabolic alterations in fibromyalgia

Previous metabolic studies, albeit sparse, have reported disruptions in amino acid metabolism, including alterations in circulating lysine, proline, glutamine and histidine, as observed here (Russell et al. 1989). These changes have been suggested to reflect a state of increased oxidative stress, mitochondrial dysfunction, or compensatory metabolic shifts aimed at sustaining energy production in a state of chronic stress (Menzies et al. 2020). Studies have also demonstrated altered lipid metabolism, in line with the changes in the Chylomicrons and VLDL signals observed here (Menzies et al. 2020). While not specifically in FM, a potential link between serum 3-hydroxybutyrate (3-HB) and chronic pain has been identified (Tamaki, Saito, and Tomita 2023). As a ketone body, 3-HB serves as an alternative energy source when glucose availability is low, which we observe in these participants (Mierziak, Burgberger, and Wojtasik 2021). The increase in 3-HB alongside decreased glucose suggests an enhanced reliance on fatty acid oxidation, possibly reflecting adaptive metabolic reprogramming due to mitochondrial inefficiency. This energy-deficient state may contribute to fatigue, cognitive dysfunction, and low mood and is consistent with inflammatory metabolic signatures reported in other conditions as well as a direct result of immune-challenges in animal models (Dunstan et al. 2024).

The observed decrease in serum histidine is notable, given its anti-inflammatory properties and role as a histamine precursor, potentially impacting immune regulation and neurotransmission. Indeed, histidine supplementation has been associated with decreases in BMI, proinflammatory cytokines, and markers of oxidative stress, which have been linked to improved cognitive function (Thalacker-Mercer and Gheller 2020). The reductions in both proline and glutamine in the serum suggest possible glutamatergic dysfunction, as glutamine serves as a critical precursor for glutamate and GABA, while proline is derived from glutamate. This imbalance may impair excitatory-inhibitory regulation in the nervous system and contribute to peripheral nociceptor sensitisation, amplifying pain. While glutamine can cross the blood brain barrier, brain glutamine is predominantly synthesised locally by astrocytes (Castegna and Menga 2018). Indeed, we observed a weak positive correlation between serum and CSF glutamine levels and a strong positive correlation between peripheral and central levels of proline (Figure S6.7), indicating a degree of peripheral-central metabolic interplay. Notably, both proline and glutamine were among the top CSF metabolites distinguishing FM, reinforcing the presence of central metabolic perturbations. The decrease in CSF glutamine suggests local dysfunction that may exacerbate glutamatergic dysregulation, contributing to central sensitisation and heightened pain responses (Chiang et al. 2008). These findings highlight that metabolic dysregulation in FM extends across both peripheral and central compartments, supporting its classification as a disorder with integrated systemic and CNS involvement.

6.4.2. Neuroinflammation in fibromyalgia

Our findings also highlight the presence of central inflammation in FM. While several inflammatory markers were increased in serum, the most robust and consistent finding was elevated CRP in the CSF, which remained significant after adjusting for age and BMI. Chronic low-grade neuroinflammation has been proposed as a driver of central sensitisation, and elevated CSF CRP may contribute to increased glial activation, nociceptive hypersensitivity, and impaired descending pain modulation (Ji et al. 2018). The association between neuroinflammation and pain in FM has been supported by previous studies demonstrating increased CSF levels of IL-8, CXCL-10, and IL-18 among others, as well as neuroimaging studies showing microglial activation in pain-processing regions of the brain (Kadetoff et al. 2012; Backryd, Tanum, et al. 2017; Albrecht et al. 2019). Importantly, we did not observe any significant differences in NfL levels in either the serum or CSF after adjusting for age. This suggests that while inflammatory processes may contribute to pain amplification and central sensitisation, they are not sufficient to cause measurable neuronal damage. The absence of elevated NfL reinforces the idea that FM is not a neurodegenerative disorder but rather a condition characterised by functional dysregulation in pain processing pathways.

6.4.3. Lower nociceptive thresholds in fibromyalgia

Our study corroborates findings that FM patients exhibit significantly lower pain thresholds across multiple sensory modalities, including pressure, thermal, and pinprick pain. It is possible that this heightened pain sensitivity is a result of increased abundance of inflammatory mediators that lower the activation threshold of nociceptors and hence, contribute to a sustained pain state (Bains et al. 2023). While initially demonstrating a significant increase in plasma CRP and IL-1RA, these differences did not appear in the smaller

age and BMI matched subset. However, an interesting finding was the increased baseline level of CRP in females, potentially contributing to the susceptibility profile of these individuals. The metabolic aberrations identified also withheld throughout the matching process, despite the smaller sample size, and warrant further investigation into potential nociceptive sensitisation in FM. Although less pronounced, conditioned pain modulation (CPM) was reduced in FM patients compared to controls, indicating an imbalance between descending pain inhibition and facilitation. To further assess pain processing, we examined temporal summation of pain (TSP), a measure of spinal-level pain facilitation. FM participants exhibited an increased TSP ratio, indicating exaggerated wind-up – a phenomenon where repeated noxious stimuli elicit a progressively stronger pain response (Hachisuka et al. 2018). This heightened facilitation may be driven by dysregulation of the glutamine-glutamate cycle within the central nervous system, along with neuroinflammation, as suggested by elevated CSF CRP levels. However, the causes of these perturbations remain unclear.

6.4.4. Pain thresholds are not reflected in clinical pain or functional measures, but are associated with female sex and tender points

Increased sensitivity to pain is often linked to heightened anxiety (Robin et al. 1987). The emotional modulation of pain is a well-documented, with negative emotional states amplifying pain perception and positive states ameliorating it. Indeed, at the group level, the FM cohort exhibited higher levels of anxiety, depression, and sleep deprivation, along with greater levels of pain intensity. However, when the cohort was stratified into two clusters using unsupervised clustering based on QST outcome measures (as a proxy for pain thresholds), there were no significant differences in these clinical scores between individuals with high and low pain thresholds. Interestingly, there were also no differences in clinical

measures of pain intensity. This suggests that while emotional factors may be associated with chronic pain – likely in a bidirectional manner – they do not account for variations in pain thresholds. Conversely, the greater proportion of females in cluster 2 (low pain thresholds) suggests that biological sex influences pain sensitivity. The higher prevalence of females among FM patients has been attributed to emotional factors (Robin et al. 1987), but our findings challenge this assumption, as there were no differences across mood scores. While more females were present in the low-threshold group, the biological underpinnings remain unclear. Indeed, it is important to note that sex was not the primary driver of metabolite differences across the pain threshold clusters, as there was no significant difference between males and females in these metabolites.

6.4.5. Metabolic associations with pain thresholds

Given the variability in QST scores between the two FM clusters, and the absence of corresponding differences in emotional factors, we focused on exploring potential biological mechanisms. Serum and CSF metabolites that distinguished FM from controls, including glutamine, histidine, proline, and lysine, were more perturbed in patients with lower pain thresholds. The most striking differences were observed in serum glutamine, histidine, and CSF proline, where cluster 2 (lower pain threshold) exhibited significantly greater reductions in all compared to cluster 1. These findings suggest that metabolic dysfunction may underlie heightened pain sensitivity in FM, potentially through alterations in neurotransmitter availability, oxidative stress, or mitochondrial function. Interestingly, CSF glutamine was not different between the clusters, potentially reflecting the more profound effect on peripheral sensitisation. The variability across QST has caused uncertainty over the clinical utility of QST in FM. However, our results suggest that QST identifies biologically distinct subgroups,

reinforcing its value as a meaningful measure of pain processing dysfunction. Further research is needed to validate these subtypes and determine whether they predict treatment responses or long-term outcomes. If confirmed, this stratification approach could pave the way for more personalised FM management strategies, shifting away from a one-size-fits-all approach toward targeted interventions based on underlying pathophysiological mechanisms.

6.5. Conclusion

Our findings suggest that glutamine contributes to both peripheral and central mechanisms of pain amplification, supporting its potential as a metabolic target in chronic pain syndromes such as fibromyalgia. The distinct nociceptive threshold clusters observed, with one group closely resembling controls, indicate biologically meaningful subtypes that could warrant recognition as distinct clinical entities. This stratification may enable more targeted therapeutic strategies, provided these subgroups reflect distinct pathophysiological mechanisms. Importantly, QST was conducted under controlled conditions, minimising inter-examiner variability, though its broader clinical utility remains uncertain due to potential site-specific differences. Metabolomic insights further highlight FM's complexity, potentially linking metabolic alterations to cognitive and fatigue-related symptoms. Future longitudinal studies are needed to validate these subtypes and assess their relevance for personalised treatment approaches.

Chapter 7: Osteoarthritis pain inversely correlates with histidine and glutamine following CSF and serum profiling

Chapter Summary

Building on prior chapters that identified metabolic and inflammatory subtypes in long COVID and fibromyalgia, this chapter focuses on osteoarthritis (OA), a leading cause of chronic pain and disability worldwide. Despite its prevalence, OA remains a condition with limited objective biomarkers for pain severity or progression. In this study, we applied ^1H NMR-based metabolomics alongside targeted protein analysis to cerebrospinal fluid (CSF) and serum samples from individuals with OA and pain-free controls, with the aim of identifying metabolic signatures linked to pain and disability. By examining both central and peripheral compartments, this work sought to uncover disease-relevant pathways and assess their potential clinical utility.

Multivariate analyses revealed clear distinctions between OA and control participants, with Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) models achieving high diagnostic accuracies in both CSF (89%) and serum (87%). Notably, reductions in serum histidine and glutamine emerged as robust discriminators of OA status and were inversely correlated with clinical indices of pain, fatigue, and sleep disturbance, including the Numeric Rating Scale (NRS), Insomnia Severity Index (ISI), and Symptom Impact Questionnaire (SIQR). In the CSF, elevated lactate and glutamate alongside reduced glucose and glutamine levels were consistent with altered energy metabolism and central sensitisation mechanisms. These patterns suggest a convergence of peripheral and central metabolic dysfunction in OA.

The diagnostic performance of metabolomics was further enhanced by integrating serum and CSF data with targeted protein measures, achieving a combined classification accuracy of 90%, which remained robust even after matching for age and BMI. Subgroup analyses confirmed that histidine and glutamine reductions were consistent across pain severity strata, and female participants demonstrated more pronounced metabolic alterations, aligning with their higher reported symptom burden. These findings support the hypothesis that sex-specific metabolic adaptations may contribute to OA pathophysiology and pain perception.

Overall, this chapter demonstrates the value of metabolomics in delineating the biochemical basis of OA-associated pain and functional impairment. The consistent involvement of histidine and glutamine across central and peripheral compartments mirrors patterns observed in fibromyalgia and inflammatory subtypes of long COVID, reinforcing the notion of shared metabolic vulnerabilities across chronic pain conditions. These results provide a compelling rationale for future investigations into histidine supplementation or metabolic modulation as a strategy for OA pain management and further strengthen the case for precision medicine approaches in musculoskeletal disease.

7.1. Introduction

Osteoarthritis (OA) is a complex joint disease characterized by cartilage degradation, synovial inflammation, and subchondral bone changes (Goldring and Goldring 2007; Kim, Yoo, and Kim 2018; Wei and Bai 2016). It is the most prevalent joint disorder in older adults and a major cause of disability (Kim, Yoo, and Kim 2018; Li et al. 2013). OA pathogenesis involves multiple factors, including mechanical influences, aging, and genetic predisposition (Goldring and Goldring 2007). Synovial inflammation and subchondral bone alterations are increasingly

recognised as key contributors to OA onset and progression (Hügle and Geurts 2016; Mathiessen and Conaghan 2017). These changes can precede visible cartilage degeneration and are associated with pain and functional impairment (Mathiessen and Conaghan 2017). The subchondral bone undergoes uncoupled remodelling, leading to osteosclerosis in advanced stages (Hügle and Geurts 2016). Current treatments are primarily palliative, and there is a pressing need for disease-modifying therapies targeting the underlying molecular mechanisms of OA (Martel-Pelletier 1998; Poulet and Staines 2016). Current treatments aim to relieve symptoms and improve joint function, but no curative therapy exists (Altman 1987). Emerging strategies targeting cytokine inhibition and signalling pathways offer potential avenues for disease modification (Malemud, Islam, and Haqqi 2003), but personalised approaches to prevention and treatment, tailoring interventions to distinct OA phenotypes are likely to be necessary (Mobasheri and Batt 2016).

Biomarkers have emerged as a critical focus in OA research (Attur et al. 2013). Recent studies have focused on the role of biomarkers in assessing OA pain severity and subtypes. While serum and synovial fluid biomarkers, such as extracellular matrix molecules, have been extensively studied, the potential of cerebrospinal fluid (CSF) biomarkers is an emerging area of interest (Kalogera et al. 2023; Mobasheri 2012). CSF biomarkers are increasingly recognised as valuable tools in osteoarthritis (OA) research, despite the primary pathology occurring in joints. CSF biomarkers offer unique insights into central nervous system involvement in OA pain. Indeed, studies have identified elevated levels of inflammatory proteins in CSF of OA patients, indicating neuroinflammation (Kosek et al. 2018a; Palada et al. 2020). Interestingly, some CSF biomarkers, particularly those with neuroprotective effects, are associated with reduced pain intensity and milder symptoms (Palada et al. 2020). Specific proteins like

monocyte chemoattractant protein 1 (MCP1) and interleukin (IL)-8 have been found elevated in CSF, suggesting their role in neuroimmune signalling (Kosek et al. 2018a). However, hitherto there has been no investigation of the metabolome profile of CSF from individuals with OA.

Sex differences are a recognised feature of OA, with women experiencing higher prevalence, pain intensity, and poorer clinical outcomes compared to men. Women account for 60% of OA cases globally (Segal, Nilges, and Oo 2024) and report more severe pain, functional limitations, and poorer surgical outcomes (Glass et al. 2014; Segal, Nilges, and Oo 2024). These differences are influenced by hormonal factors, joint anatomy, central pain processing, and psychosocial aspects (Perruccio et al. 2019; Sluka et al. 2012). The interplay between inflammatory markers and pain also varies by sex, suggesting distinct biological mechanisms underlying OA progression (Perruccio et al. 2019). Addressing these disparities is crucial for tailoring interventions and improving treatment outcomes across sexes (Tschon et al. 2021; Valdrighi et al. 2022).

Here, we sought to investigate the relationship between serum and CSF metabolites and a panel of proteins, including markers of neurodegeneration and cytokines, to characterise OA pathology and its association with pain and disease severity. Metabolomics provides a comprehensive view of metabolic changes in OA, revealing alterations in amino acid, lipid, and energy metabolism (Adams, Setton, and Nettles 2012; Zhai, Randell, and Rahman 2018). Nuclear magnetic resonance (NMR) spectroscopy of biofluids, particularly synovial fluid, has shown promise in identifying OA biomarkers (Clarke, Anderson, and Peffers 2020). Thus, we

chose to integrate metabolomics and protein data from blood and CSF samples to perform a multiomics analysis to improve our understanding of OA pathogenesis, potentially improving early diagnosis, prognosis, and treatment (Ratneswaran, Rockel, and Kapoor 2020). Our study also explored sex differences in metabolic and inflammatory profiles, aiming to identify biomarkers with potential utility in personalised OA diagnosis and treatment.

7.2. Materials and methods

7.2.1. Participants

Participants aged 18–80 years who were able to understand and respond to the questionnaires were recruited as part of The Danish Pain Research Biobank (DANPAIN Biobank), as previously described (Blichfeldt-Eckhardt et al. 2023). For the current study, samples were included from two groups: individuals with osteoarthritic pain scheduled for hip or knee arthroplasty surgery at a regional hospital (n=81), and pain-free volunteers recruited locally (n=70). Detailed recruitment procedures and cohort characteristics have been described previously (Blichfeldt-Eckhardt et al. 2023).

7.2.2. Sample Collection

See Chapter 6 for details. Briefly, serum and CSF samples were obtained following standard protocols. All samples were centrifuged at 2,000 G for 10 minutes at 4°C, aliquoted, and stored at –80°C.

7.2.3. Clinical and Pain Assessments

Functional and psychological impacts were assessed using validated instruments, including the Symptom Impact Questionnaire (SIQR) (Friend and Bennett 2015), Major Depression Inventory (MDI), Generalized Anxiety Disorder (GAD-7), Insomnia Severity Index (ISI),

EuroQol visual analogue scale (EQ VAS), and the Oxford Hip or Knee score (OHS/OKS) (Harris et al. 2013). Pain intensity and interference were quantified using a Numeric Rating Scale (NRS) (Ornetti et al. 2011) and Pain Disability Index (PDI) (Tait et al. 1987), respectively. Further details can be found in previously published work and in the previous chapter (Blichfeldt-Eckhardt et al. 2023).

7.2.4. Analysis of neuroimmune biomarkers

The measurement of neuroimmune biomarkers in CSF and plasma/serum was performed as previously described (see Chapter 6.2.5 for full methodological details). Briefly, cytokines and soluble receptors including TNF, LT α , IL-8, IL-6, IL-1 α , IL-1 β , IL-1Ra, IL-6R, gp130, TNFR1, and TNFR2 were quantified using multiplex assays (Mesoscale Discovery) (Martin et al. 2016). hsCRP was measured using a high-sensitivity ELISA (TECAN, IBL International GmbH) with a detection limit of 0.02 mg/L. Serum suPAR was assessed using the suPARnostic® AUTO Flex ELISA (ViroGates A/S), with a detection limit of 0.4 μ g/L. Neurofilament light (NfL) and glial fibrillary acidic protein (GFAP) in CSF and serum were measured using a Simoa® HD-X Analyzer (Quanterix), blinded to clinical data (Nielsen et al. 2020). Detection and quantification limits, as well as quality control procedures and performance metrics, were as detailed in Chapter 6.2.5.

7.2.5. Sample Processing and 1H NMR data collection

Sample preparation and spectral acquisition were performed exactly as described in Chapter 6.2.6. Serum and CSF samples were prepared and analysed in a single batch alongside those from the fibromyalgia cohort, using identical protocols, instrumentation, and quality control measures.

7.2.6. ¹H NMR Data Processing

Topspin 4.1.4 (Bruker) was used to phase, baseline correct, and reference all spectra as described in Chapter 6.2.7. ACD/Labs Spectrus Processor Academic Edition 12.01 (Advanced Chemistry Development, Inc.) was used to manually bin each resonance signal, and the resulting integrals of these bins were sum normalised and exported to R version 4.3.0 (R_Core_Team 2024). Metabolites assignment was conducted as previously described (Chapter 6.2.7.).

7.2.7. Multivariate Statistical Analysis

Multivariate analyses were performed to identify key metabolite differences between groups, with rigorous cross-validation to ensure robustness. Specifically, the ropls package in R (Thevenot et al. 2015) was used to conduct orthogonal partial least squares discriminant analysis (OPLS-DA) between pain-free and OA predictor variables (i.e. all serum and CSF metabolites and proteins from the targeted panels, z-scaled). Models were validated through external cross-validation, whereby the data was separated into train (90%) and test sets (10%), 10 times for every 100 external fold, producing 1000 models in total. The training data was used to learn discriminatory patterns in the predictor variables, whereas the test set was used for assessing model prediction parameters (accuracy, sensitivity, and specificity). Significant separation between OA and pain-free samples was determined by comparing to a null distribution, where classes were shuffled randomly to achieve the same sized dataset for direct comparison. The key variables for distinguishing between classes were identified using Variable Importance in Projection (VIP) scores and were investigated using univariate analysis as described below.

7.2.8. Univariate Statistical Analysis

Univariate analyses such as Student's T test and two-way ANOVA were carried out in R (v 4.3.0) and visualised using ggplot2. The significance threshold was set to $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ and all results were presented as mean $\pm$ standard error of the mean (SEM). All correlations were tested via Pearson correlational analyses, using the Benjamini-Hochberg method to correct for false discovery rate at 0.05. Univariate Receiver Operating Characteristic (ROC) analyses and multivariate ROC analyses on a combination of features using logistic regression were determined using the pROC package in R.

7.3.0. Results

7.3.1. Distinct Serum and CSF Metabolite Profiles Differentiate Osteoarthritis from Controls.

We initially sought to discover whether OPLS-DA models could be built using the NMR spectra alone to discriminate OA from pain-free individuals using serum or CSF. As previous studies have shown metabolic aberrations in serum and synovial fluid from OA patients (McNearney et al. 2000; Farah et al. 2022; Mehta et al. 2023), we expected to see a metabolic signature of OA serum. While cytokine alterations in OA CSF have been demonstrated (Kosek et al. 2018b), no previous metabolomics analysis of OA CSF has been conducted. Here, metabolomic analysis of both serum and CSF revealed a clear separation between OA and the pain-free control group. OPLS-DA models demonstrated robust discrimination, with diagnostic accuracies of $87\% \pm 1.9\%$ for serum and $89\% \pm 1.9\%$ for CSF profiles (Figure 7.1A (i) and 7.1B (i), respectively) and performed significantly better than random chance (serum random: $50.9\% \pm 4.4\%$ accuracy, $p < 0.001$; CSF random: $50.4\% \pm 5.2\%$, $p < 0.001$, Kolmogorov-Smirnov test, Figure S7.1). Key metabolites driving this separation included increases in very-low-density lipoprotein (mobile -CH₃ in chylomicron/VLDL) and 3-hydroxybutyrate, alongside

decreases in lysine, alanine, and glutamine concentrations in serum (Figure 7.1A (iii-vii)). In CSF, lactate and glutamate were elevated, whereas glutamine and glucose were reduced (Figure 7.1B (iii-vi)). These findings suggest distinct metabolic alterations in both systemic and, more surprisingly, central compartments in OA.

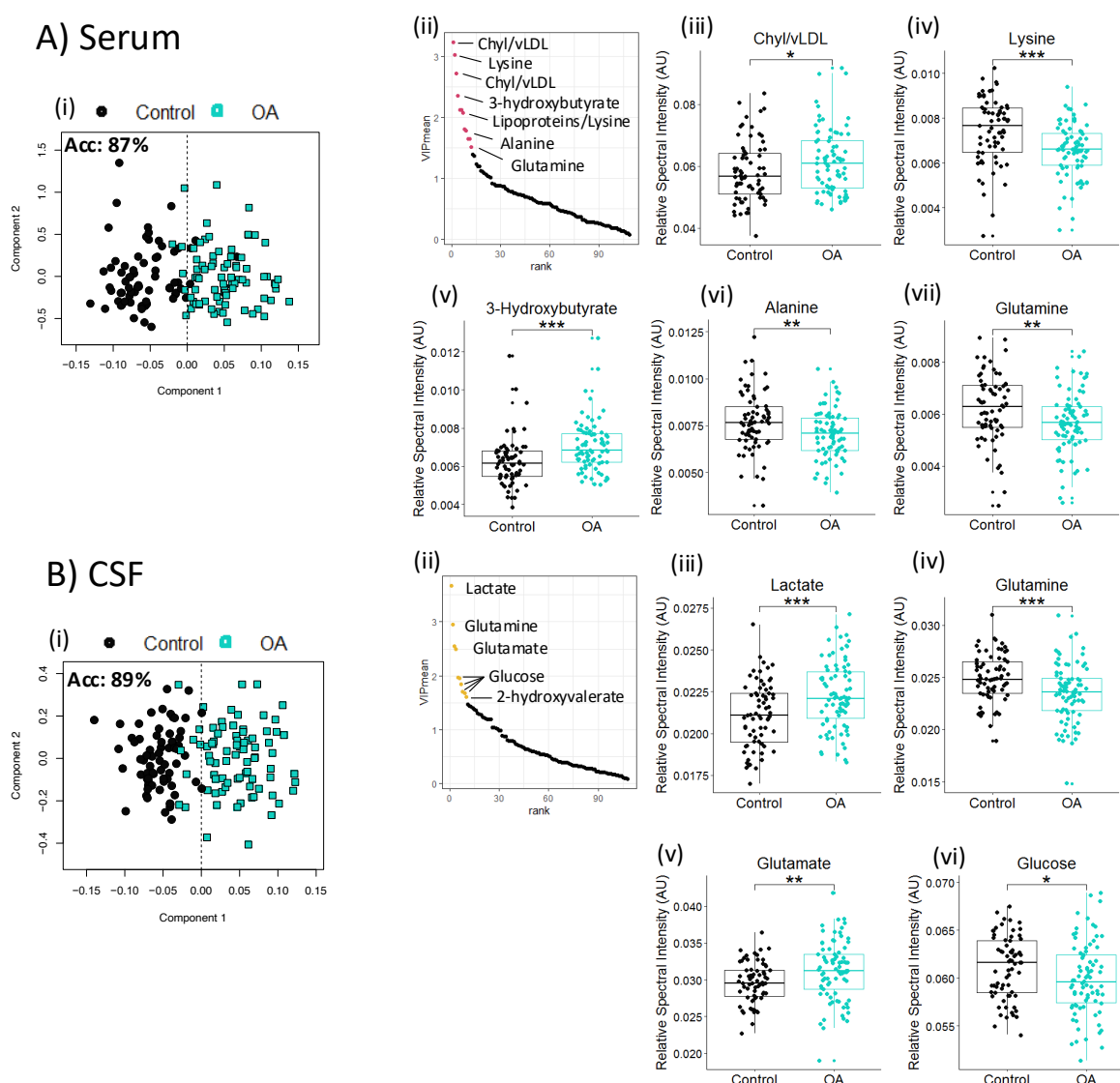


Figure 7.1: Distinct serum and CSF metabolite profiles separate osteoarthritis from control.
A (i) OPLS-DA scores plots showing discrimination between osteoarthritis and control serum metabolite profiles. The average accuracy (Acc) from the ensemble of OPLS-DA models (10-fold external cross-validation strategy with 100 repetitions) is shown. (ii) Variable importance in projection (VIP) plot with metabolites scoring 1.5 and above in colour. These metabolites

were selected for univariate analysis as shown in (iii-iv), displayed descending order. B (i) OPL-DA scores plot illustrating separation between control and osteoarthritis CSF metabolite profiles. (ii) VIP score plot with top VIPs coloured. (iii-vii) Metabolites with the top VIP scores, in order of descending score. Data presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Note that in serum the VIPs are increases in VLDL, 3-hydroxybuterate, and a decrease in the concentration of lysine, alanine, and glutamine. For CSF lactate and glutamate are increased, but glutamine and glucose are decreased. Surprisingly, the diagnostic accuracies of the model were higher of CSF (89%) compared to (87%) for serum.

7.3.2. Combined Serum and CSF Metabolite Analysis with Target Protein Panel Enhances Diagnostic Accuracy

Integrating serum and CSF metabolite data with a targeted protein panel, including cytokines and neurodegeneration markers, increased the model's diagnostic accuracy to $90\% \pm 2.2\%$ (Figure 7.2A). After finding these metabolic and inflammatory differences in OA biofluids, we wanted to ensure these were a result of disease and not confounding factors such as differences in age, sex, or BMI between OA and pain-free control groups. There was no significant difference in sex between the groups (Blichfeldt-Eckhardt et al. 2023); however, both age and BMI were significantly increased in the OA group (Blichfeldt-Eckhardt et al. 2023). This combined metabolic and targeted protein approach maintained high discriminatory power even after matching participants for age and BMI, although accuracy was slightly reduced in this smaller subset (Figure 7.2B). Notable metabolites that remained significantly altered in both full and matched cohorts included decreases in serum histidine and glutamine (Figure 7.2C). To validate the importance of these variables in discriminating between OA and pain-free controls, a multivariate ROC analysis coupled with logistic regression was performed on only the variables that remained significantly altered after matching for age and BMI. This gave an area under the curve (AUC) of 0.906 (91%) (Figure 7.2D). These results highlight the

potential of a combined metabolite and protein profiling approach for more accurate OA diagnosis.

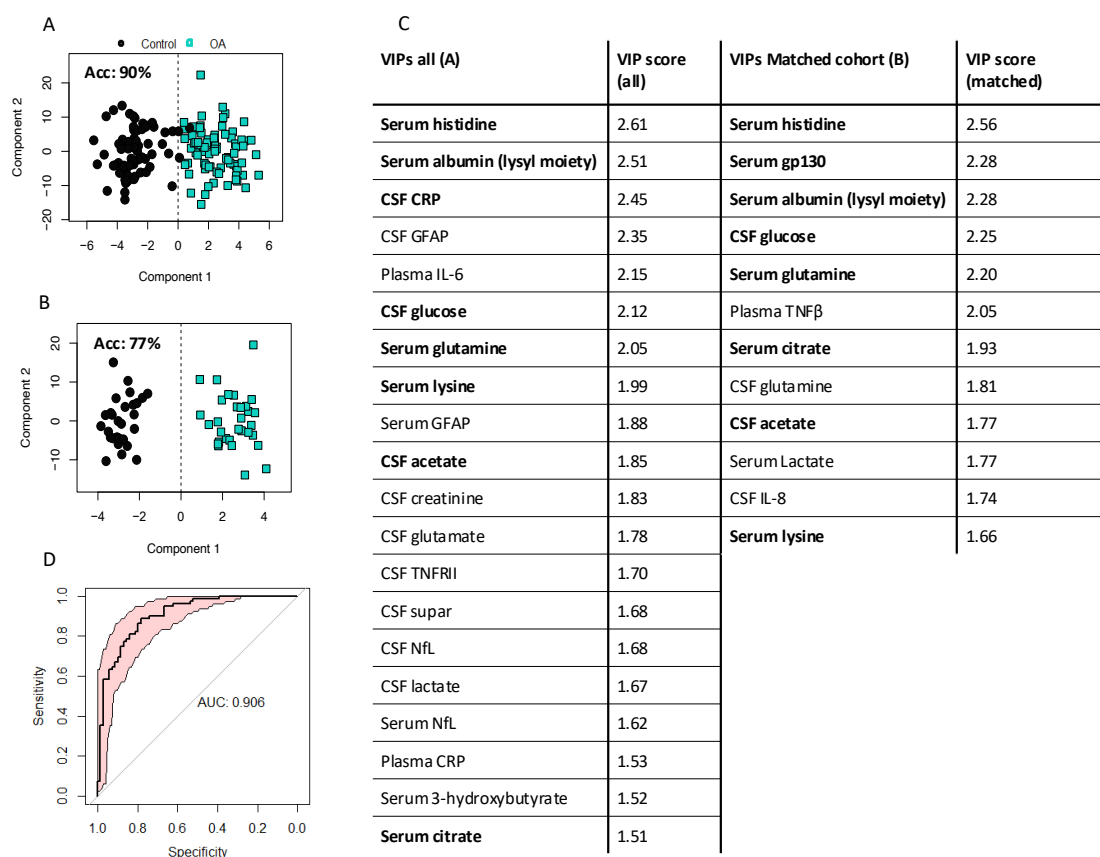


Figure 7.2: OPLS-DA of combined serum and CSF metabolites and the target protein panel increases the model accuracy. A) OPLS-DA scores plot shows z-scaled CSF and serum metabolites and target proteins distinguish between osteoarthritis (OA) and control. B) OPLS-DA scores plot shows separation between OA and control is retained in a subset of participants matched for age and BMI. C) Metabolites with VIP scores above 1.5 in the full model and in the age/BMI-matched model. **Emboldened** metabolites signify the metabolites that remained significantly altered in both the full and matched cohorts even though they may not have contributed to the matched model. D) ROC curve of the VIPs that remained significantly altered in OA vs control after matching for age and BMI. It is of note that the combination of a target protein panel including cytokines and markers of neurodegeneration increased the accuracy of diagnosis to 90% when the values for both serum and CSF were combined. Matching for Age and BMI reduced the accuracy of the models (though the ROC AUC was till

90%). The most notable VIP in the matched cohort, that were conserved in the unmatched, were decreases in serum histidine and serum glutamine.

7.3.3. Key Metabolites and Proteins in OA After Adjusting for Age and BMI

To better understand the relationship between the key variables distinguishing between OA and pain-free controls with the disease state, we performed univariate analysis (two-tailed T tests) on those metabolites and proteins that remained significantly altered in OA after controlling for age and BMI. Among these, serum histidine and glutamine levels were consistently decreased (Figure 7.3A and 7.3C). Additionally, CRP levels were markedly elevated in the CSF (Figure 7.3H), while acetate concentrations were reduced in CSF (Figure 7.3G). Statistical results of these metabolites in the smaller matched subset are shown in the Supplementary Figure 7.S2. These findings reinforce the role of systemic and central metabolic disruptions in OA pathology.

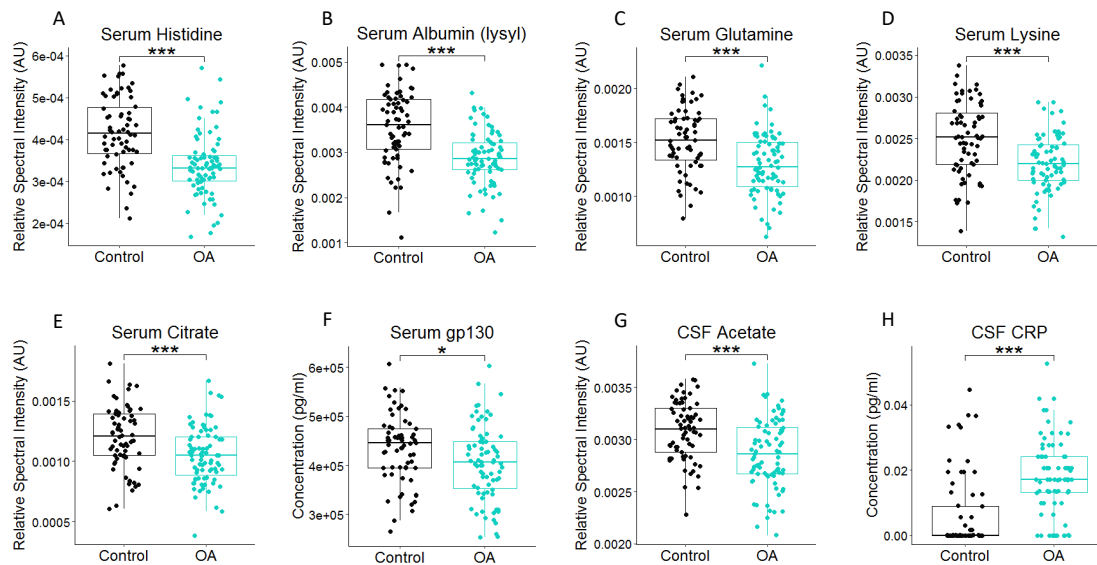


Figure 7.3: Univariate analysis of the VIPs that remained significantly altered after matching for age and BMI. Data presented as mean $\pm$ SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Note that among the serum metabolites that were altered in the matched cohort, CSF CRP was markedly elevated in the CSF and acetate was decreased.

7.3.4. Correlations Between Metabolic Changes, Cytokines, and Clinical Measures

Next, we sought to characterise the interplay between the key differentiating metabolite and inflammatory protein markers identified. Pearson correlation analysis revealed significant associations between key metabolites, cytokines, and clinical outcomes in OA (Figure 7.4). In particular, serum histidine, albumin (resonance lysyl moiety), and glutamine levels were inversely correlated with several clinical scores, including the Symptom Impact Questionnaire (SIQR), Widespread Pain Index (WPI), and Insomnia Severity Index (ISI). These correlations suggest that specific metabolic and inflammatory alterations are linked to symptom severity and functional impairment in OA. Moreover, many of the key metabolites, including peripheral metabolites, were significantly correlated with cytokine levels and markers of neuronal damage (Supplementary figure S7.5).

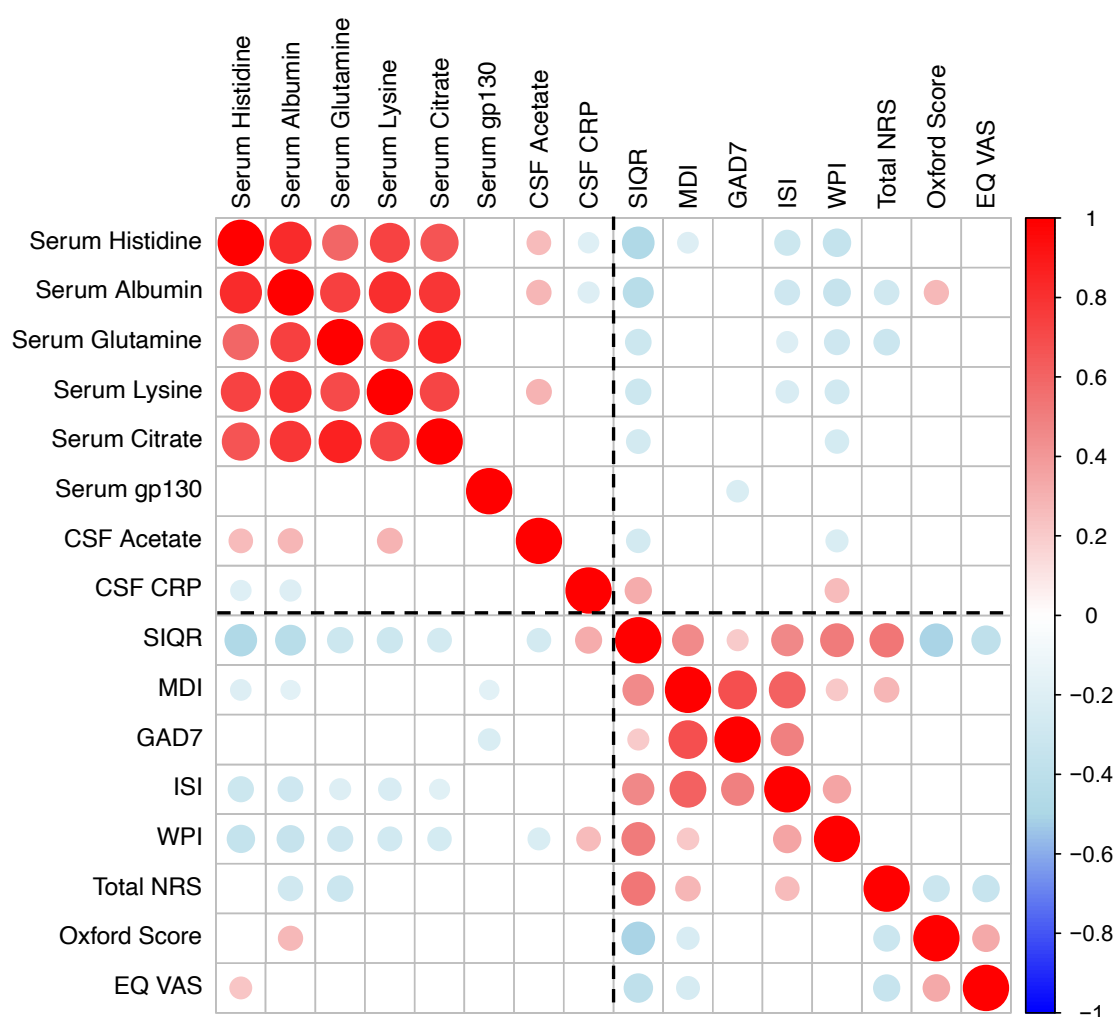


Figure 7.4: Correlation plot of the metabolites and cytokines with the top VIP scores (most responsible for the separation between osteoarthritis and control in the OPLS-DA model). The colour scale of the circles corresponds to the Pearson Correlation Coefficient (R value) as indicated in the bar on the right. The size of circles is proportional to the p value, with larger circles indicating greater significance. Dotted lines separate the VIP metabolites/cytokines from clinical scores. SIQR: Symptom Impact Questionnaire Revised, MDI: Major Depression Inventory, GAD7: General Anxiety Disorder-7, ISI: Insomnia Severity Index, WPI (Widespread Pain Index), Total NRS: Numeric Rating Scale totaled from average pain score, pain during exercise, pain at rest, and strongest pain Score, Oxford Score (hip/knee score), EQ-VAS (EuroQol-Visual Analogue Score). Note that the levels of serum histidine, glutamine and albumin levels are all inversely correlated with the SIQR, WPI and ISI scores.

7.3.5. Serum Metabolites and Pain Severity in OA

Measures of pain intensity are rarely associated with disease severity in terms of the damage or progression of joint destruction (Bacon et al. 2020; Neogi et al. 2009; Wang et al. 2018). Indeed, here we see no association between those suffering high pain, as measured by a Numeric Rating Scale (NRS), and radiographic measures of OA severity (Kellegren Lawrence scores). However, analysis of the discriminatory serum metabolites stratified by pain intensity revealed significant differences between high and low pain groups within OA. High vs low pain was defined using the upper and lower quartiles of total NRS score as thresholds (Figure 7.5A). Decreases in serum histidine, glutamine, lysine, and albumin were observed in the high-pain group, while serum GFAP, likely derived from chondrocytes (Kanazawa et al. 2017), was increased (Figure 7.5B). Notably, CSF GFAP levels were not elevated, and there was no difference in age between high and low pain individuals (Figure 7.5D). Logistic regression using a combination of these variables alone could predict whether a sample belonged to the high or low pain group with an ROC AUC of 82.4% (Figure 7.5C). Serum histidine also correlated with indirect measures of pain severity, including the Pain Disability Index (PDI), SIQR, and EuroQol Visual Analogue Score (EQ-VAS) (Figure 7.5E (i-iii)). These findings underscore the potential of specific serum metabolites as biomarkers of pain intensity in OA.

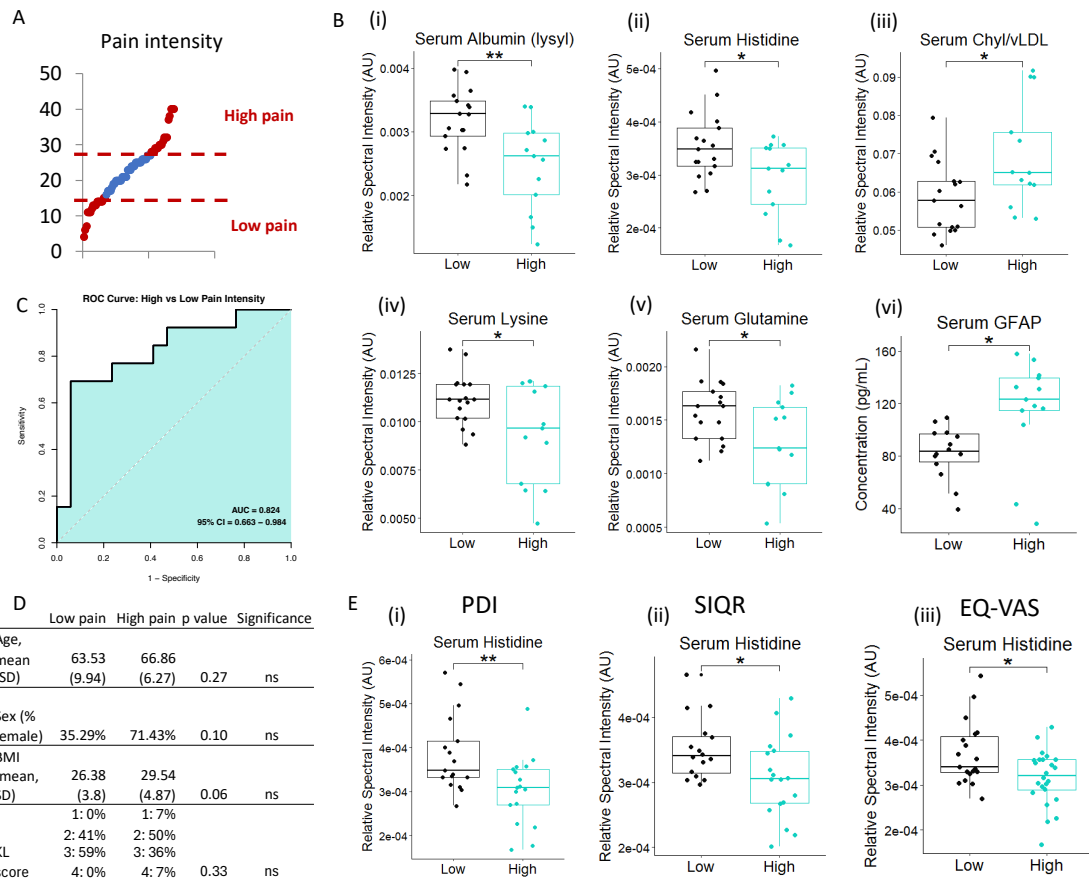


Figure 7.5: Serum metabolites distinguish between high and low pain intensity in osteoarthritis. A) Graph to illustrate how the high vs low pain was defined. The upper and lower quartiles of the total Numeric Rating Scale (NRS) were used, with a higher score indicating higher pain intensity. B (i-vi) the VIPs that distinguish between osteoarthritis and control that remained significantly altered in high vs low pain intensity within OA. C) ROC curve generated from the metabolites in B to distinguish between high vs low pain intensity. D) Table summary to show group characteristics. Continuous variables assessed by T test, while categorical variables were assessed by Fisher's exact test or the Monte Carlo extension for the Kellegren Lawrence (KL) score, which comprises four levels of increasing radiographic severity. E (i-iii) serum histidine remained significantly decreased in more indirect measures of pain severity - the pain disability index (PDI), Symptom Impact Questionnaire (SIQR), and The EuroQol-Visual Analogue Score (EQ-VAS). For this investigation into the association of pain with serum metabolites serum albumin, serum histidine, serum glutamine, and serum lysine were all decreased in the high pain group. Serum GFAP, likely derived from chondrocytes, was increased in the high pain group, but there was no increase in CSF GFAP.

7.3.6. Sex Differences in OA Severity and Metabolomic Profiles

Sex is known to be a major risk factor for the onset and severity of osteoarthritis. As such, it was important to investigate whether any of the identified biomarkers of disease depended on sex. Sex-stratified analyses revealed that female participants with OA presented with more severe disease, as evidenced by higher scores on the SIQR, ISI, and Major Depression Inventory (Figure 7.6). OPLS-DA models demonstrated distinct metabolite profiles for female and male participants, suggesting that sex-specific metabolic alterations may contribute to OA severity. Metabolomic changes were more pronounced in females, with larger decreases in serum histidine and glutamine compared to males (Supplementary Figures S7.3 and S7.4).

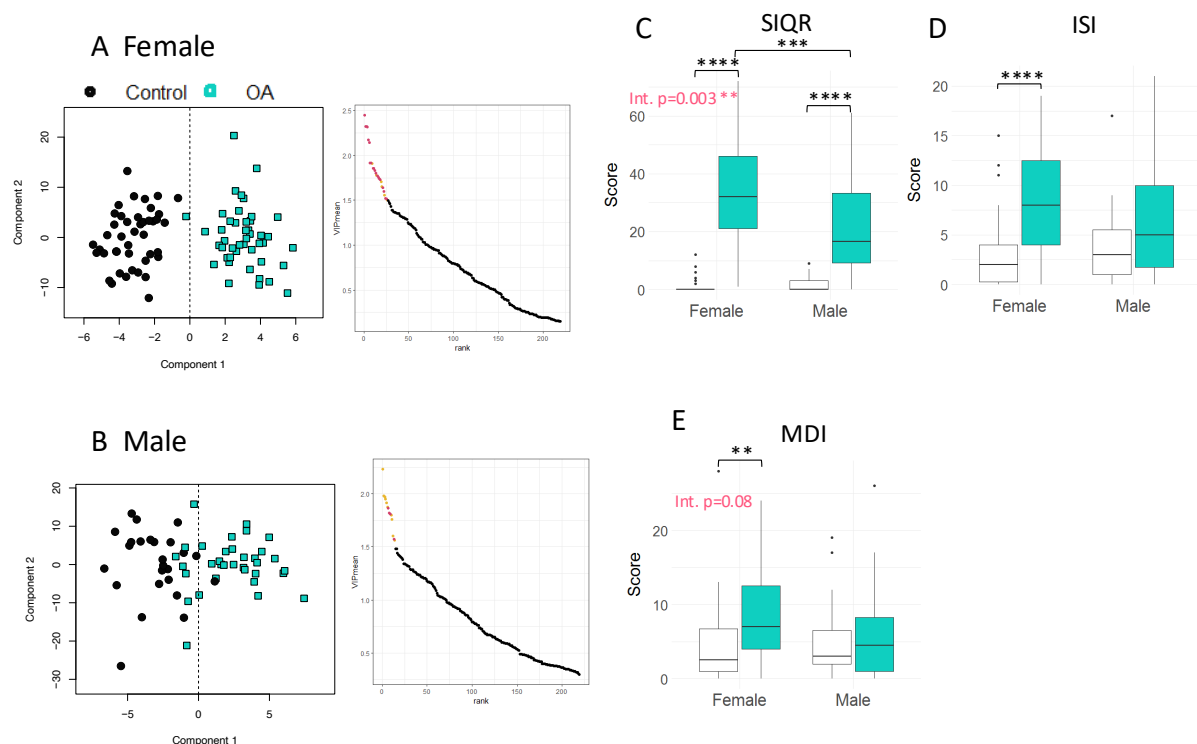


Figure 7.6: Females presented with a more severe form of osteoarthritis. OPLS-DA scores plots of A) female and B) male osteoarthritis vs controls combined serum and CSF metabolome. VIP plots are shown adjacent, with pink representing serum metabolites and

*yellow representing CSF metabolites. Only metabolites with a VIP score above 1.5 are shown in colour. C) revised symptom impact questionnaire D) Insomnia severity index E) major depression inventory scores separated into males and females. Data represented as mean +/- SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ indicating significant post hoc Tukey tests following a significant main effect of disease as determined by two-way ANOVA. Interaction p values (sex*diagnosis), where appropriate, are shown in pink. Note that this subanalysis revealed that the women included in our study present with more severe disease and the metabolites changes were all enhanced in females compared to the males (c.f. the decrease in serum histidine was more marked in the female participants).*

7.4.0 Discussion

This study provides novel insights into the distinct metabolic and inflammatory profiles in OA using a combination of serum and CSF biomarkers, advanced metabolomic techniques, and targeted protein panels. By integrating these data, we uncovered valuable insights into the pathophysiological mechanisms underlying OA, highlighted associations between biomarkers with pain severity, and identified significant sex differences in disease presentation.

7.4.1. Metabolic Alterations in OA

Our results reveal distinct metabolic signatures in OA, characterised by increases in VLDL and 3-hydroxybutyrate and decreases in lysine, alanine, histidine, and glutamine in serum, along with elevated lactate and glutamate and reduced glutamine and glucose in CSF. These results are consistent with growing evidence suggesting that metabolic dysregulation, particularly in amino acid and lipid metabolism, is linked to inflammation and joint degradation in OA (Gkretsi, Simopoulou, and Tsezou 2011). The observed metabolic alterations align with prior research indicating systemic inflammation, oxidative stress, and chondrocyte metabolic shifts in response to environmental stressors and mitochondrial dysfunction (Tootsi et al. 2020). Chondrocyte metabolism is known to be affected by environmental stressors, which can lead

to shifts between metabolic pathways and mitochondrial dysfunction (Zheng et al. 2021). Furthermore, obesity and metabolic syndrome, often comorbid with OA, exacerbate these metabolic changes through complex interactions of biomechanical, inflammatory, and metabolic factors (June et al. 2016). Lipid deposition in joints and altered cholesterol metabolism have been observed in early OA stages (Gkretsi, Simopoulou, and Tsezou 2011). These findings support the emerging concept of OA as a metabolic disease, with lipids and adipokines playing crucial roles in cartilage degradation (Masuko et al. 2009; Wu et al. 2022). Similarly, reductions in amino acids such as lysine and glutamine may indicate their increased utilisation in pro-inflammatory and metabolic pathways (Wu et al. 2022).

The differentiation between serum and CSF metabolite profiles provides further support that OA also involves systemic and central metabolic changes. Evidence suggests that elevated lactate and glutamate levels in CSF may reflect altered energy metabolism and excitotoxicity in OA, contributing to central pain sensitisation and neuroinflammatory responses. Studies have found increased levels of glutamate and other excitatory amino acids in synovial fluid of OA patients (Damyanovich, Staples, and Marshall 1999; McNearney et al. 2000), however, this is the first report of an imbalance in the CNS. Metabolic changes in synovial fibroblasts and fluid, particularly in the glutamine-glutamate pathway, have been associated with inflammatory responses in OA (Farah et al. 2022). Exposure of rat spinal cord slices to CSF from OA patients has been shown to affect neuronal excitability, potentially altering nociceptive processing at the spinal level (Lu, Peter, and S 2012). Elucidating the altered composition of OA CSF here suggests that glutamate signalling may contribute to peripheral nociceptive transduction and inflammation in OA (Wen, Chang, and Jean 2015). Additionally, reactive astrocytes and their release of lactate have been implicated in chronic pain

development and central sensitisation (Lluch et al. 2014; James, Mercedes, and G 2021; Liu-Bryan 2015). These findings underscore the importance of considering systemic and central contributions to OA pathology and the potential utility of targeting metabolic pathways in therapeutic interventions.

7.4.2. Diagnostic Potential of Biomarker Integration

Combining serum and CSF metabolites with a targeted protein panel improved the diagnostic accuracy of OA models, reaching 90%. This integration highlights the value of a multi-modal biomarker approach in capturing the complex and multifactorial nature of OA. Previous research has shown the limitations of single biomarkers in diagnosing OA due to the heterogeneity of the disease (Lotz et al. 2013). By incorporating markers of inflammation, neurodegeneration, and joint degradation, our model achieved a higher discriminatory power, and highlighted unique relationships between metabolites and proteins in CSF and serum. The persistence of certain metabolites, such as reduced serum histidine and glutamine, in both the full and matched cohorts suggests their robustness as potential biomarkers for OA diagnosis. Histidine, an essential amino acid, has been demonstrated to have anti-inflammatory properties in a number of studies; it inhibits local inflammation induced by histamine and carrageenan in rats (Farshid, Tamaddonfard, and Morvaridi 2011), and reduces IL-8 secretion in intestinal epithelial cells (Son, Satsu, and Shimizu 2005b). Histidine also exhibits anti-inflammatory effects in human coronary arterial endothelial cells by inhibiting nuclear factor-kappa B activation (Hasegawa et al. 2012). Histidine has also shown potential as a supplement for various conditions, including organ preservation in cardiac surgery, and its administration may increase ammonia levels and affect amino acid metabolism (Holeček 2020a). Indeed, we show here that serum histidine levels are inversely

correlated with cytokines both in the serum and CSF (Figure S7.5). Others have also highlighted histidine as a potential metabolomic biomarker for knee OA in which the ratio of branched-chain amino acids to histidine seems to be important (Zhai et al. 2010). A clinical trial on rheumatoid arthritis patients showed no significant advantage of histidine over placebo, although there was suggestive evidence of benefit in patients with more active and prolonged disease, and results obtained in rheumatoid arthritis ought not to be extrapolated to OA (Pinals et al. 1977).

7.4.3. Pain-Associated Metabolic Changes

One of the key findings of this study is the association of specific serum metabolites with OA pain intensity. Decreases in serum histidine, glutamine, lysine, and albumin (lysyl moiety) were observed in participants with high pain intensity, alongside an increase in serum GFAP. Animal models have demonstrated the involvement of pro-inflammatory mediators, neuropeptides, and various chemical mediators in OA pain (Rui-xin, Ren, and Dubner 2013). Given the chronicity of OA, there is evidence to suggest an adaptive neuropathic component in OA pain, potentially explaining treatment-resistant cases (Dimitroulas et al. 2014). Neuroimaging studies have shown functional and structural brain changes associated with sensitization in OA patients, although the evidence is conflicting (Pedersini et al. 2021). These findings highlight the multifactorial nature of OA pain and the need for targeted mechanism-based treatments.

The observed reduction in histidine and glutamine in high-pain groups may reflect their roles in modulating oxidative stress and inflammatory responses. Histidine and glutamine play important roles in modulating immune responses and oxidative stress. Histidine has been

shown to scavenge reactive oxygen species (ROS) and inhibit inflammatory pathways in various studies (Holeček 2020b; Peterson et al. 1998). It can reduce fluid accumulation and protect intestinal tissue during inflammation (Peterson et al. 1998), as well as inhibit IL-8 secretion in intestinal epithelial cells (Son, Satsu, and Shimizu 2005a). Glutamine serves as a substrate for glutathione synthesis, which is crucial for antioxidant activities and immune cell function (Amores-Sánchez and Medina 1999; Nagashima, Soejima, and Saito 2013). It is essential for cell proliferation and can enhance the function of stimulated immune cells (Newsholme 2001). Both amino acids have demonstrated potential in modulating respiratory burst in neutrophils (Moinard et al. 2002). Their immunomodulatory effects make them promising candidates for therapeutic interventions in various conditions, including inflammation, infection, and oxidative stress-related disorders (Holeček 2020b; Yoneda, Andou, and Takehana 2009). The elevation of serum GFAP, potentially derived from chondrocytes, suggests a link between joint damage and systemic markers of neurodegeneration, further supporting the interconnectedness of peripheral and central mechanisms in OA pain (Gil et al. 2023).

The correlation between these metabolic changes and clinical pain scores, such as the NRS and SIQR, provides a compelling case for the use of serum metabolites as objective measures of pain severity. This could address the current reliance on subjective pain assessments in clinical practice and trials, improving the precision of pain management strategies.

7.4.4. Sex Differences in OA

Our findings reveal significant sex differences in OA presentation, with women exhibiting more severe disease symptoms and greater metabolic changes compared to men. This aligns

with the broader literature indicating that women are disproportionately affected by OA, with higher pain prevalence and intensity, worse functional outcomes, and distinct inflammatory profiles (Segal, Nilges, and Oo 2024).

The greater reductions in serum histidine and glutamine observed in women may reflect sex-specific differences in inflammatory and metabolic processes. Oestrogen significantly influences cartilage metabolism, immune responses, and pain perception, particularly in postmenopausal women, thereby exacerbating OA pathology (Gokhale, Frenkel, and Dicesare 2004). Systematic reviews indicate a relationship between endogenous oestrogen and cartilage turnover, with evidence suggesting that oestrogen replacement therapy may lower OA prevalence (Felson and Nevitt 1998; Tanamas et al. 2011). Oestrogen affects chondrocyte function and may regulate joint inflammation, highlighting its potential role in OA management (Karsdal et al. 2012). Furthermore, metabolic imbalances influenced by oestrogen can alter cartilage turnover, contributing to OA progression (Bay-Jensen et al. 2013). Recent studies emphasize the importance of timing and dosage of oestrogen treatment in improving cartilage health. Overall, the direct and indirect effects of oestrogen on cartilage underscore its relevance in OA pathology and potential therapeutic strategies (Tankó et al. 2008). Research suggests a significant relationship between OA pain and psychosocial factors, particularly depression and anxiety. Studies indicate that approximately 20% of OA patients experience depressive and anxiety symptoms (Stubbs et al. 2016), with a moderate positive correlation between pain severity and both anxious and depressive symptomatology (Fonseca-Rodrigues et al. 2021). Depression, fatigue, and increased weight are linked to higher OA pain (Somers et al. 2009). Central pain processing mechanisms, including central sensitization, may contribute to chronic pain and psychological distress in

OA (Harth and Nielson 2019; Sofat, Ejindu, and Kiely 2011). Psychosocial factors such as pain catastrophizing, self-efficacy, and social support can influence OA pain perception (Creamer and Hochberg 1998; Somers et al. 2009). While the causal relationship between OA pain and psychological factors remains unclear, evidence suggests that addressing psychosocial aspects through interventions like self-management programs and cognitive-behavioural approaches may help reduce OA pain and disability (Somers et al. 2009; Wang and Ni 2022).

The enhanced metabolite changes in women underscore the importance of considering sex as a biological variable in OA research and treatment. Tailored interventions that address the unique metabolic and inflammatory pathways in women could improve clinical outcomes and reduce the sex disparity in OA burden.

7.4.5. Implications for Clinical Practice and Research

This study provides a foundation for the development of biomarker-based diagnostic and therapeutic strategies in OA. Multi-modal biomarker panels integrating serum and CSF metabolites with targeted proteins could improve diagnostic accuracy, stratify patients by disease severity, and inform personalised OA management. The identification of robust biomarkers, such as histidine and glutamine, highlights potential therapeutic targets that warrant further investigation. Interventions aimed at restoring these metabolic pathways could mitigate inflammation and pain, offering a new avenue for disease-modifying treatments. Additionally, the sex-specific differences observed in this study call for the inclusion of sex-stratified analyses in future OA research to ensure equitable and effective treatment development.

7.4.6. Limitations and Future Directions

While this study advances our understanding of OA biomarkers, it has several limitations. The cross-sectional design precludes causal inferences, and the sample size, particularly in the unmatched analyses, may limit generalisability. Longitudinal studies are needed to confirm the temporal relationship between metabolic changes and OA progression and to evaluate the predictive value of identified biomarkers. Experimental studies exploring the functional roles of key metabolites and proteins in OA pathophysiology are also warranted. Additionally, integrating imaging biomarkers and expanding the range of measured inflammatory and metabolic markers could enhance the diagnostic and prognostic capabilities of biomarker panels.

7.4.7 Conclusion

In summary, this study identifies distinct metabolic and inflammatory profiles in OA. Specifically, reduced serum histidine and glutamine emerged as key discriminators, correlating inversely with pain intensity and disability scores and should be further explored for their role in OA pathophysiology and pain modulation as well as possible targets for personalised treatment strategies.

Chapter 8: General Discussion

This thesis presents an integrated exploration of metabolic and inflammatory alterations across long COVID, fibromyalgia (FM), osteoarthritis (OA), and post-viral syndromes. Through a combination of preclinical and clinical studies, it demonstrates that complex chronic syndromes are underpinned by measurable metabolic and inflammatory changes affecting both central and peripheral systems. While earlier chapters focused on long COVID and FM in isolation, this final discussion draws these strands together to explore potential points of convergence. In particular, it examines how persistent symptoms in long COVID may reflect a shared pathophysiological landscape with FM. Specifically, patients with long COVID who do not report improvements in symptoms show a shift towards a metabolic profile resembling that observed in FM. Notably, this is the first time such a similarity has been highlighted, characterised by decreased serum histidine and glutamine, two markers consistently altered in FM and uniquely found to be changed only in the long COVID patients who did not recover. This convergence on shared metabolic signatures suggests that overlapping clinical phenotypes are mirrored by underlying biological processes. Thus, by combining metabolic and protein markers from different biofluids with clinical assessments, this work identifies candidate biomarkers and biological subtypes, providing insights into potential diagnostic tools and personalised therapeutic strategies for managing complex chronic conditions that arise following viral illness.

This discussion will further examine the shared metabolic signatures between long COVID and FM, considering how these insights advance our understanding of chronic post-viral syndromes. Comparison to the preclinical models from Chapter 2 will be explored to elucidate potential mechanisms linking immune activation to metabolic shifts, including sex-specific

responses. Additionally, the distinct yet overlapping features of OA, where known immune pathology drives chronic disease, will be addressed to contextualise the similarities and differences among these conditions. By integrating mechanistic insights from animal models with clinical data, this discussion highlights how the thesis contributes to our understanding of the biological basis of chronic symptoms in post-viral syndromes, as well as how it points to where future research efforts should be focussed.

8.1. Commonalities between murine models of sickness behaviour and clinical cohorts suggest ongoing immune perturbation underlies chronic symptomatology

The use of animal models in this thesis sought to provide mechanistic insights into immune activation and metabolic dysfunction, with a focus on understanding the interactions between the periphery and the central nervous system (CNS). Animal models offer the unique advantage of being able to control conditions much more effectively than in clinical cohorts, allowing the dissection of specific immune-metabolic interactions without the complexity inherent in human diseases. However, it is essential to acknowledge that these models, particularly those using Toll-like receptor (TLR) agonists, are inherently simplistic. They simulate infectious illness to generate mechanistic insights rather than replicating the full complexity of chronic human disease, where multiple immune pathways and other factors often interact over extended periods. Despite this limitation, the models employed were instrumental in identifying shared metabolic perturbations induced by immune activation, including those found in long COVID, fibromyalgia (FM), and osteoarthritis (OA). Specifically, consistent findings included low blood glucose, increased 3-hydroxybutyrate (3HB), altered amino acid metabolism, and elevated GlycA in the condition relative to the control group. These changes are summarised in Table 8.1 and are discussed in turn below. Such shared

metabolic signatures suggest that immune activation is a primary driver of persistent metabolic disturbances despite the varying presentations of chronic disease. By focusing on immune challenges and conserved metabolites, these models support the hypothesis that ongoing TLR activation in long COVID may sustain metabolic disruptions, reinforcing the interconnected nature of inflammatory and metabolic responses.

Serum metabolites	LC baseline	LC follow-up non-recovered	Fibromyalgia	Osteoarthritis	Acute TLR
Glucose	↓*	ns	↓***	↓*	↓**
3-hydroxybutyrate	ns	ns	↑***	↑***	↑**
GlycA	↑**	ns	↑**	ns	↑*
Glutamine	ns	↓*	↓***	↓**	ns
Histidine	ns	↓*	↓***	↓***	ns
Proline	↓***	↓**	↓***	↓**	ns

Table 8.1. Key serum metabolites driving disease pathology in long COVID (baseline and at follow up if not improved), fibromyalgia, osteoarthritis and murine TLR models (combined TLR challenge at 24 hours post TLR agonist injection). These are taken from the cohorts in Chapter 4, 6, 7, and 2, respectively. Arrows depict the change in blood metabolite relative to each respective control group, as determined by T test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

8.2. Conserved sex differences across murine and clinical cohorts

A consistent observation across both animal models and human cohorts was the presence of sex differences in immune and metabolic responses. In the animal models, female mice demonstrated greater resilience to TLR-induced sickness behaviour compared to males, despite exhibiting a more robust cytokine response to the same immune stimulus. This increased cytokine production in females points to a biologically meaningful difference,

rather than a reporting bias or hypervigilance to account for the increased prevalence of females in long COVID and chronic pain. This interpretation is further supported by the lack of association between anxiety phenotypes and disease severity, as demonstrated in Chapter 6, where lower pain thresholds in females were not linked to heightened anxiety measures. The magnitude of cytokine differences between male and female mouse models is particularly noteworthy, as it could explain the clinical observation that women are more susceptible to these chronic pain syndromes and post-viral fatigue (Sorge and Totsch 2017). Indeed, baseline CRP values in the Chapter 6 control group revealed higher levels in females compared to males. These findings underscore the importance of considering sex-specific immune-metabolic interactions when investigating chronic diseases, as understanding these differences may improve the accuracy of diagnostics and the effectiveness of therapeutic approaches. In addition to sex differences, another key aspect that emerged from both the animal models and clinical cohorts is the identification of conserved metabolic alterations, indicating the cause of some of the metabolic changes seen in clinical cohorts may be linked to an activated immune system.

8.3. Conserved metabolic alterations across acute TLR models, Long COVID, Fibromyalgia, and Osteoarthritis

8.3.1. Low blood glucose

One of the key conserved metabolic alterations identified across both animal models and clinical cohorts (including long COVID, FM, and OA) is low blood glucose. In the animal models, acute immune activation via TLR challenge led to a significant decrease in glucose levels, indicating its rapid utilisation as an energy substrate during the innate immune response. This depletion of glucose is a well-recognised feature of the acute phase response,

as immune cells exhibit heightened metabolic demands when activated (Chan et al. 2020a; Piarulli and Lapolla 2020). Furthermore, previous studies have linked hypoglycaemia with fatigue, cognitive impairment, and mood alterations (Warren and Frier 2005; Frier 2021, 2001). In chronic conditions such as long COVID and FM, persistent low blood glucose was also observed, suggesting that ongoing immune activation may continue to drive glucose consumption. This finding aligns with the hypothesis of chronic low-grade inflammation in post-viral syndromes, where metabolic shifts persist beyond the acute phase of infection (Maamar et al. 2022). However, it should be noted that there is no obvious record of clinical hypoglycaemia in these patients; this may be more of a subtle abnormality that is detected on the group level and aided in defining a metabolic fingerprint of disease. Nonetheless, the consistently low glucose levels observed across conditions highlight the interconnectedness between immune activation and metabolic dysfunction and suggest that an immune-mediated metabolic shift may underly some of the key features of post-viral syndromes.

8.3.2. Increased blood 3-hydroxybutyrate

The observation of low blood glucose across both animal models and clinical cohorts highlights the potential for metabolic shifts as compensatory mechanisms in response to systemic stress. One such adaptation is the consistent increase in circulating 3-hydroxybutyrate, observed not only in animal models but also in patients with fibromyalgia (FM) and osteoarthritis (OA). Elevated 3-hydroxybutyrate suggests a shift towards ketogenesis, typically triggered by reduced glucose availability or heightened energy demand. This metabolic reprogramming enables the utilisation of ketone bodies as alternative energy substrates when conventional glucose metabolism is impaired (Mierziak, Burgberger, and Wojtasik 2021). Although this phenomenon is frequently studied in the context of fasting or

ketogenic diets, the findings from the TLR-activation mouse model indicate that it may also be a fundamental part of the acute sickness response.

In chronic conditions such as FM and OA, the persistence of elevated 3-hydroxybutyrate levels may reflect a longer-term metabolic adaptation – potentially driven by sustained immune activation, mitochondrial dysfunction, or the energetic demands of prolonged low-grade inflammation. Comparable changes have also been reported in ME/CFS, where proteomic analyses of peripheral blood mononuclear cells revealed increased expression of key enzymes in ketone metabolism, including 3-hydroxybutyrate dehydrogenase type 1 (BDH1) and acetyl-CoA acetyltransferase 2 (ACAT2) (Sweetman et al. 2020).

In long COVID, although these specific protein changes were not observed in blood, notable alterations in ketone body profiles were found in urine, suggesting a systemic shift in energy metabolism may still be occurring. This divergence may reflect tissue-specific metabolic responses or differences in the duration and nature of inflammatory signalling in long COVID compared to FM or ME/CFS. Nonetheless, the involvement of ketone metabolism – whether reflected in blood, urine, or cellular pathways – points to a shared thread of disrupted energy homeostasis across post-viral syndromes. Elucidating the role of 3-hydroxybutyrate not only as a biomarker but also as a potential modulator of immune-metabolic interactions could open new avenues for therapeutic intervention aimed at restoring metabolic flexibility and resilience in long COVID and related conditions.

8.3.3. Increased blood GlycA

Another conserved metabolic alteration identified across the clinical cohorts is the elevation of GlycA, observed consistently in long COVID and FM but notably absent in OA. GlycA is a

composite marker of chronic low-grade systemic inflammation, reflecting the glycosylation of acute-phase proteins (Connelly et al. 2017). Its elevation in long COVID and FM initially suggests that these conditions are characterised by sustained systemic immune activation. Supporting this, increased GlycA levels were also observed in murine models following TLR agonist challenge, indicating that this marker may form part of the acute-phase response triggered by innate immune stimulation. The cross-species consistency strengthens the case for GlycA as a biologically meaningful indicator of immune-metabolic disturbance in post-viral and inflammatory syndromes. However, given that OA also exhibits features of systemic inflammation in our data, the relationship between GlycA levels and chronic inflammatory states may be more complex than previously thought. This nuance highlights the need for further research to delineate the specific inflammatory pathways and glycosylation patterns that drive GlycA elevation in different chronic conditions. The presence of elevated GlycA in both long COVID and FM nonetheless underscores the potential health risks associated with chronic inflammation, as increased GlycA levels have been linked to a higher risk of cardiovascular and metabolic diseases (Connelly et al. 2017). Monitoring GlycA may thus provide valuable insights into long-term health outcomes in these patients, but a deeper understanding of its regulation and significance across inflammatory disease states remains essential.

8.3.4. Decreased amino acid availability

Altered amino acid metabolism emerged as a key conserved metabolic signature across the clinical cohorts, with notable reductions in glutamine, histidine, and proline. These amino acids were significantly decreased in fibromyalgia (FM) and osteoarthritis (OA), with lower levels of glutamine and histidine in OA also correlating with more severe pain, suggesting a

consistent pattern of amino acid depletion associated with chronic inflammatory and pain states. In long COVID, while these amino acids were not significantly altered at baseline, those who failed to recover by the 9-month follow-up exhibited marked reductions in circulating glutamine, histidine, and proline. This metabolic profile closely resembled that seen in FM, reinforcing the hypothesis that long COVID may evolve into a chronic pain-like phenotype in a subset of individuals. Conversely, those who reported symptom resolution at follow-up showed normalised amino acid profiles, with glutamine, histidine, and proline levels comparable to healthy controls – supporting the potential of these amino acids as state markers of recovery.

Mechanistically, these amino acid reductions may reflect mitochondrial dysfunction, a feature increasingly recognised in chronic inflammatory conditions (Palmer 2022). In ME/CFS, impaired oxidative phosphorylation has been linked to decreased serum glutamine and histidine, which serve as key substrates for mitochondrial energy production and redox balance (Armstrong et al. 2012; Fluge et al. 2016). Fluge and colleagues (2016) also identified increased expression of pyruvate dehydrogenase (PDH) inhibitory factors, suggesting a metabolic shift from glucose oxidation towards greater reliance on amino acid catabolism to sustain the TCA cycle. Whether similar mechanisms contribute to amino acid depletion in long COVID or FM remains to be clarified, but the consistent pattern across conditions implies mitochondrial stress as a unifying feature underlying persistent symptoms such as fatigue and pain.

From a therapeutic perspective, targeting mitochondrial dysfunction through amino acid supplementation, particularly with glutamine and histidine, could offer a potential avenue for

intervention. Enhancing mitochondrial function and supporting metabolic resilience may alleviate some of the chronic symptoms observed in post-viral syndromes and chronic pain conditions. Future studies should investigate whether restoring amino acid levels can mitigate the downstream effects of chronic immune activation and support recovery from these debilitating conditions.

8.4. HPA Axis Dysregulation and Cortisol Alterations

The consistent metabolic alterations observed across chronic conditions such as long COVID, FM, and ME/CFS highlight potential mechanisms underlying persistent symptoms, including mitochondrial dysfunction and disrupted energy metabolism. While these findings provide insight into the biological changes that may underpin chronic disease states, the factors that predispose some individuals to incomplete recovery remain unclear. One potential contributor is an ongoing stress response causing dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and downstream metabolic regulation.

The relationship between stress and cortisol is complex and context dependent. Acute stress typically triggers an increase in cortisol release as part of the body's adaptive response via the HPA axis (Michaud et al. 2008; Pisanski, Nowak, and Sorokowski 2016). Interestingly, elevated cortisol in this context has been associated with heightened pain sensitivity, including higher pain ratings and lower pain thresholds (Choi, Chung, and Lee 2012). However, under conditions of chronic stress, such as in Post-Traumatic Stress Disorder (PTSD), numerous studies have reported reduced cortisol levels in plasma, saliva, and urine (Boscarino 1996; Wahbeh and Oken 2013; Mason et al. 1986; Yehuda et al. 1990). This paradoxical decrease is thought to reflect enhanced negative feedback within the HPA axis, potentially due to

prolonged glucocorticoid receptor activation. Such sustained signalling may lead to receptor desensitisation or the upregulation of inhibitory pathways that suppress cortisol production (Tomas, Newton, and Watson 2013; Young, Kwak, and Kottak 1995).

In the context of long COVID, emerging evidence has also indicated decreased cortisol levels in affected individuals, potentially driven by chronic stress (Klein et al. 2023). Interestingly, this thesis provides novel mechanistic insights by identifying an increase in 11-deoxycortisol, the immediate precursor of cortisol. This finding suggests a potential deficiency in 11 β -hydroxylase, the enzyme responsible for converting 11-deoxycortisol to cortisol (Lightman, Birnie, and Conway-Campbell 2020). Given there were no differences in the time of day the samples were taken from long COVID and control participants, this enzymatic disruption may reflect a specific impairment within the steroidogenic pathway, warranting further investigation to clarify its role in the chronic phase of long COVID.

The relationship between cortisol and pain in FM and OA remains unclear, with mixed findings reported. Some studies in FM have noted reduced basal cortisol levels, reflecting the patterns seen in PTSD and chronic stress conditions. Indeed, low cortisol levels are also associated with increased risk of chronic pain development in trauma patients (Trevino et al. 2022). However, higher cortisol levels have been linked to greater pain intensity in osteoarthritis (Carlesso, Sturgeon, and Zautra 2016) and stress-induced cortisol increases can enhance pain sensitivity, particularly in visceral pain (Benson et al. 2019), indicating a complex association with pain pathology and therefore, a less clear relationship with between stress, immune activation and systemic cortisol.

To better understand the trajectory of cortisol alterations, longitudinal studies are critical. Monitoring changes over time could reveal whether the observed disruptions are transient or represent a sustained shift in HPA axis regulation. It would also be valuable to assess diurnal patterns of cortisol secretion, as time-of-day measurements could help distinguish between blunted circadian rhythms and more generalised suppression. Investigating whether cortisol levels – and their diurnal variation – normalise in parallel with symptom improvement may offer further insight into the dynamics of recovery. Such findings could deepen our understanding of the interplay between chronic inflammation, stress, and metabolic dysfunction, guiding the development of therapeutic strategies that target both HPA axis normalisation and metabolic resilience.

8.5. Limitations and Future Directions

While this thesis provides important insights into the metabolic and inflammatory alterations associated with long COVID, FM, OA, and post-viral syndromes, several limitations must be acknowledged. First, the observational nature of the clinical studies limits the ability to determine whether the identified metabolic and immune alterations are causal, a consequence of disease, or an epiphenomenon. Although the findings suggest consistent patterns of dysregulation across multiple chronic conditions, disentangling cause from effect requires longitudinal and interventional studies. Similarly, while the animal models provided valuable mechanistic insights – particularly regarding the acute-phase response and energy metabolism – they do not fully replicate the chronic, multifactorial nature of human disease where immune, metabolic, and psychosocial factors interact over extended periods.

A key goal of this work was to explore the diagnostic potential of metabolomic profiling in long COVID. While distinct metabolic signatures were identified – most notably, an 81% AUC for long COVID vs. controls – independent validation in external cohorts is essential. Planned work includes testing these models in the UK Biobank, which will enable assessment of generalisability and replication of the diagnostic performance. It is also notable that the baseline metabolome of this cohort was not able to predict recovery. While we saw an interesting spontaneous recovery of the metabolome of individuals reporting symptom improvement at the 9-12 month follow up, the metabolites remaining perturbed in the non-recovered cohort had shifted from the initial baseline measures, resembling a more FM-like profile. Whether this is a reflection of the pathology driving persistence, or whether this is a result of lifestyle factors such as an increasingly sedentary life, is unclear. Indeed, the evolving picture of disease may complicate the use of metabolomics in diagnosis. A further complication arises from the heterogeneity of such syndromes. Indeed, key findings from this thesis demonstrate distinct subgroups within long COVID, as well as within fibromyalgia, each relating to a different symptom profile. Again, larger scale studies are likely to reveal additional subgroups, and external validation will be key to confirming these findings.

While comparing each disease group to their respective controls highlighted key similarities across chronic conditions, technical limitations impacted the scope of this work. Due to equipment failure, long COVID and chronic pain samples were acquired using different NMR platforms (700 MHz vs. 950 MHz) and were not processed in parallel. This prevented direct comparison between long COVID and FM – an analysis that could have yielded deeper insights into shared versus divergent mechanisms. Complicating this further, the control groups differed significantly: while long COVID cohorts were carefully matched, the FM/OA controls

were younger, had a higher proportion of males, and lower BMI, introducing potential confounders that required statistical adjustment. Despite these challenges, the consistent metabolic findings across cohorts warrant further exploration. Moreover, the identification of distinct subgroups within these chronic conditions supports a more nuanced approach to disease management – one that prioritises biological and symptom-based stratification over broad diagnostic categories such as “long COVID” or “FM”.

Building on the findings of this thesis, several directions for future research emerge. First, interventional studies targeting key metabolic pathways – such as histidine supplementation or ketone modulation – are needed to determine whether these alterations are functionally relevant and therapeutically actionable. Additionally, the consistent sex differences observed in symptom burden and metabolic profiles point to the need for sex-stratified analyses in both mechanistic and therapeutic studies.

The role of the HPA axis in chronic symptom persistence remains an important area of inquiry. While this thesis identified reduced cortisol levels in long COVID, follow-up measurements are still pending. These are planned imminently and will help clarify whether this pattern persists or resolves over time according to improvement status. The chronic pain cohorts will also undergo lipidomic profiling in the same lab to assess whether similar HPA axis dysregulation is present. Future work should also incorporate diurnal cortisol sampling to capture alterations in circadian rhythm, not just basal output, and explore how these changes track with symptom evolution.

Another priority is the investigation of autoimmune mechanisms, including the presence of brain-targeted autoantibodies, to explore links between autoreactivity and cognitive symptoms in long COVID and FM, taking sex into consideration. While the metabolic data

presented in this thesis point to ongoing immune activation, the underlying source of inflammation remains unclear. Several hypotheses warrant exploration – including sterile inflammation driven by chronic stress (a possibility supported by cortisol findings here), the persistence of viral fragments, or low-grade infection by less obvious pathogens such as L-form bacteria, which have been implicated in ME/CFS and FM. Investigating these potential causes, in addition to understanding the factors that drive susceptibility of individuals, is key to informing directed therapeutic or preventative strategies.

8.6. Concluding remarks

This thesis provides new insights into the metabolic and inflammatory mechanisms underlying long COVID, FM, and OA, through an integrated multi-omics approach. The findings reveal a convergence in metabolic changes across these conditions, suggesting that chronic immune activation drives a pattern of metabolic reprogramming, with consistent alterations in glucose handling, amino acid utilisation, and systemic inflammatory markers. Importantly, sex differences emerged across both preclinical and clinical datasets, with female subjects exhibiting more pronounced immune responses and symptom severity, underscoring the need to account for sex-specific biology in both mechanistic studies and therapeutic development.

Historically, conditions such as CFS and FM were often dismissed as psychosomatic due to the absence of consistent structural pathology or clear diagnostic tests. However, accumulating evidence – including the data presented here – demonstrates reproducible biological abnormalities in affected individuals, particularly involving immune, endocrine, and metabolic systems. While establishing definitive causality remains challenging, it is clear that

these syndromes are not simply psychological in origin. Rather, they reflect complex, multifactorial disturbances in physiological regulation that can be objectively measured and potentially targeted. Recognising these disorders as biologically grounded opens the door to more empathetic clinical care and research-driven therapeutic innovation.

By identifying candidate biomarkers and biological subtypes within chronic post-viral and inflammatory syndromes, this thesis lays important groundwork for more precise diagnostics and individualised treatment approaches. These findings support a shift in how we understand and classify such conditions, and they highlight the potential for targeted interventions to address the lingering burden of post-viral illness.

Appendix A (for Chapter 2, Murine TLR models)

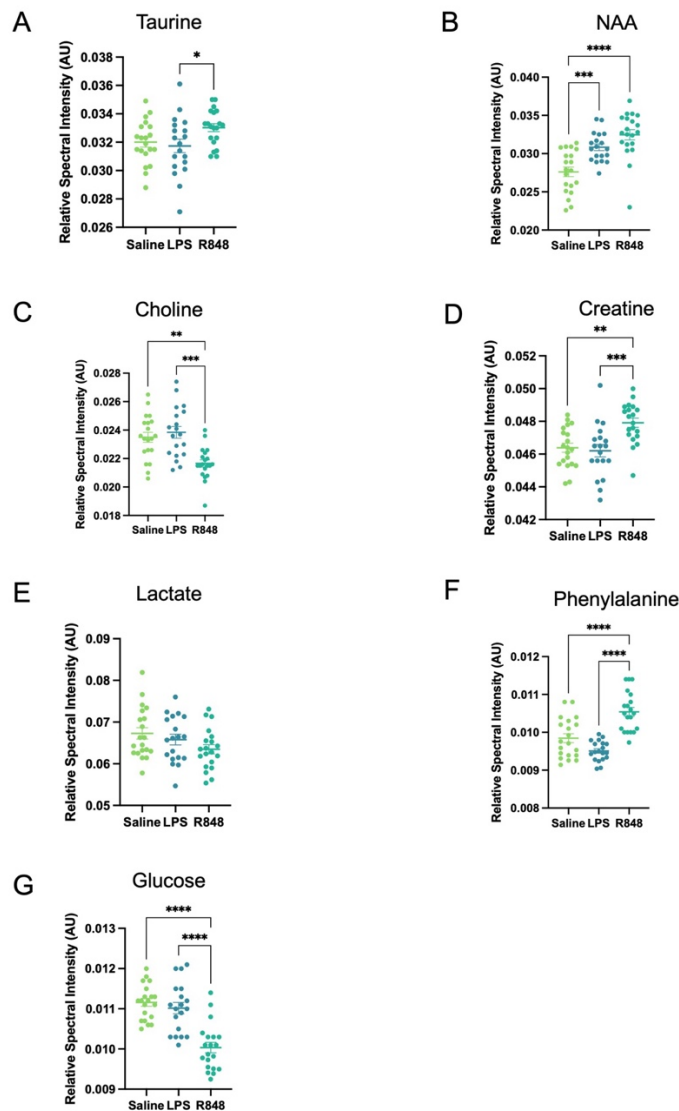


Figure S2.1: Distinct brain metabolite profiles following TLR4 and TLR7 activation. Relative spectral intensities of key brain metabolites measured by ^1H NMR spectroscopy in the prefrontal cortex of mice 24 hours after intraperitoneal administration of saline, TLR4 agonist (LPS, 0.83 mg/kg), or TLR7 agonist (R848, 5 mg/kg). The selected metabolites shown here – (A) Taurine, (B) N-acetylaspartate (NAA), (C) Choline, (D) Creatine, (E) Lactate, (F) Phenylalanine, and (G) Glucose – represent those with the greatest discriminatory power between groups. Several metabolites, including NAA, phenylalanine, and glucose, show significant upregulation in the R848 group compared to LPS and saline, suggesting divergent metabolic responses to TLR7 versus TLR4 stimulation. Data are presented as mean $\pm$ SEM; statistical significance determined by one-way ANOVA with post hoc testing. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

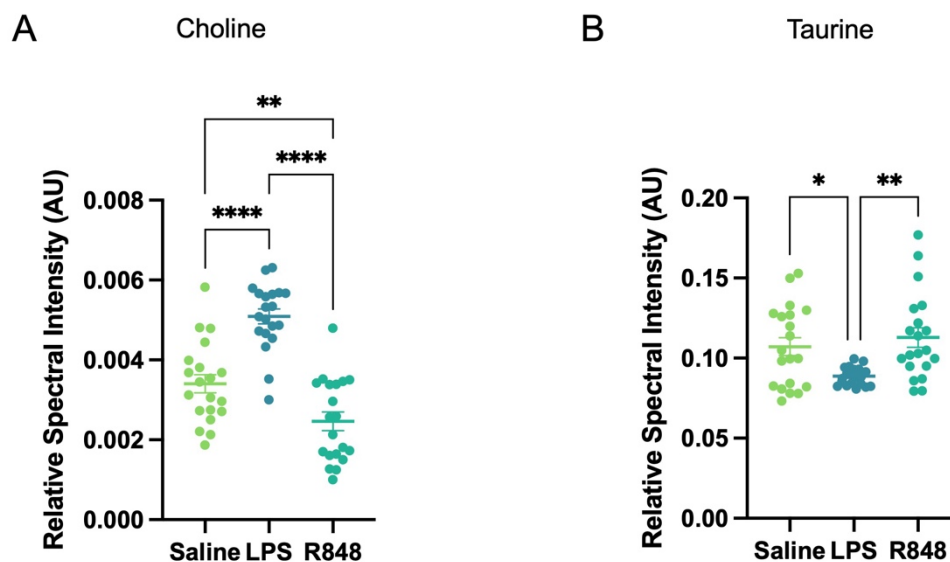


Figure S2.2: Distinct liver metabolite alterations following TLR4 and TLR7 activation. ^1H NMR spectroscopy of liver tissue reveals changes in metabolite levels 24 hours after intraperitoneal injection of saline, TLR4 agonist (LPS, 0.83 mg/kg), or TLR7 agonist (R848, 5 mg/kg). (A) Choline and (B) Taurine were among the most discriminatory metabolites across treatment groups. Choline levels were significantly elevated in both LPS and R848 groups relative to saline, with a further increase observed in the TLR7-stimulated group, suggesting enhanced membrane turnover or altered phospholipid metabolism. Taurine levels were also significantly increased following immune activation, with R848 showing a stronger effect than LPS. Data represent relative spectral intensity (arbitrary units) and are presented as mean $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA with post hoc testing. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

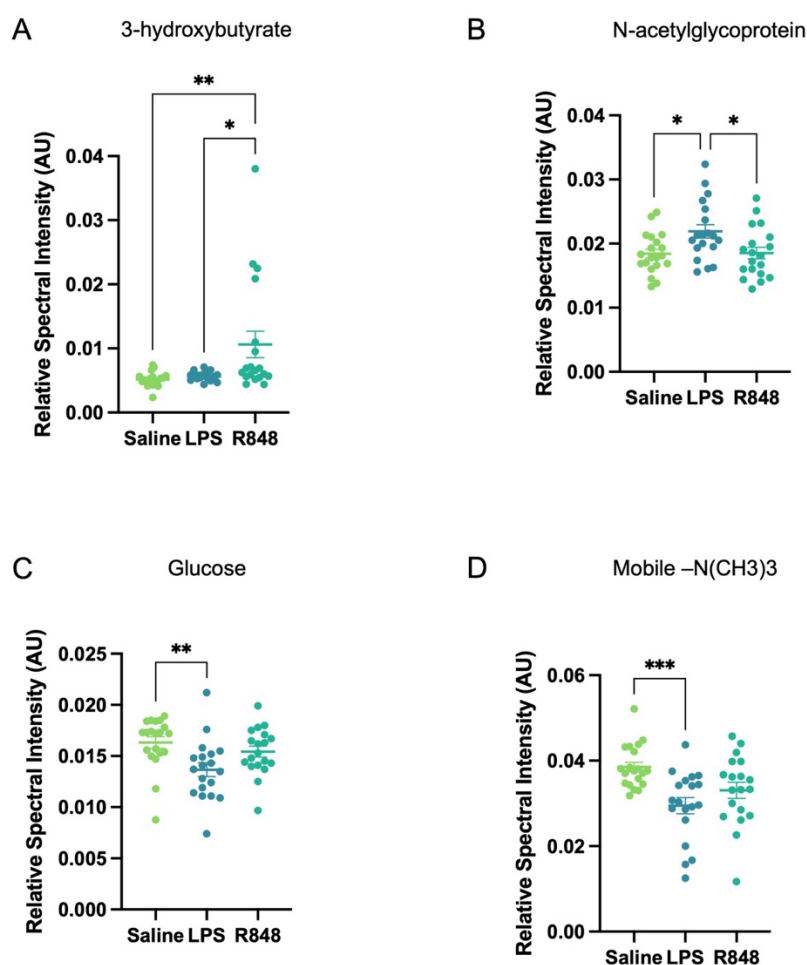


Figure S2.3: Plasma metabolite alterations following TLR4 and TLR7 activation. ¹H NMR analysis of plasma samples collected 24 hours after treatment with saline, TLR4 agonist (LPS, 0.83 mg/kg), or TLR7 agonist (R848, 5 mg/kg) identified metabolites that discriminate between immune activation pathways. (A) 3-Hydroxybutyrate levels were significantly elevated in response to R848, suggesting alterations in ketone body metabolism. (B) N-acetylglycoprotein, a marker of systemic inflammation (GlycA), was modestly increased following both TLR4 and TLR7 stimulation. (C) Glucose levels were significantly decreased in LPS-treated animals, consistent with immune-related metabolic demand. (D) Mobile -N(CH₃)₃, reflecting signals from choline-containing compounds, was significantly increased in R848-treated animals. Together, these findings reveal distinct plasma metabolic responses to TLR4 and TLR7 activation. Data are presented as mean ± SEM; statistical significance was assessed by one-way ANOVA with post hoc testing. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Appendix B (for Chapter 3, Protocol & Cohort)

Symptoms	Control (n=64) first 6 weeks	Long COVID (n=65) first 6 weeks	Significance	Long COVID (n=65) after 6 weeks
I never experienced any symptoms	2 (3.1%)	0		0
Fever or feeling hot	29 (45.3%)	46 (70.8%)	**	16 (24.6%)
Chills and shivers	27 (42.2%)	44 (67.7%)	**	17 (26.2%)
A persistent cough (coughing a lot for more than an hour, or 3 or more coughing episodes in 24 hours)	27 (42.2%)	38 (58.5%)		20 (30.8%)
Mild fatigue (tired but still able to perform most daily activities)	35 (54.7%)	27 (41.5%)		26 (40.0%)
Severe fatigue (struggle to get out of bed)	11 (17.2%)	46 (70.8%)	****	41 (63.1%)
Headache	33 (51.6%)	48 (73.8%)	*	34 (52.3%)
Nausea and/or vomiting	2 (3.1%)	23 (35.4%)	****	17 (26.2%)

Dizziness or lightheadedness	8 (12.5%)	37 (56.9%)	****	33 (50.8%)
Mild shortness of breath or difficulty breathing doing ordinary activity	13 (20.3%)	31 (47.7%)	**	33 (50.8%)
Severe shortness of breath or difficulty breathing – only comfortable at rest	0	26 (40.0%)	****	19 (29.2%)
Sore or painful throat	30 (46.9%)	36 (55.4%)		19 (29.2%)
Loss of smell/taste	17 (26.6%)	31 (47.7%)	*	17 (26.2%)
An unusually hoarse voice	5 (7.8%)	18 (27.7%)	**	16 (24.6%)
Unusual chest pain or tightness in your chest	3 (4.7%)	38 (58.5%)	****	37 (56.9%)
Unusual abdominal pain or stomach ache	1 (1.6%)	17 (26.2%)	****	13 (20.0%)
Diarrhoea	2 (3.1%)	21 (32.3%)	****	12 (18.5%)
Reflux	2 (3.1%)	14 (21.5%)	**	14 (21.5%)
Unusually strong muscle aches or pains	11 (17.2%)	45 (69.2%)	****	33 (50.8%)

Any skin rashes, blisters, sores, or skin swelling	1 (1.6%)	17 (26.2%)	****	18 (27.7%)
Confusion, disorientation, drowsiness	6 (9.4%)	29 (44.6%)	****	25 (38.5%)
Memory problems	6 (9.4%)	33 (50.8%)	****	43 (66.2%)
Difficult with attention	11 (17.2%)	35 (53.8%)	****	43 (66.2%)
Difficulty with word-finding (or understanding)	2 (3.1%)	33 (50.8%)	****	39 (60.0%)
Tingling, numbness, or lack of feeling in any limbs	0	22 (33.8%)	****	25 (38.5%)
Scalp pain	0	9 (13.8%)	**	12 (18.5%)
Any unusual eye-soreness or discomfort (light-sensitivity, excessive tears, pink/red eye)	2 (3.1%)	30 (46.2%)	****	26 (40.0%)
Any visual disturbance	0	11 (16.9%)	***	21 (32.3%)
Loss of appetite	7 (10.9%)	27 (41.5%)	***	19 (29.2%)
Joint pain	7 (10.9%)	33 (50.8%)	****	30 (46.2%)
Did not respond	7 (10.9%)	3 (4.6%)		3 (4.6%)

Table S3.1. Prevalence of symptoms reported during the acute and long phases of COVID-19 infection in individuals with Long COVID and controls. *This table presents the frequency and percentage of self-reported symptoms among participants during the acute phase (first 6 weeks) and long phase (after 6 weeks) of COVID-19 infection. Participants were categorised into those with Long COVID (n=65) and controls (n=64). Symptoms are listed in the leftmost column. The next three columns show the number and percentage of individuals reporting each symptom in the control group (during the first 6 weeks), the Long COVID group (during the first 6 weeks), and the Long COVID group (after 6 weeks). The Significance column indicates the statistical significance of the difference between controls and the Long COVID group in the acute phase (first 6 weeks), using T-tests for continuous variables and chi-squared analysis for categorical variables. $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$, $p < 0.0001 = ****$.*

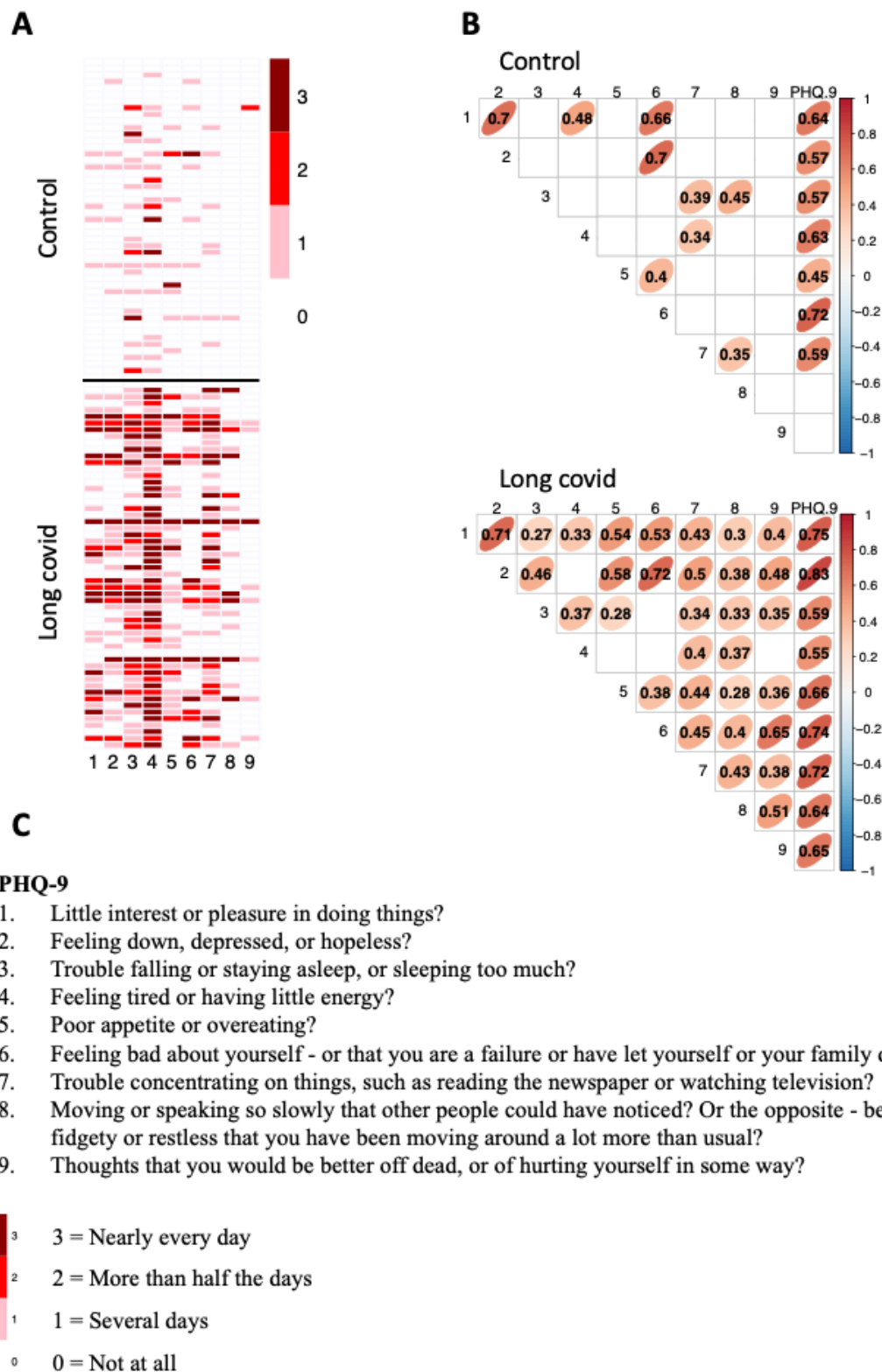
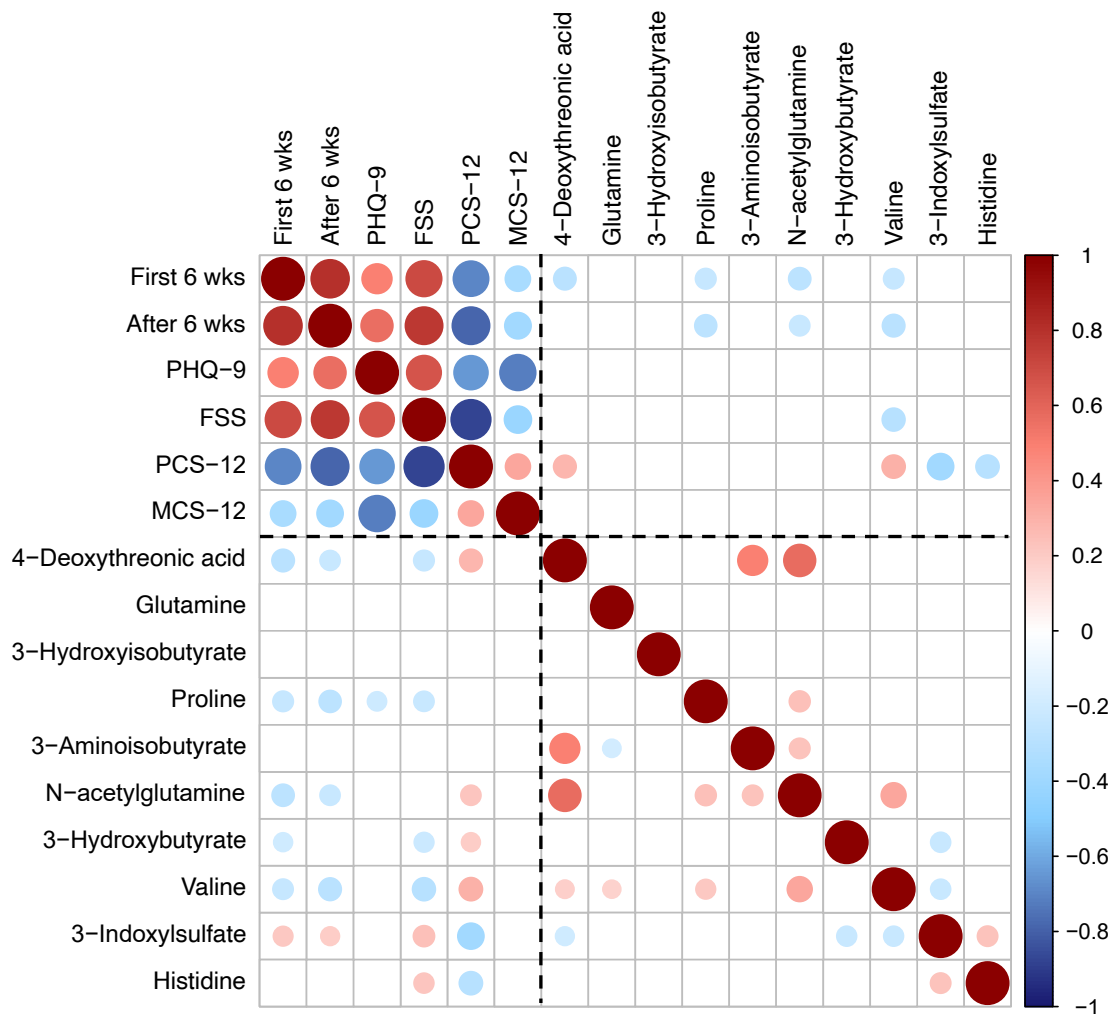


Figure S3.1. Depressive symptoms in individuals with Long COVID and controls assessed using the PHQ-9. (A) Heatmap showing individual responses to the 9-item Patient Health Questionnaire (PHQ-9) in control and Long COVID participants. Each column corresponds to

one PHQ-9 item (labelled 1–9, see panel C for questions), and each row represents an individual. Responses are colour-coded according to severity: 0 = not at all (lightest), 1 = several days, 2 = more than half the days, and 3 = nearly every day (darkest). **(B)** Correlation matrices (Spearman's ρ) for PHQ-9 items and total PHQ-9 scores in control (top) and Long COVID (bottom) groups. Only significant positive correlations are shown. The colour and size of each ellipse indicate the strength of the correlation, with stronger correlations shown in darker red and larger ellipses. **(C)** PHQ-9 questionnaire items. Participants were asked how often they had been bothered by each symptom over the past two weeks, with responses ranging from 0 (not at all) to 3 (nearly every day).

Appendix C (for Chapter 4, Long COVID)



Supplementary Figure 4.1: Correlation plot of the most discriminatory urine metabolites and questionnaire data. The colour scale of the circles corresponds to the Pearson Correlation Coefficient (R value) as indicated in the bar on the right. The size of circles is proportional to the p value, with larger circles indicating greater significance. Dotted lines separate the metabolites from questionnaire scores. First/after 6 weeks refers to the number of symptoms experienced at these times. PHQ-9: Patient Health Questionnaire, FSS: Fatigue Severity Scale, PCS-12: Physical Component Summary of the 12-Item Short Form Health Survey (SF-12), MCS-12: Mental Component Summary of the 12-Item Short Form Health Survey. Note that valine correlated with PCS scores and the levels of histidine and Indoxysulfate were inversely correlated the PCS scores.

Appendix D (for Chapter 5, EXPLAIN)

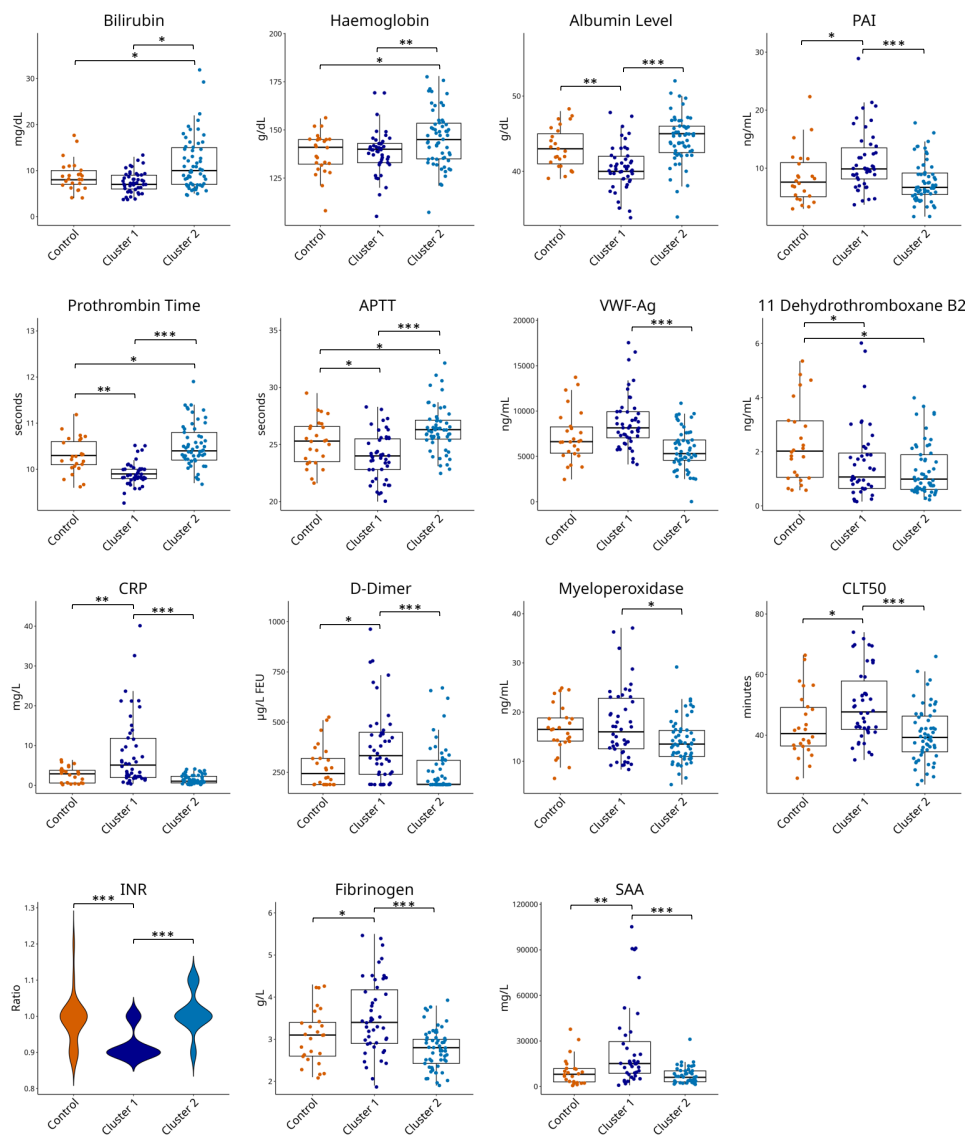


Figure S5.1 Haematological, inflammatory, and coagulation-related biomarkers in controls and two Long COVID subgroups. Box-and-whisker plots (and violin plot for INR) showing levels of all variables used for unsupervised clustering, presented across all groups: healthy controls (orange), Long COVID Cluster 1 (dark blue), and Long COVID Cluster 2 (light blue). Clustering input involved: Liver function and blood proteins: Bilirubin, Haemoglobin, Albumin; Coagulation and endothelial activation markers: PAI, Prothrombin Time, APTT, INR, VWF-Ag, Fibrinogen, CLT50, and D-Dimer; Inflammatory and oxidative stress markers: CRP, Serum Amyloid A (SAA), and Myeloperoxidase; Platelet activation marker: 11-Dehydrothromboxane B2. Statistical comparisons were performed between groups using appropriate tests (ANOVA or Kruskal-Wallis with post-hoc testing). Significance is denoted as: $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$.

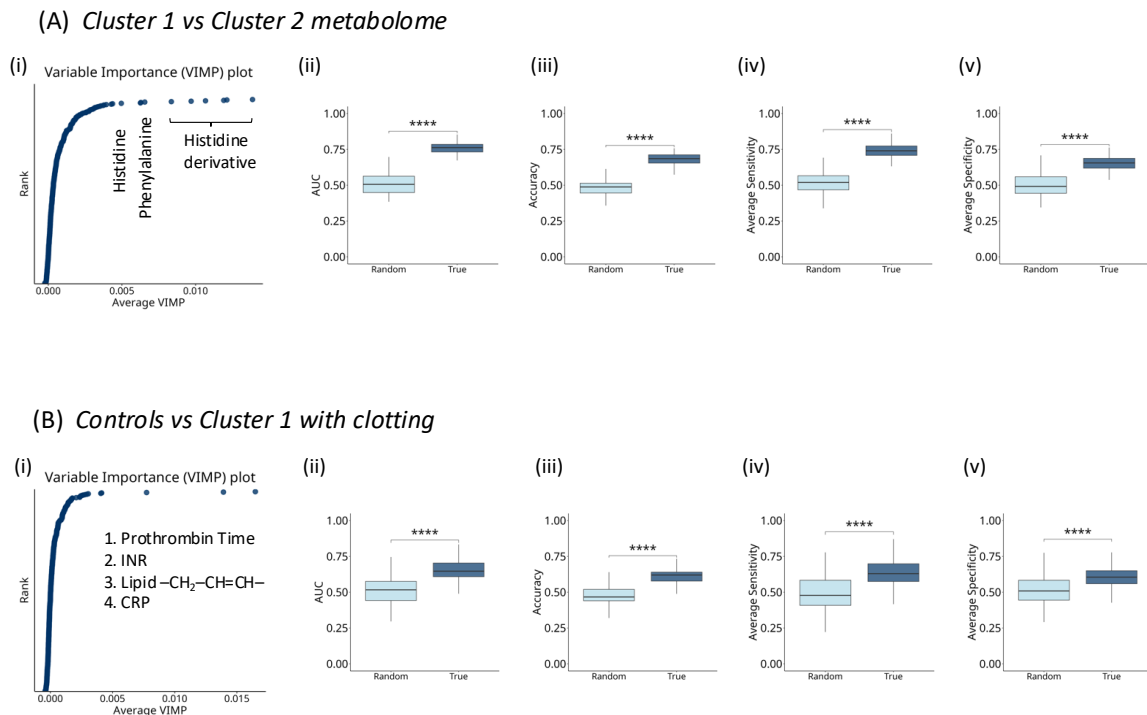


Figure S5.2. Discriminative features and classification performance distinguishing Long COVID subgroups and controls. (A) Cluster 1 vs Cluster 2 metabolome. (i) Variable Importance (VIMP) plot identifying the most discriminative metabolites separating Long COVID Cluster 1 (characterised by elevated levels of markers associated with inflammation and hypercoagulability) and Cluster 2 (more similar to controls in these measures). Notably, serum histidine, phenylalanine, and a histidine derivative show high importance. (ii–v) Performance of a Random Forest Classification model trained to distinguish Cluster 1 from Cluster 2 using metabolomic data alone. Boxplots show comparisons between true model performance (dark blue) and a randomly permuted model (light blue) for: (ii) Area under the ROC curve (AUC), (iii) Accuracy, (iv) Average sensitivity, (v) Average specificity, **** $p < 0.0001$ for all comparisons. (B) Controls vs Cluster 1 with clotting-related variables. (i) VIMP plot highlighting top discriminative features for distinguishing healthy controls from Long COVID Cluster 1. The top variables include Prothrombin Time, INR, a lipid signal ($-\text{CH}_2-\text{CH}=\text{CH}-$), and CRP. (ii–v) Classification model performance metrics as in (A), demonstrating strong discriminatory power using these features. **** $p < 0.0001$ for all comparisons. Boxplots display distribution of metric values across repeated model runs. Collectively, these analyses suggest distinct metabolic and clotting profiles in Long COVID subtypes, which can be robustly classified using key biomarkers.

Appendix E (for Chapter 6, Fibromyalgia)

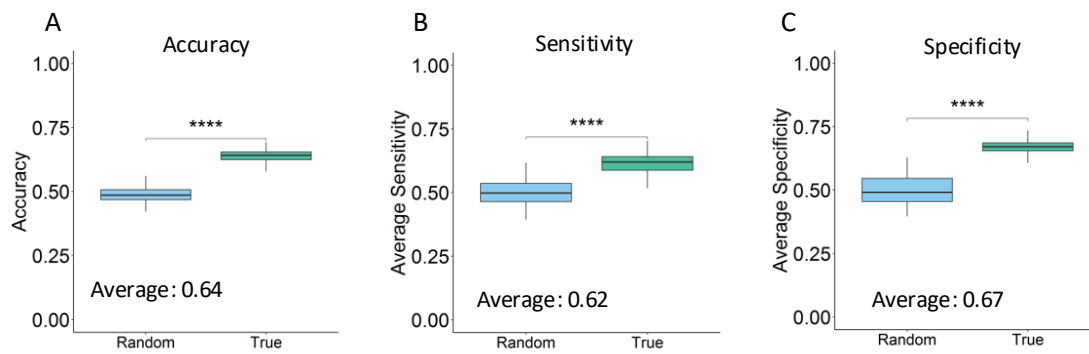


Figure S6.1. Model performance metrics from the initial Random Forest (RF) model distinguishing individuals with fibromyalgia (FM) from pain-free controls using CSF and serum metabolomic data. Boxplots show cross-validated performance metrics comparing the true model (green) to a label-permuted random model (blue). Metrics include: **(A)** Classification accuracy (average: 0.64), **(B)** Sensitivity (true positive rate; average: 0.62), and **(C)** Specificity (true negative rate; average: 0.67). The true model significantly outperformed the random control across all metrics (**** $p < 0.0001$).

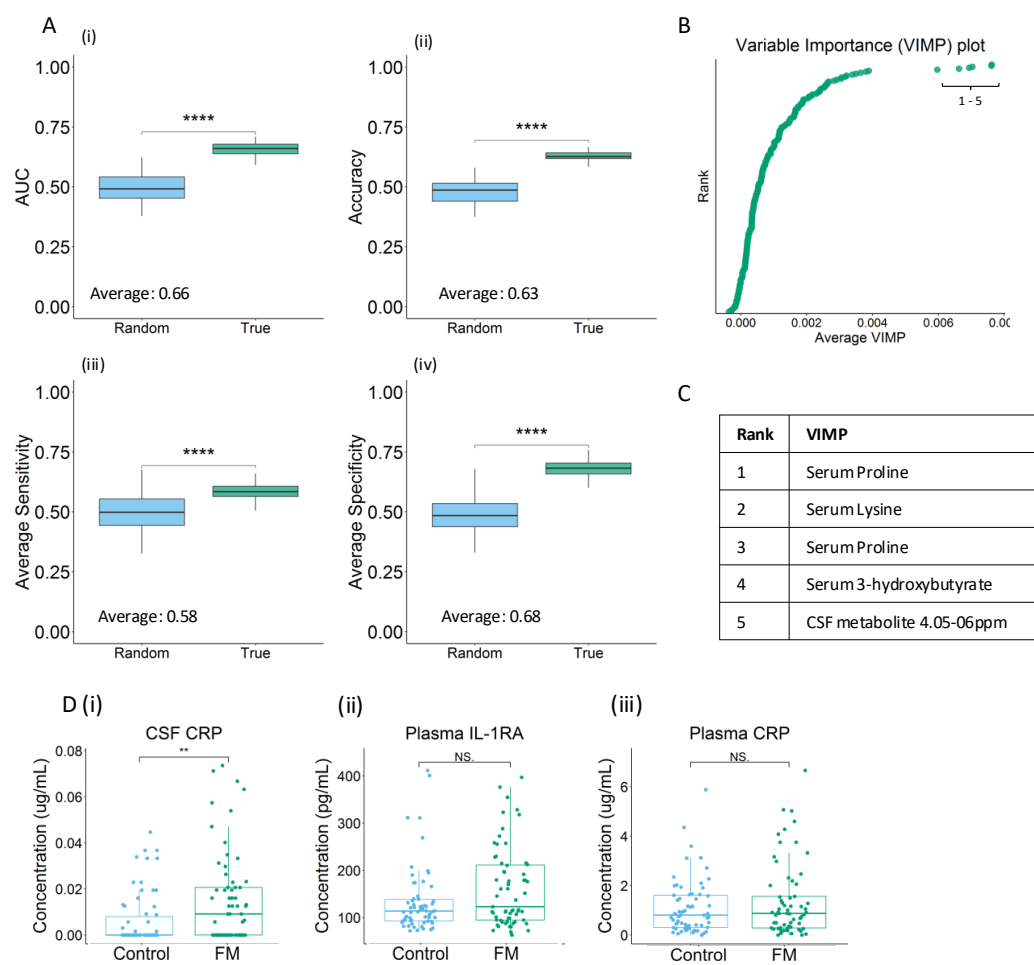


Figure S6.2. Random Forest model distinguishing fibromyalgia (FM) from age- and BMI-matched controls retains significance. Matching was performed using the ‘MatchIt’ package in R, and the model was built using all available blood and CSF metabolites and proteins. A) Performance metrics of the Random Forest model comparing true and label-permuted random models across: (i) Area under the ROC curve (AUC; average: 0.66), (ii) Classification accuracy (average: 0.63), (iii) Sensitivity (average: 0.58), (iv) Specificity (average: 0.68). True models significantly outperformed random models in all metrics (**** $p < 0.0001$). B) Variable Importance (VIMP) plot showing the ranked importance of features contributing to model accuracy. C) Top five metabolites ranked by average VIMP score: serum proline, serum lysine, serum proline (distinct spectral bin), serum 3-hydroxybutyrate, and a CSF metabolite in the 4.05–4.06 ppm range. D) Univariate comparisons between FM and matched controls: (i) CSF CRP remained significantly elevated in FM, (ii) Plasma IL-1RA trended toward significance, (iii) Plasma CRP showed no significant difference.

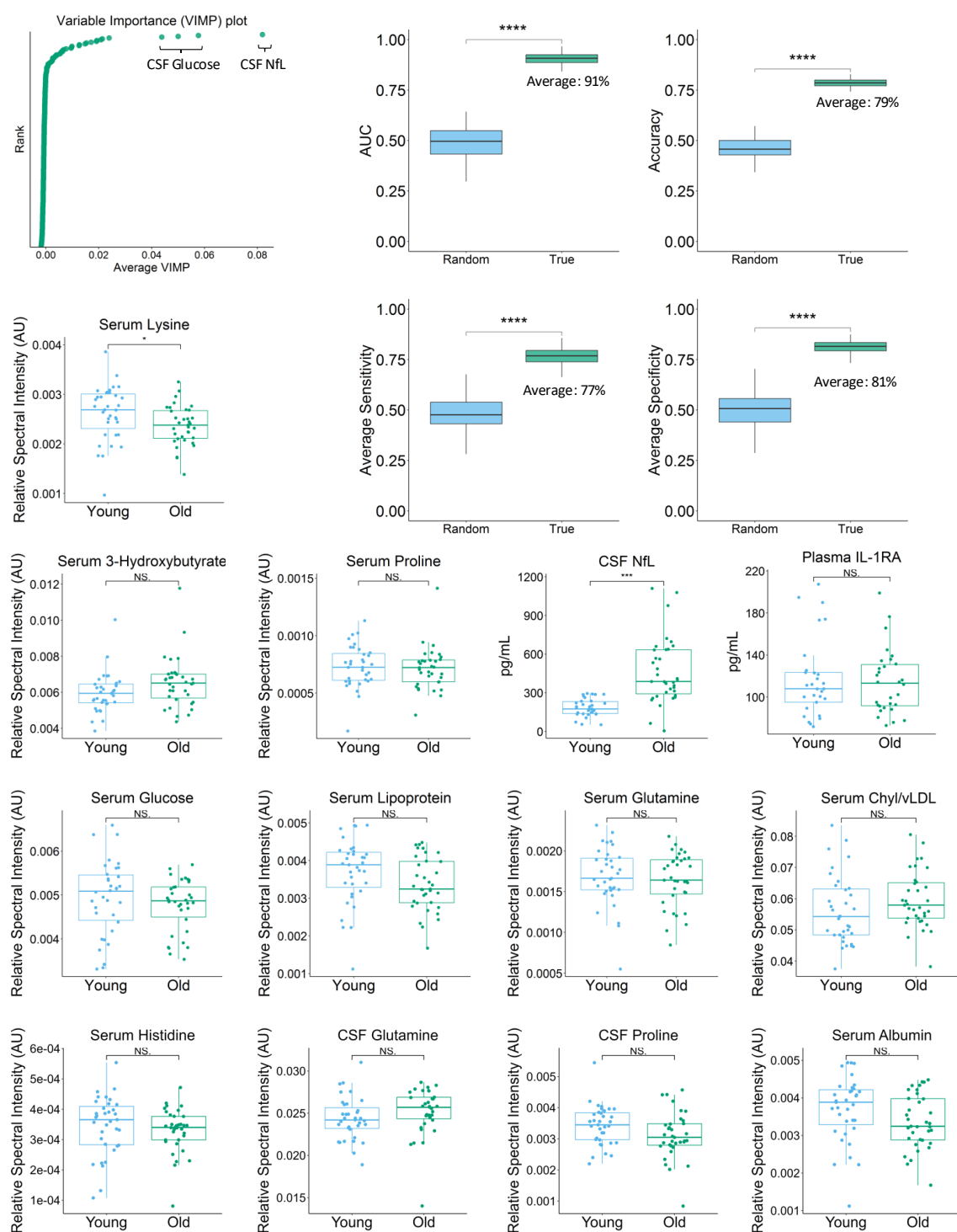


Figure S6.3. Random Forest model testing the effect of age on metabolomic profiles in pain-free controls. A) Random Forest classifier was used to distinguish control participants above and below the age of 33, a threshold selected to ensure equal class sizes for "young" and "old" groups. Top row: Performance metrics of the model show significant separation between age groups, with high predictive value: AUC (average: 0.91), Accuracy (average: 0.79), Sensitivity

(average: 0.77), Specificity (average: 0.81), all outperforming the random model ($****p < 0.0001$). Top left: Variable Importance (VIMP) plot identifies CSF glucose and CSF neurofilament light chain (NfL) as the top-ranked features contributing to age classification. Bottom panels: Univariate comparisons of selected serum and CSF metabolites and inflammatory markers between younger and older FM participants. Only serum lysine was significantly different between groups ($p < 0.05$); all other comparisons were not statistically significant.

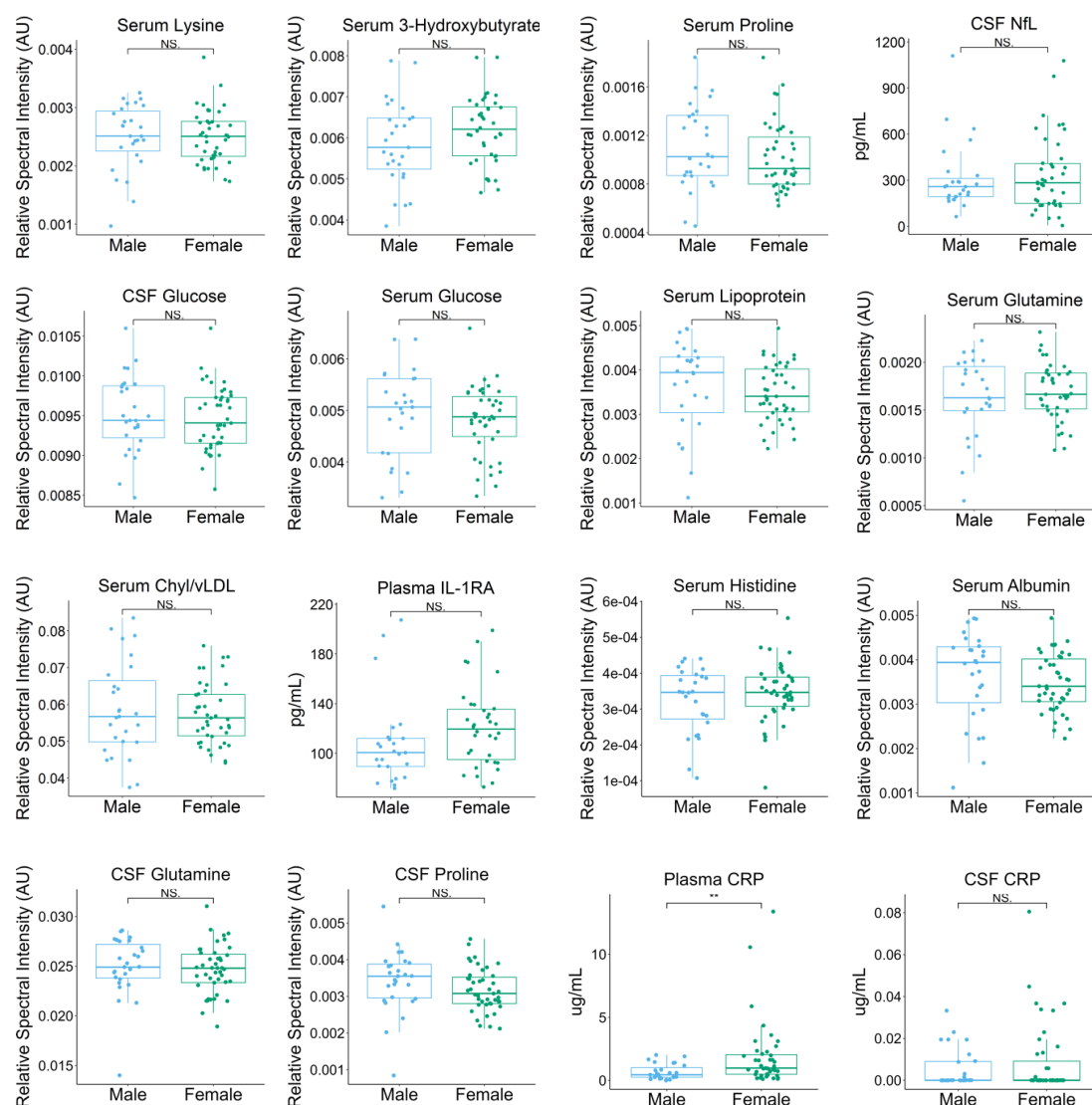


Figure S6.4. Sex-based analysis of top-ranked metabolites in pain-free controls reveals minimal differences apart from plasma CRP. Boxplots show serum and CSF metabolite and protein levels in pain-free male and female participants. These include the top variables identified in the fibromyalgia (FM) versus control model. None of the variables that contributed to FM classification differed significantly between sexes, indicating that sex is

*metrics and CSF IL-1 receptor antagonist (IL-1RA) concentrations across control participants and the two FM sensory subgroups identified via hierarchical clustering (Cluster 1: relatively preserved pain thresholds; Cluster 2: hyperalgesic profile). Significant differences were observed across several QST parameters, including: Right cPPT and Right cPTT (cuff pressure pain threshold/tolerance), TSP ratio (temporal summation of pain), HDT and HPT (heat detection and pain thresholds), Pinprick and TSP pain areas. Cluster 2 consistently showed greater pain sensitivity and larger pain areas than both controls and Cluster 1. CSF IL-1RA levels were elevated in FM participants overall but did not significantly differ between clusters, suggesting that central inflammatory signalling may not fully explain sensory phenotype divergence. p values: $p < 0.05$ (), $p < 0.01$ (), $p < 0.001$ (), $p < 0.0001$ (****), NS = not significant.*

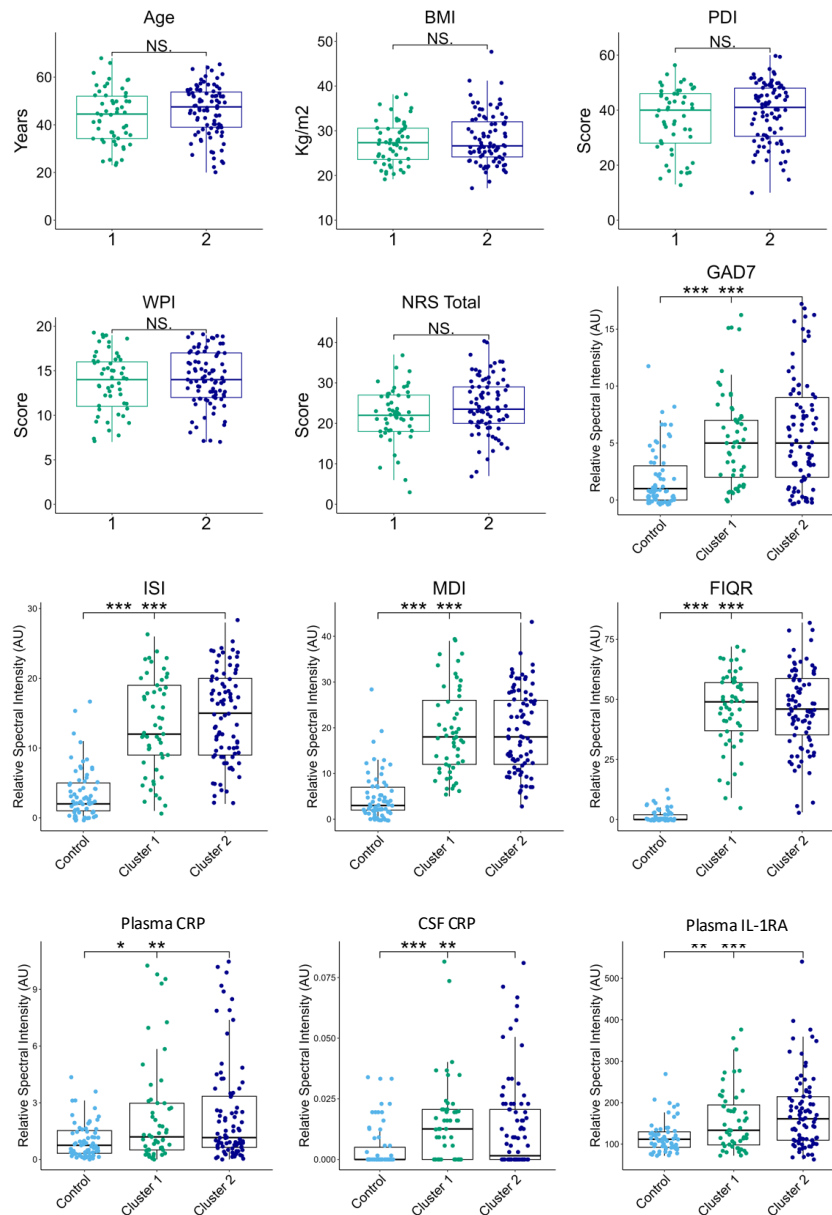
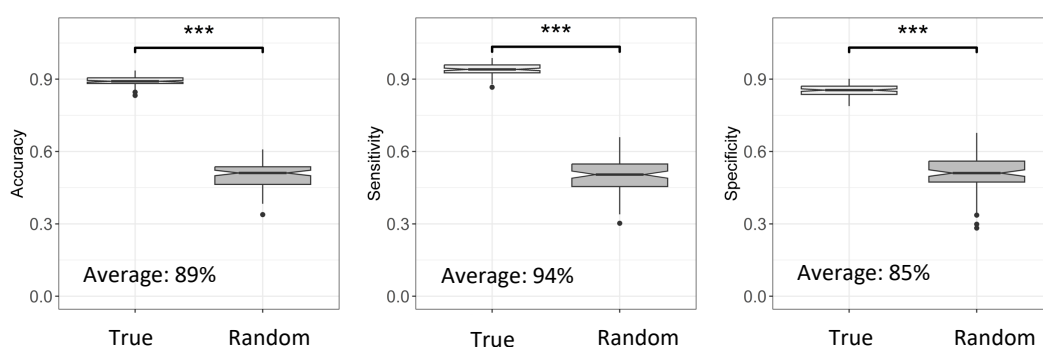


Figure S6.6. Comparison of clinical, psychological, and inflammatory measures across fibromyalgia (FM) sensory subgroups and controls. Boxplots show demographic variables, symptom burden scores, and inflammatory marker levels across pain-free controls and the two FM subgroups (Cluster 1 and Cluster 2, defined by sensory phenotype). Top row: No significant differences in age or body mass index (BMI) were observed between FM clusters, confirming these are unlikely to confound sensory subtype differences. Second row: Clinical pain measures – Pain Disability Index (PDI), Widespread Pain Index (WPI), and Numerical Rating Scale (NRS) total – did not significantly differ between clusters. Middle row: Both FM subgroups exhibited significantly higher scores for anxiety (GAD-7), insomnia (ISI), depression (MDI), and functional impact (FIQR) compared to controls, but these scores did not differ significantly between Cluster 1 and Cluster 2. Bottom row: Plasma and CSF CRP levels, as well

*as plasma IL-1RA, were significantly elevated in both FM subgroups relative to controls, with no difference between clusters. These findings suggest that while psychological and inflammatory burden is consistently elevated in FM, it does not account for the observed divergence in sensory profiles. p values: $p < 0.05$ (), $p < 0.01$ (), $p < 0.001$ (), $p < 0.0001$ (****), NS = not significant.*

Appendix F (for Chapter 7, Osteoarthritis)

CSF



Serum

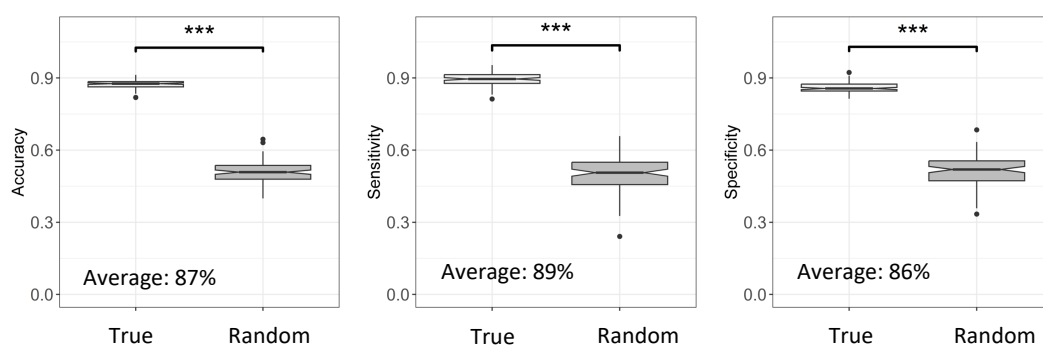


Figure S7.1. OPLS-DA classification model performance compared to random label permutation. Box plots show the classification accuracy of orthogonal partial least squares–discriminant analysis (OPLS-DA) models generated from CSF and serum metabolomics data. True group labels (OA vs. control) are compared to null distributions derived from multiple random permutations of group labels. The consistently higher accuracy, sensitivity, and specificity of the true models versus the random models demonstrates that the observed group separation exceeds what would be expected by chance, supporting the robustness of the OPLS-DA findings.

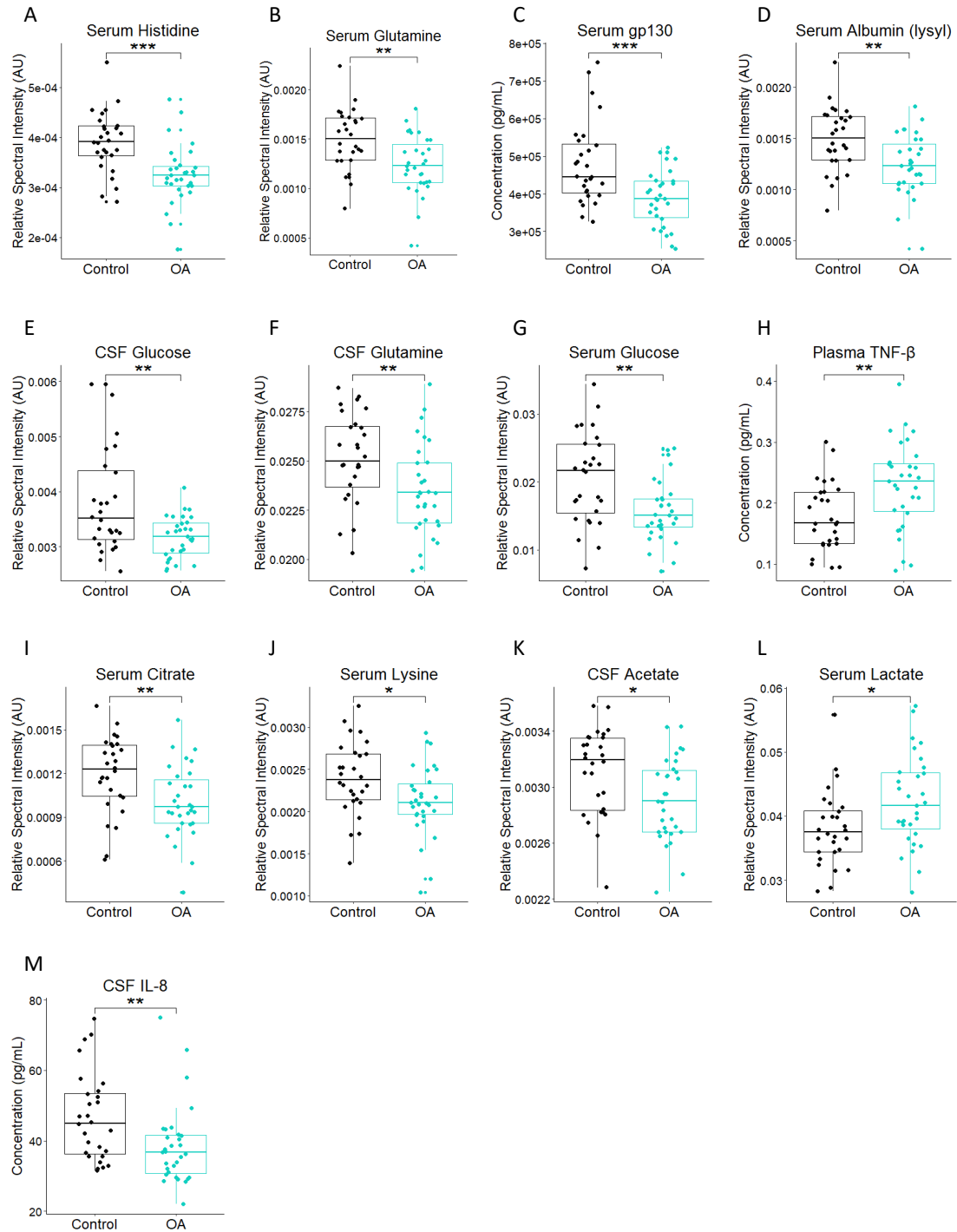


Figure S7.2. Key serum and CSF metabolites differentiating osteoarthritis and control participants in age- and BMI-matched subgroups. Boxplots display metabolite concentrations in osteoarthritis patients ($n=31$) and pain-free controls ($n=28$) matched for age and BMI. Metabolites were selected based on their discriminative value in multivariate analyses from the full cohorts. Asterisks indicate statistical significance based on unpaired t-tests: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. These findings highlight the robustness of histidine,

glutamine, and related metabolite alterations even after accounting for demographic confounders.

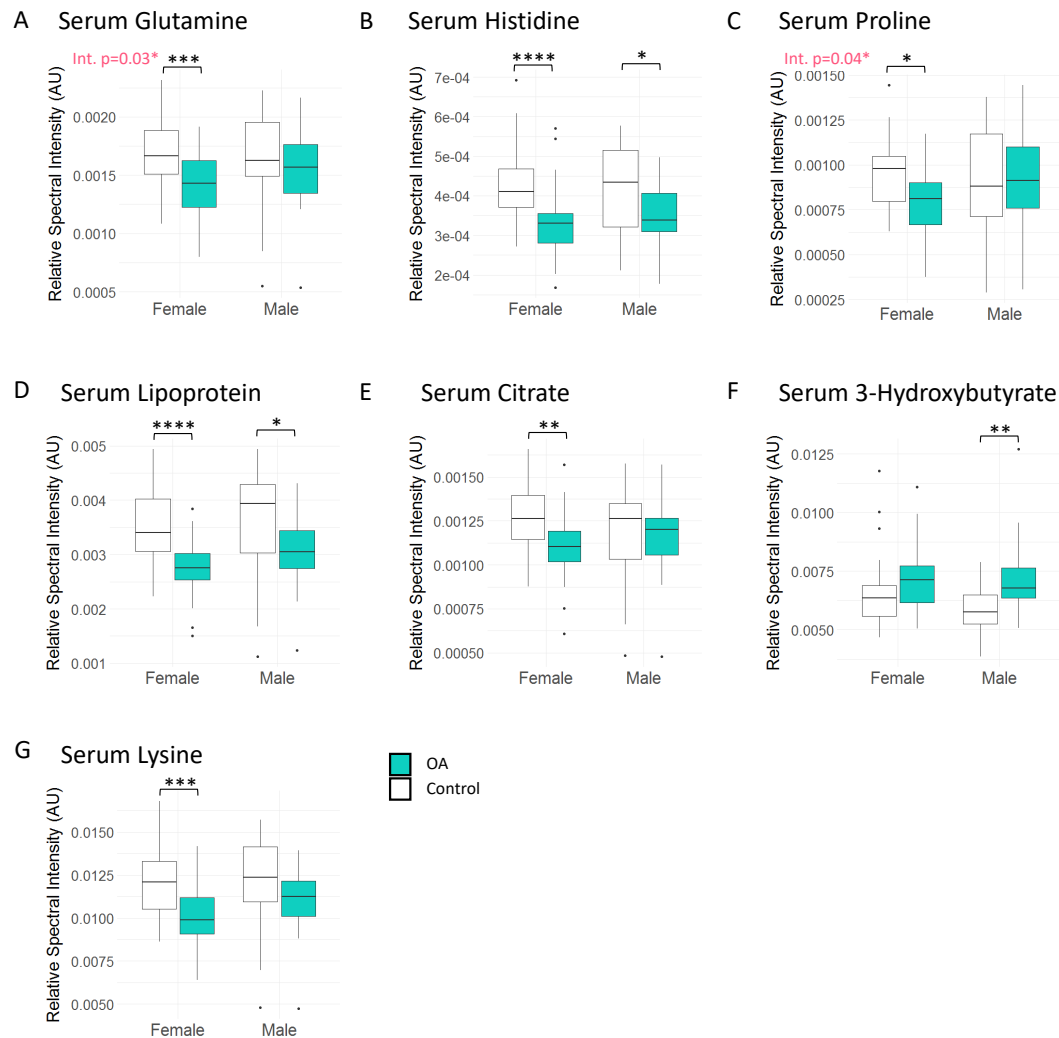


Figure S3. Sex-stratified analysis of key serum metabolites contributing to osteoarthritis classification models. Box plots depict the relative levels of serum metabolites identified as top variable importance in projection (VIP) scores from orthogonal partial least squares–discriminant analysis (OPLS-DA) models stratified by sex. Data were analysed using two-way ANOVA; interaction *p*-values are shown in red. Post hoc Tukey tests were applied where significant main effects were present. Asterisks denote statistical significance: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. These results suggest sex-specific metabolic alterations in OA, particularly in glutamine, histidine, and lipoprotein-related measures.

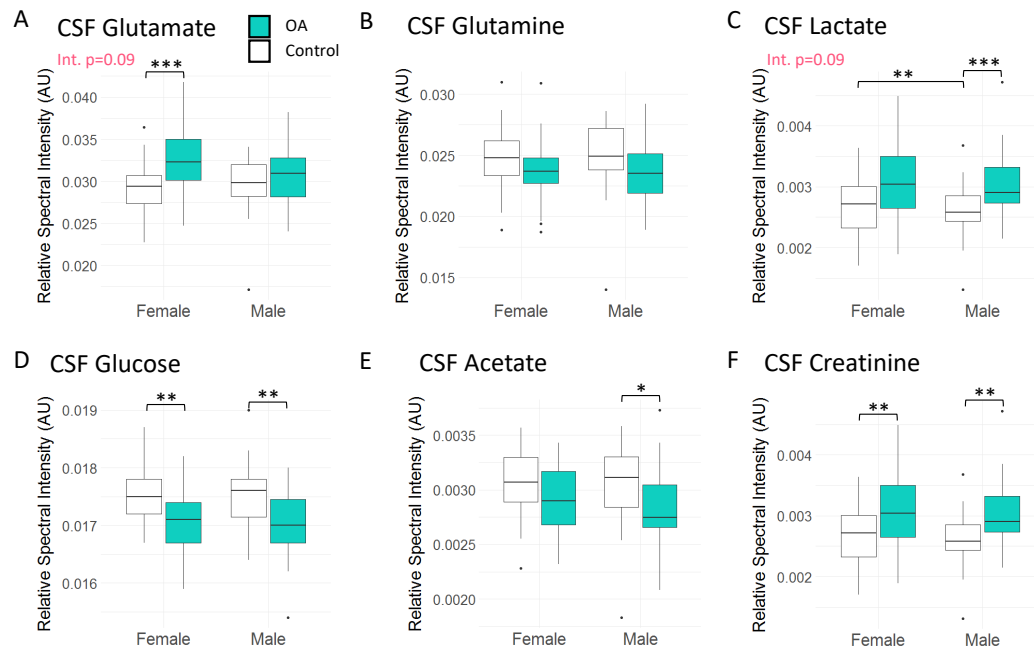


Figure S4. Sex-stratified analysis of key cerebrospinal fluid metabolites contributing to osteoarthritis classification models. Box plots display mean ($\pm$ SEM) CSF levels of glutamate, glutamine, acetate, glucose, lactate, and creatinine from sex-specific OPLS-DA models differentiating osteoarthritis patients and pain-free controls. Data were analysed using two-way ANOVA with factors for disease status and sex. Significant main effects were followed by post hoc Tukey tests. Asterisks indicate levels of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Interaction p-values are shown in red. Results highlight sex-specific alterations in central energy metabolism and neurotransmitter-related pathways in OA.

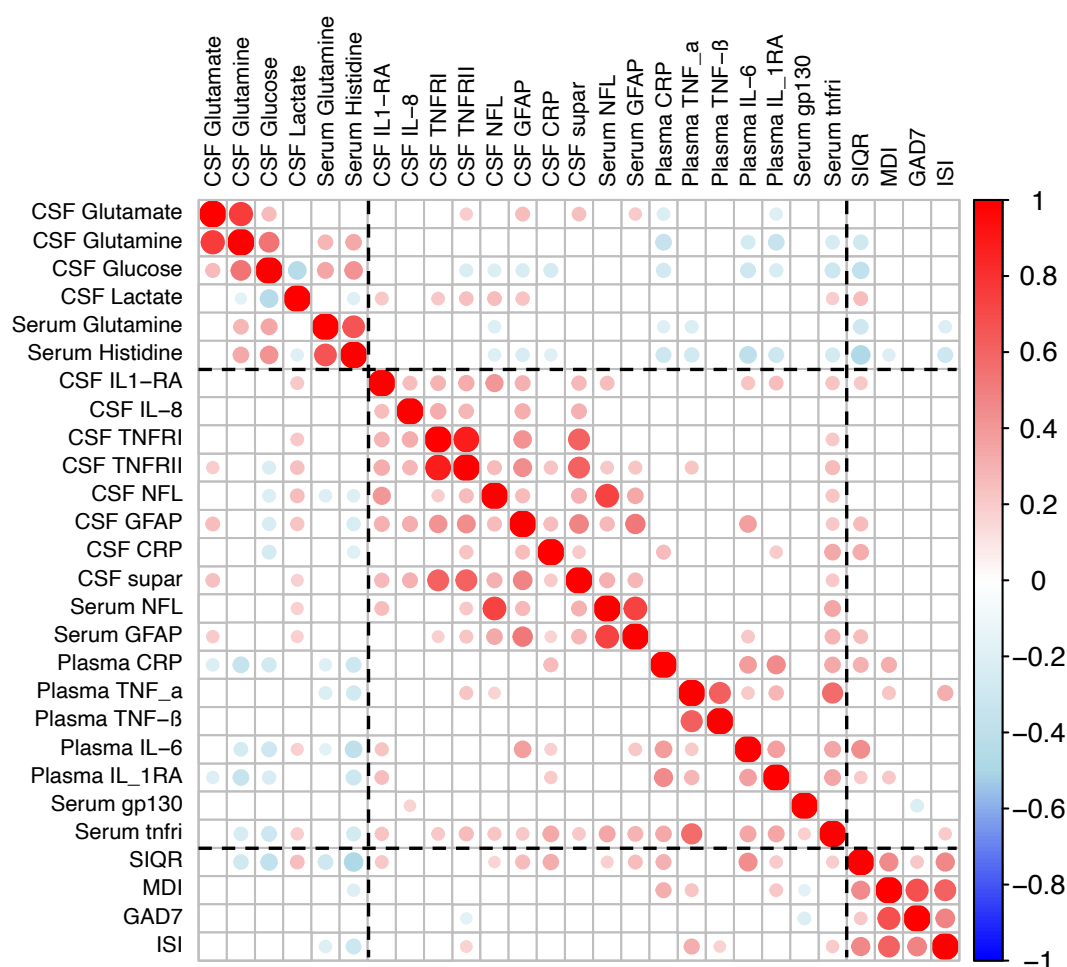


Figure S5. Correlations between key metabolites and protein biomarkers in osteoarthritis and control participants. Heatmap displaying Pearson correlation coefficients between select cerebrospinal fluid (CSF) and serum metabolites and inflammatory or neurodegenerative protein biomarkers across all study participants (OA and controls). Metabolites were selected based on their variable importance in projection (VIP) scores from the OPLS-DA model. Strong correlations were observed between glutamate, glutamine, and lactate with several central and peripheral immune markers, including TNFR1, TNFR2, IL-1RA, and GFAP, suggesting metabolic-immunological interactions relevant to OA pain pathophysiology.

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