

The discovery and validation of biomarker candidates of immune dysfunction in Motor Neuron Disease

By

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Bachelor of Medical Science (Honours)

A thesis submitted for the degree of

Doctor of Philosophy

November 10th, 2025

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ABSTRACT

Urine shows potential as a source of biomarkers, offering opportunities to reduce patient heterogeneity in clinical trials for Motor Neuron Disease (MND). To reduce heterogeneity, multiple biomarkers are needed to reflect disease pathology (Rogers et al., 2023), complementing serum neurofilament light. Our laboratory has identified urinary neopterin as a pro-inflammatory biomarker that increases over disease progression (Shepherd et al., 2022) which might have value as a predictive marker of response to anti-inflammatory therapy; however, improved analytical methods are needed. We aim to discover and validate immune biomarkers of MND prognosis in urine using Liquid Chromatography-Mass Spectrometry (LCMS), gene ontology (GO) analysis, and baseline neurological data.

From a cohort of 78 people with MND at baseline (first visit to neurologist) and 39 controls, two studies were completed. Neopterin was measured using multiple reaction monitoring (MRM) on a Thermo Altis QQQ mass spectrometer (MS). Neopterin quantified by LCMS was compared to an inhibition ELISA (Shepherd et al., 2022) in a subset of 44 MND and 19 controls. Differentially expressed urinary proteins were identified in a subset of 39 MND and 26 controls using a Thermo Orbitrap Exploris-480 MS in Data-Dependent Acquisition (DDA) mode for spectral library generation and Data-Independent Acquisition (DIA) mode for differential abundance analysis using Spectronaut. PANTHER was used for GO pathway analyses to identify candidates associated with neuroinflammation in MND. 10 candidate urinary proteins were verified using parallel reaction monitoring (PRM) and assessed for prognostication using clinical data (ALSFRS-R, Δ FRS, onset, age, gender, and time to death/ventilation) and SPSS.

Urinary neopterin quantified by LCMS/MRM correlated significantly with an inhibition ELISA in 44 people with MND ($r=0.78$, $p<0.0001$) (Shepherd et al., 2022). Neopterin levels were increased in 78 people with MND at baseline compared to 39 controls (170.7 ± 73.2 vs 135.2 ± 44.7 μ mol neopterin/mol creatinine, $p=0.0017$) and was a weak, but independent predictor of prognosis (HR: 1.005, 95% CI:1.001-1.010, $p=0.019$). LCMS in DDA mode discovered 1,970 protein groups in the urinary proteome, with DIA mode detecting 1,350 protein groups common between MND and controls, including ≥ 257 differentially changed proteins found in MND ($q<0.01$, >1.5 -fold difference). GO analysis highlighted key changes in immune alteration, cell death, and growth-factor receptor activity pathways. 10 protein candidates associated with neuroinflammation, neurodegeneration, and muscle degeneration were verified by PRM, identifying significant differences in 7 proteins at baseline. Cathepsin D

(P07339, $r=-0.34$, $p=0.035$) and Fibulin-5 (Q9UBX5, $r=-0.35$, $p=0.031$) were significantly correlated to ALSFRS-R scores, however, when compared to prognostic factors neither could add value beyond the Δ FRS (HR: 2.60, 95% CI:1.49-4.55, $p<0.001$) or baseline urinary p75^{ECD} quantified by ELISA (HR: 1.27, 95% CI:1.08-1.49, $p=0.004$). Ongoing validation is required in larger sample sizes and different MND phenotypes.

Urine is a valuable biofluid for discovering immune-related biomarkers and candidate proteins in MND. Ongoing validation studies using PRM mass spectrometry and stable isotope standards (SIS) will quantify predictive candidates of inflammation with absolute quantitative accuracy to assess their prognostic value. Panels of urine-based prognostic biomarkers will hopefully improve patient selection in clinical trials and reduce MND heterogeneity.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text; and has been completed without the use of generative artificial intelligence tools.

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Signed.....

Vassilios Karnaros, June 2025.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to everyone who has supported me throughout the course of this thesis and my academic journey at Flinders University. I am deeply grateful to my supervisor, Associate Professor Mary-Louise Rogers, for her invaluable guidance, continuous encouragement, and endless constructive feedback throughout every stage of my candidature. This work would not have been possible without your support, particularly in securing the funding that made it a reality. Your mentorship has been instrumental in shaping both this thesis, and my development as a researcher.

Special thanks go to my co-supervisor, Associate Professor Timothy Chataway, whose exceptional expertise in mass spectrometry laid the foundation for this project. Your insightful and technical knowledge was not only instrumental in shaping the design of this project, but also in inspiring my own passion for the field. I am truly grateful for the wisdom, patience, and passion you brought to every discussion.

I would like to extend my heartfelt thanks to Associate Professor David Schultz, the head neurologist of the 4th Generation MND clinic, for his ongoing support and commitment to advancing research into MND. I am profoundly grateful to the healthy individuals and people living with MND who have generously donated biological samples for this research as per HREC #229.11 approved by SALN. Their selfish contribution, made with the knowledge that they themselves would not benefit, reflects their extraordinary strength and altruism. This work would not have been possible without their generosity.

Further, my sincere appreciation to the Flinders University proteomics facility, including Dr. Alex Colella, Ms Nusha Chegeni, and Ms Kamelya Aliakbari for their invaluable contributions. I am especially thankful for the ongoing generosity with their time, expertise, and support throughout this project. Thank you to my fellow past and present lab members and friends, Dr. Shephanie Shepheard, Dr. Megan Fowler, and Ms Danielle Renfrey for their invaluable company and support during what was often a challenging and stressful journey, and to Yuri Ogawa for her expertise in MatLab scripts that contributed to this research.

Also, a big thank you to our collaborators, Associate Professor Beben Benyamin from the University of Adelaide, Professor Michael Benatar and Joanne Wu from the University of Miami, Professor Andrea Malaspina from University College London, and Professor Paul Lingor from the Technical University of Munich.

I would like to gratefully acknowledge the support of our funding bodies including Fight MND, MND SA, and the Australian Government Research Training Program (RTP) Scholarship. I am especially thankful to Fight MND for supporting my PhD scholarship, and to MND SA for supporting my attendance at two international ALS/MND conferences where I had the honour of presenting my work by platform presentation. These funding bodies have provided key experiences in my early career development as a researcher.

On a personal note, I am especially thankful to my family, partner, friends, and their extended families for the unwavering support, love, and patience they all provided throughout this process. Thank you for your encouragement and companionship, your belief in me kept me grounded and motivated. This thesis would not have been possible without the support from these individuals, and I am deeply thankful to each one of you.

PUBLICATIONS

Urinary neopterin: A novel biomarker of disease progression in amyotrophic lateral sclerosis. S. R. Shepherd, V. Karnaros, B. Benyamin, D. W. Schultz, M. Dubowsky, J. Wu, et al. Eur J Neurol 2022 Vol. 29 Issue 4 Pages 990-999. Accession Number: 34967083 PMCID: PMC8901536 DOI: 10.1111/ene.15237. Included in *Chapter 3* within *section 3.2*.

Urinary biomarkers for amyotrophic lateral sclerosis: candidates, opportunities and considerations. M.-L. Rogers, D. W. Schultz, V. Karnaros and S. R. Shepherd. Brain Communications 2023 Vol. 5 Issue 6 Pages fcad287. DOI: 10.1093/braincomms/fcad287. Included in *Chapter 1* within *section 1.4*

PRESENTATIONS

(2024). "Platform Communications: Abstract Book 35th International Symposium on ALS/MND (Complete printable file)." Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration 25(sup1): 1-92. **C125 Detection and discovery of urinary biomarkers of immune dysfunction for amyotrophic lateral sclerosis.** V. Karnaros, B. Benyamin, D. Schultz, J. Wu, A. Malaspina, T. Chataway, M. Benatar and M.L. Rogers.

(2023). "Theme 10 - Disease Stratification and Phenotyping of Patients." Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration 24(sup1): 230-244. **DSP-06 Detection and discovery of urinary biomarkers of immune dysfunction for amyotrophic lateral sclerosis.** V. Karnaros, S. Shepherd, B. Benyamin, D. Schultz, A. Malaspina, M. Benatar, T. Chataway and M.L. Rogers.

(2023). Australian and New Zealand MND Research Symposium: rapid fire presentation. **Urinary biomarkers of immune dysfunction for motor neuron disease.** V. Karnaros, S. Shepherd, B. Benyamin, D. Schultz, A. Malaspina, M. Benatar, T. Chataway and M.L. Rogers.

(2023). Australian and New Zealand MND Research Symposium: rapid fire presentation. **Urinary biomarkers of immune dysfunction for motor neuron disease.** V. Karnaros, S. Shepherd, B. Benyamin, D. Schultz, A. Malaspina, M. Benatar, T. Chataway and M.L. Rogers.

(2022). Australian and New Zealand MND Research Symposium: poster presentation. **Optimising the measurement of urinary neopterin as a biomarker of motor neuron disease.** V. Karnaros, S. Shepherd, B. Benyamin, D. Schultz, A. Malaspina, M. Benatar, T. Chataway and M.L. Rogers. Best in clinical and patient focus award.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AGC	Automatic gain control
ALS	Amyotrophic Lateral Sclerosis
ALSFRS-R	Revised ALS functional rating scale
AUC	Area under the curve
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factors
BH4	5,6,7,8-tetrahydrobiopterin
BSA	Bovine serum albumin
C18	Carbon 18
C1q	Complement factor 1q
C3	Complement factor 3
C4	Complement factor 4
C9orf72	Chromosome 9 open reading frame 72 (gene/protein)
cGAS/STING	Cyclic GMP-AMP synthase/stimulator of interferon genes
CHIT-1	Chitinase-1
CI	Confidence interval
CID	Collision induced dissociation
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CV	Coefficient of variation
Da	Dalton
DAMPs	Damage-associated molecular patterns
DDA	Data-dependent acquisition
DIA	Data-independent acquisition
DNA	Deoxyribonucleic acid
DTI	Diffusion tensor imaging
DTT	Dithiothreitol
ECD	Extracellular domain
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FA	Formic acid
FDA	Food and Drug Administration
FDR	False discovery rate
fMND	Familial Motor Neuron Disease
FTD	Frontotemporal dementia
FUS	Fused in sarcoma
GCC	Gold Coast Criteria
GO	Gene ontology
H-ESI	Heated electrospray ionisation

HCD	Higher energy collisional dissociation
HI	Healthy individuals
HPLC	High performance liquid chromatography
HPLC-FD	High performance liquid chromatography-fluorescent detection
HPLC-MS	High performance liquid chromatography-mass spectrometry
HR	Hazard ratio
HRP	Horseradish peroxidase
IAA	Iodoacetamide
IFN	Interferon
IFN- γ	Interferon-gamma
IFN-1	Type 1 interferon response
IGF	Insulin-like growth factor
IL	Interleukin
KANK1	KN motif and ankyrin repeat domain-1
kDa	Kilodalton
LCMS	Liquid chromatography mass spectrometry
LLoD	Lower limit of detection
LLoQ	Lower limit of quantification
LMN	Lower motor neuron
m/z	Mass-to-charge ratio
MCP-1	Monochemoattractant protein-1
MeOH	Methanol
MHC	Major histocompatibility complex
MND	Motor Neuron Disease
MRI	Magnetic resonance imaging
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS1	Precursor ion scan
MS2	Product ion scan
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NfH	Neurofilament heavy chain
NfL	Neurofilament light chain
NH ₂ TP	7,8-Dihydroneopterin triphosphate
NK	Natural killer cell
NMJ	Neuromuscular junction
OPTN	Optineurin
OS	Oxidative stress
p75 ^{ECD}	Neurotrophin receptor p75 extracellular domain
PANTHER	Protein Analysis Through Evolutionary Relationships
PAV	Permanent assisted ventilation
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PFN1	Profilin 1

pNfH	Phosphorylated neurofilament heavy chain
ppm	Parts per million
pre-fMND	Pre-familial MND
PRM	Parallel reaction monitoring
PSM	Peptide specific match
Q1	Quadrupole 1
Q3	Quadrupole 3
QQQ	Triple quadrupole
rEEC	Revised EI Escorial criteria
RF	Radiofrequency
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROC	Receiver operator characteristic
ROS	Reactive oxygen species
RT	Retention time
S/N	Signal-to-noise
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIS	Standard isotope standard
SOD1	Glycine to alanine substitution, position 93 on the superoxide dismutase gene
SQSTM1	Sequestome-1
TDP-43	Transcriptive response DNA binding protein 43 (protein coded by TARDBP)
TGA	Therapeutic goods administration
TGF- β	Transforming growth factor-beta
THP-1	Human leukemia monocytic cell line
TIC	Total ion current
TMB	3,3',5,5'-tetramethylbenzine
TNF- α	Tumour necrosis factor-alpha
TUBA4a	Tubulin 4A
UBQLN2	Ubiquilin-like protein 2
UMN	Upper motor neuron
Uosm	Urinary osmolality
UPS	Ubiquitin proteasome system
USG	Urine specific gravity
VCP	Valosin-containing protein
VSMC	Vascular smooth muscle cells
WHO	World health organisation
XIC	Extracted ion chromatogram
z	Charge

CHAPTER 1.

INTRODUCTION

The review article summarising **Urinary biomarkers for Amyotrophic Lateral Sclerosis: candidates, opportunities, and considerations**. M.-L. Rogers, D. W. Schultz, V. Karnaros and S. R. Shepheard. Brain Communications 2023 Vol. 5 Issue 6 Pages fcad287. DOI: 10.1093/braincomms/fcad287 includes part of this introduction from *section 1.4* regarding urinary neopterin as a candidate biomarker for MND.

1.1 Motor Neuron Disease

This thesis is concerned with the discovery and validation of biological markers of immunological dysfunction in Motor Neuron Disease (MND). This chapter provides a comprehensive review on biomarkers for MND, with an emphasis on biomarkers that can characterise the immune response.

1.1.1 Neurodegenerative diseases

Motor Neuron Disease (MND) is also known as Amyotrophic Lateral Sclerosis (ALS). MND is a neurodegenerative disease causing progressive muscular paralysis by the death of motor neurons, revoking the innervation of the skeletal muscles responsible for walking, talking, breathing, and swallowing (Andersen and Al-Chalabi, 2011). MND involves the death of both upper motor neurons (UMN) and lower motor neurons (LMN) (Garg et al., 2017). The cell bodies of UMNs reside in the primary cortex and cranial nerves of the brainstem and are responsible for the initiation of voluntary movements, while LMN cell bodies reside in the grey matter of the anterior horn of the spinal cord (Valetdinova et al., 2015). The axonal processes of these upper and lower motor neurons form parts of the corticospinal, pyramidal, and extrapyramidal tracts, augmenting the roots of the spinal cord which synapse with individual skeletal muscle fibres to constitute the neuromuscular junction (NMJ), facilitating voluntary muscle movements (Desai and Swash, 1999). MND is characterised by the progressive degeneration of both upper and lower motor neurons in the motor cortex, brainstem, and spinal cord, leading to muscle atrophy causing premature death from respiratory failure (Hardiman et al., 2017).

Jean-Martin Charcot was the first to describe the pathological findings of individuals passing from a degenerative muscle disease in 1869, identifying the atrophy of muscle fibres and hardening of the anterior and lateral corticospinal tracts (lateral sclerosis) in MND (Kumar et al., 2011). The disease was named ALS, referred to as MND in Australia and the United Kingdom (Faravelli et al., 2014). The detrimental consequences of MND were seen in 1939 when the famous baseball player Lou Gehrig succumbed to the hallmark degeneration of upper and lower motor neurons (Martin et al., 2017) shortly following his diagnosis (Lewis and Gordon, 2007). From this point MND was distinguished from other pure upper or lower motor neuron diseases (Garg et al., 2017) by their long duration and gradual decline, compared to the rapid progression, degeneration, and dysphonia involved in MND, debilitating patients within two to three years of diagnosis (Longinetti and Fang, 2019, Andersen and Al-Chalabi, 2011). Pure LMN diseases include progressive muscular atrophy

and bulbar muscular atrophies (Martin et al., 2017), compared to the degeneration of UMNs observed in primary lateral sclerosis (Faravelli et al., 2014).

There is no known cure and so far, only two disease-modifying outcomes have been approved for treatment of sporadic MND, riluzole and edavarone. Riluzole is approved for use in Australia through the pharmaceutical benefits scheme (PBS) and therapeutic goods administration (TGA) (Khamaysa and Pradat, 2022), slowing disease progression to extend life by two to three months (Masrori and Van Damme, 2020). Edaravone is the only other treatment approved by the TGA for use in Australia, which has inconclusive and poor clinical evidence (O'Neill et al., 2024b), and tofersen is a treatment available only to people who carry mutations in the SOD1 gene (Miller et al., 2022), but is not yet approved by the TGA. This demonstrates the limited translation success of treatments from animal models to human trials (van Eijk et al., 2020). One factor contributing to the lack of breakthrough therapies is the difficulty in clinical trial design to demonstrate the efficacy and benefits of new drug candidates in phase 2 (Oskarsson et al., 2018, Goyal et al., 2020). This challenge has been attributed to poor patient selection protocols in a disease that is highly heterogeneous in the clinic, where there is a paucity of tools to identify individuals that share a similar MND phenotype and stratify individuals based on anticipated disease trajectory.

Previous research in our MND and neurotrophic research laboratory has identified the extracellular domain of the p75 receptor (p75^{ECD}) (Shepherd et al., 2017) as a novel urinary biomarker for MND, increasing with disease and showing potential usefulness in clinical trials for MND (Gold et al., 2019). Expression of the p75 neurotrophin receptor is known to be upregulated in MND compared to healthy individuals (Seeburger et al., 1993). The extracellular domain is cleaved from sick and dying neurons in neurodegenerative diseases (Kenchappa et al., 2010) and removed from the body by renal filtration, excreted as urinary p75^{ECD} (Shepherd et al., 2014). Urinary p75^{ECD} is joined by serum neurofilament light (NfL) as a generalised marker of neurodegeneration (Lu et al., 2015, Benatar et al., 2020, Benatar et al., 2024b) that has been used in phase 2 clinical trial design (Gold et al., 2019, Cadavid et al., 2019, Vucic et al., 2020b, Benatar et al., 2024c, Koch et al., 2024, Benatar et al., 2025). Building upon our previous studies, I propose that identifying predictive markers associated with neuroinflammation in MND will be useful to select homogenous patients with similar disease characteristics for clinical trials based on aspects of disease pathology (Malaspina et al., 2015).

1.1.2 MND Epidemiology – what is the incidence and prevalence of MND

Recent epidemiological studies continue to identify a worldwide increase in incidence and prevalence of MND (Longinetti and Fang, 2019). Global registers reported an average incidence of 1.96 per 100,000 person-years with 95% confidence (Chiò et al., 2013). This has increased by 36% over 25 years for a traditionally rare neurodegenerative disorder, where for 77% of studies, (Leighton et al., 2019) saw incidences peak between 60 and 75 years of age, suggesting this increase is a result of the reduced mortality rate of competitive diseases, and a general increase in lifespan in developed nations. The average age at diagnosis is 54 ± 13 years of age. Disease prevalence averages to 4.48 per 100,000 person-years with 95% confidence as the lifetime risk for MND is increasing (MND Australia, 2024), presenting for 1 in every 472 women and 54% higher in men for 1 in every 350 (Alonso et al., 2009).

The economic, societal, and individual burdens are substantial as the rapid progression of muscular atrophy and paralysis debilitates 90% of MND patients within 2 to 5 years of diagnosis (Longinetti and Fang, 2019). Receiving a diagnosis of MND is not only devastating for the individual, as families, carers, and healthcare providers are impacted throughout the complex course of disease. Australia presents two new diagnoses, and two deaths of the disease each day (Australia, 2019), with an average survival rate of 27-30 months following diagnosis (MND Australia, 2024). The average annual cost per patient has tripled to \$63,693 AUD in 2017 (Meng et al., 2018), averaging the total Australian cost to \$2.37 billion in 2015 (Deloitte Access Economics (Report), 2016). This highlights the need to explore disease pathology and enrich homogenous populations for clinical trials based on therapeutic advancements for MND.

1.1.3 Causes of MND

MND is characterised by the progressive degeneration and death of upper and lower motor neurons, causing dyspnoea and death (Oskarsson et al., 2018, Hardiman et al., 2017). It is a clinically and pathologically heterogeneous disorder which includes both sporadic and hereditary origin, where axons and motor neurons are uniquely vulnerable to pathological changes that can occur (Hardiman et al., 2017, Goyal et al., 2020).

1.1.4 Aetiology and risk factors

The aetiology behind MND is largely unknown. Ongoing research has generated a new hypothesis of multiple risk factors contributing towards disease development (Martin et al., 2017, Chio et al., 2018). Following evidence underlying the genes and pathology of MND,

Al-Chalabi and colleagues suggested that the development of MND is a multistep process (Chio et al., 2018). This multistep hypothesis revisits the role of genetic mutations, proposing that up to six steps are involved in MND pathogenesis depending on the type of associated gene mutation, if any, and the course of phenotype expression. For example, exposure to one or more critical events during the neonatal period (Vucic et al., 2020a), or somatic nucleic acid changes from smoking, could represent an initial step for the development of MND (Chio et al., 2018). This multistep model was confirmed in people living with MND in Australia, South Korea, and Japan (Vucic et al., 2020a). Genetic risk factors have been explored in the past; however, 90% of cases are sporadic arising from isolated events of unknown causes. Numerous hypotheses include factors associated with disease pathology, suggesting exposure to environmental chemicals and toxins, immune response damage, premature development of motor neurons, and loss of growth factors regulating motor neuron viability (Ingre et al., 2015).

Following the American baseballer Lou Gehrig's diagnosis in 1939 (Lewis and Gordon, 2007), athletes have frequently been proposed to be more susceptible to developing MND from the influence of physical activity on free radicals and overstimulation of motor neurons (Veldink et al., 2005). Playing professional football has been shown to increase the risk of developing MND by four times (Lehman et al., 2012). There may be a proportional relationship between MND development and physical exercise, likely to cause injury to motor neurons in patients with high-risk genotypes. Transcriptomic analysis identified that high-risk MND genotypes predisposing individuals to MND development include the G4C2-repeat expansion of the *C9orf72* gene, discussed below, activated and altered in expression in response to acute physical activity (Julian et al., 2021). The upregulation of vascular endothelial growth factor 2 (Pronto-Laborinho et al., 2014) has been flagged as a possible risk, alongside the prevalence of head trauma and injury associated with high impact sports (Blecher et al., 2019). Known environmental risk factors include smoking (Lian et al., 2019), exposure to pesticides (Chen et al., 2019), heavy metal chemical exposure (Jang et al., 2025), and electromagnetic radiation (Yu and Pamphlett, 2017). The prolonged onset of MND makes isolating these factors difficult to understand disease aetiology (Yu and Pamphlett, 2017).

1.1.5 Genetic mutations as a cause of MND

In approximately 10% of MND cases, family history is a causative factor (Rosen et al., 1993). These cases are referred to as familial MND (fMND), compared to cases of sporadic MND with no family history. Both have the same endpoint which is the death of upper and lower

motor neurons (Oskarsson et al., 2018). The genes responsible for approximately 70% of familial cases have been identified. The highest proportion occur via five genetic dysfunctions of autosomal dominant inheritance. These include GGGGCC (G4C2) hexanucleotide repeats in chromosome 9 open reading frame 72 (*C9orf72*; 40%) (DeJesus-Hernandez et al., 2011, Renton et al., 2011), missense mutations in the SOD1 Cu/Zn superoxide dismutase 1 (*SOD1*; 20%) (Rosen et al., 1993), point mutations in TAR DNA binding protein 43 (*TDP-43*; 5%) (Neumann et al., 2006) and point mutations in the Fused in Sarcoma (*FUS*; 5%) gene (Vance et al., 2009, Kwiatkowski et al., 2009).

C9orf72 repeat expansions induce alterations in RNA/DNA processing (Brown and Al-Chalabi, 2017), as do *FUS* mutations (Brown and Al-Chalabi, 2017). *SOD1* mutations impact mitochondrial function and protein proteostasis, while *TDP-43* mutations effect proteomics, homeostasis, and RNA processing. Less common protein mutations like ubiquilin-like protein 2 (*UBQLN2*) (Deng et al., 2011), valosin-containing protein (*VCP*) (González-Pérez et al., 2012), matrin 3 (*MATR3*) (Johnson et al., 2014), profilin 1 (*PFN1*) (Smith et al., 2015), TANK-binding kinase 1 (Ahmad et al., 2016), T-cell restricted intracellular antigen-1 (*TIA1*) (Mackenzie et al., 2017), sequestome-1 (*SQSTM1*) (Bartolome et al., 2017), dynactin, tubulin 4A (*TUBA4A*) (Higelin et al., 2018), and optineurin (*OPTN*) (Toth and Atkin, 2018) dysregulate axonal transport, affect RNA metabolism, protein degradation/aggregation, and impair mitochondrial energy metabolism.

This list of gene mutations is growing as technology advances (Nguyen et al., 2018). The coiled-coil-helix-coiled-coil-helix domain containing 10 and pCMV6-CLYD mutations have been shown to impact neurotransmission from the NMJ (Genin et al., 2019) and neuronal autophagy (Dobson-Stone et al., 2020) respectfully. Mutations in *hnRNPA1* to *hnRNPA3*, *hnRNA2B1*, and *hnRNPL* genes have been found to alter proteins important to RNA metabolism in MND and FTD (Bampton et al., 2020), while mutations associated within *KANK1* are enriched in motor neurons, causing downstream *TDP-43* mislocalisation and the functional impairment of distal axons (Zhang et al., 2022). The discovery year and prevalence of over 25 gene mutations are noted in **Figure 1.1** (Brown and Al-Chalabi, 2017) alongside the newer cellular processes that have been identified (Dobson-Stone et al., 2020, Bampton et al., 2020, Zhang et al., 2022).

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Figure 1.1: Genes associated with MND

The prevalence of known MND genes is increasing rapidly, with familial causes responsible for 5-10% of MND cases. The cohort of genes associated with fMND has increased significantly between 1990 and 2020. Each circle represents the proportion of familial MND cases associated with that gene worldwide. Blue circles indicate genes associated only with familial MND, red circles indicate genes associated only with sporadic MND, and half circles indicate genes associated with both familial and sporadic MND. The hexanucleotide repeat expansion in C9orf72 is most common, reflecting approximately 45% of MND cases (Brown and Al-Chalabi, 2017).

1.1.6 Pathological changes occurring in MND

There are a multitude of pathological changes described in both sporadic and familial MND, leading to denervation and motor neuron death. Characteristic changes include the aggregation of proteins; alterations in RNA/DNA processing, transport, and function (Shi et al., 2010); mitochondrial dysfunction and glutamate excitotoxicity (Jaiswal, 2014, Wang et al., 2015); and oxidative stress (Magrane and Manfredi, 2009). In particular, inflammatory changes appear to play a role in the onset and progression of MND (McCauley and Baloh, 2019), and is a key focus of this discussion.

1.1.6.1 Protein aggregation

The ubiquitin proteasome system (UPS) and autophagy are both central protein degradation pathways in cells. The UPS degrades damaged proteins tagged with poly-ubiquitin sequences by proteolysis, and autophagy degrades cellular organelles (Nedelsky et al., 2008). Intracellular inclusions of misfolded and aggregated proteins in motor neurons and oligodendrocytes are a hallmark of MND (Blokhuys et al. 2013). For example, in 98% of MND cases, inclusions are present that contain ubiquitin and *TDP-43*. However, in *SOD1* linked mutations, *TDP-43* is absent (Scotter et al., 2015).

Pathologic *TDP-43* is subject to hyper-phosphorylation and ubiquitination, cleaving itself to construct C-terminal fragments. These have been identified in the regions of the CNS affected by MND including the hippocampus, neocortex, and spinal cord (Neumann et al., 2006). Mackenzie and Neumann (2016) showed that *TDP-43* pathology was essential for the development of MND and FTD (Mejzini et al., 2019). Additional evidence supporting UPS failure is the discovery of mutations in proteins involved in proteostasis, including *TDP-43*, *UBQLN2* and *VCP* (Zientara-Rytter and Subramani, 2019, Vicencio et al., 2020). Further mutations in other proteins *OPTN*, *SQSTM1*, and *TANK-binding kinase 1*, are implicated in pathogenesis (Watts et al., 2007), demonstrating the importance of protein processing in MND.

1.1.6.2 Alterations in RNA/DNA processing

The most rapidly expanding area of research into gene mutations are those important to RNA/DNA processing. As previously discussed, mis-localized *TDP-43* was initially discovered in sporadic MND and FTD (Mejzini et al., 2019). Over 6,000 RNA targets have been associated with *TDP-43* proteins in the CNS; disrupting RNA metabolism and contributing to MND pathogenesis (Barton et al., 2019). *FUS* encoding RNA-binding proteins are further implicated, forming intracellular stress granules to silence unnecessary mRNAs

and facilitate cell survival. Disturbances in *TDP-43* and *FUS* RNA-binding proteins also dysregulate the stress granule response (Lenzi et al., 2015). *C9orf72* repeat expansions accompany *TDP-43* pathology with other features impairing RNA metabolism and stress granule function (Chew et al., 2019). Adhering to several RNA-binding proteins and impairing function (Kumar et al., 2017), *C9orf72* repeat expansions additionally produce the repeating dipeptide protein aggregates dpoly(GR) and poly(GR), hyper-activating stress granule formation and dysregulating the stress response (Brown and Al-Chalabi, 2017).

TDP-43 is accumulated in the cytoplasm of motor neurons, and *TDP-43* pathology has been associated with activating the cytoplasmic DNA sensor cyclic guanosine monophosphate (GMP)-AMP synthase (cGAS) pathway as *TDP-43* aggregates within the mitochondria. Downstream signalling pathways of cGAS recruit stimulators of interferon genes (STING), driving the upregulation of NF- κ B and type 1 interferon pathways (i.e., IFN- α and IFN- β) and causing a downstream hyper-inflammatory response in people with MND (Yu et al., 2020). The cGAS/STING dependent upregulation of interferon-stimulated genes may cause the loss of mitochondrial homeostasis, driving inflammatory cytokine production and neurotoxic immune sensor activation and prolonging the central and peripheral pro-inflammatory response (Harapas et al., 2022).

1.1.6.3 Alterations in transport and function

Transportation of proteins and intracellular organelles to their correct destinations is an essential component of cellular function (De Vos et al., 2007). Axonal transport and compartmentalisation mechanisms in people with MND are dysregulated (Donato et al., 2019). Dying motor neurons retract their axons from the NMJ retrogradely towards the soma, dissolving muscle fibre motor endplates as acetylcholine receptors are internalised and removed (Nijssen et al., 2017). Axonal cytoskeletal dysregulation on a neurofilament level is a manifestation of both familial and sporadic MND, starving motor neurons of nutrients and oxygen required for cell survival (Didonna and Opal, 2019). As axons degrade, neurofilament heavy (NfH) and neurofilament light (NfL) proteins accumulate in the CSF and blood which have been acknowledged as potential biomarkers for MND (Xu et al., 2016, Benatar et al., 2020). *SOD1* gene mutations have demonstrated reduced efficacy of anterograde and retrograde pathways for up to months before motor neuron cell body degeneration is observed (Bilsland et al., 2010). Further, dynactin mutations and activation of the retrograde motor cytoplasmic dynein proteins (Liu et al., 2017), in addition to tubulin proteins, have been shown to provoke the retraction of axons from motor end plates and advance MND (Ikenaka et al., 2012). This is emphasised by people with fMND presenting

mutations in dynactin subunit 1 which dysregulates motor proteins including dynein and *TUBA4A*, that disrupts the structure of axons (Brown and Al-Chalabi, 2017).

1.1.6.4 Mitochondrial dysfunction and excitotoxicity

Mitochondria function is impaired in MND and excitotoxicity is also commonly observed (Echaniz-Laguna et al., 2002). Mitochondria regulate intracellular energy currencies, cellular respiration, calcium equilibrium, and apoptosis (Forsberg et al., 2011). Defects in fragmented networks, mitochondrial swelling, and augmented cristae have been localised in the soma and proximal axons of motor neurons in people with MND (Manfredi and Xu, 2005, Sasaki and Iwata, 2007). Most of the data on this pathology has been demonstrated in mice containing *SOD1* mutations (Brown and Al-Chalabi, 2017) dysregulating ATP and calcium production, axonal transport of mitochondria, and apoptosis (Boillee et al., 2006). Dysfunctional mitochondria and oxidative stress (OS) share a causal relationship; as reactive OS production increases, the endoplasmic reticulum (ER) undergoes stresses causing transcriptional abnormalities which induces the death of motor neurons (Taylor et al., 2016). Mutant phenotypes of overexpressed human *SOD1*-^{G37R} in neuroblastoma cells of N2a mice reduces the activity of oxidative phosphorylation complex I, decreasing mitochondrial membrane potentials and cytosolic ATP (Cousse et al., 2011). By displacing the electron transport chain, downstream effects reduce mitochondrial calcium uptake by reducing IP₃-induced calcium release from the ER (Cousse et al., 2011). This relationship was identified in *SOD1*-^{G37R} cells which demonstrated that as the cytosolic calcium transients were increased, severely impacting the mitochondrial respiratory chain by the loss of ATP and dysfunction of cytosolic calcium homeostasis (Cousse et al., 2011).

The most significant excitatory amino-acid neurotransmitter in the CNS is L-Glutamate (Shigeri et al., 2004). Engaged in cognition, learning, and memory, glutamate is crucial for synapse development, while regulating cell differentiation and death. Highly toxic to neurons as an endogenous excitotoxin, glutamate is strictly regulated (Sundaram et al., 2012). Upon release into the synaptic cleft, glutamate is degraded to glutamine by excitatory amino-acid transporters to avoid excitotoxic neuronal damage (Shaw and Eggett, 2000). Studies in MND patients (Lin et al., 1998) and *SOD1*-^{G93A} mice models discovered the reduction of astroglial glutamate transporter EAAT2 proteins in the motor cortex and spinal cord (Lin et al., 1998). Reduced EAAT2 increases extracellular glutamate, hyper-activating post-synaptic glutamate receptors and promoting excitotoxic neurodegeneration (Lin et al., 1998, Trotti et al., 1999). The sustained elevation of intracellular calcium promotes destructive patho-biochemical cascades causing OS and neuronal degeneration (Shaw and Eggett, 2000).

Oxidative pathways decompose the glutamate-cystine antiporter, reducing glutathione to form aberrant neurotoxic reactive oxygen species (ROS) (Shaw and Eggett, 2000). Aberrant EAAT2 mRNAs are located in the spinal cord, motor cortex, and CSF of people with MND (Lin et al., 1998), causing the localised accumulation of ROS inducing upper and lower motor neuron programmed cell death (Shaw and Eggett, 2000). Evidence validating excitotoxicity in MND is the disease-modifying agent Riluzole, and its ability to attenuate disease progression (albeit by two to three months) by inhibiting the release of glutamate.

These mitochondrial abnormalities are the largest facilitators of OS in motor neurons. Imbalanced free radical and cellular antioxidant homeostasis is a consequence of OS, altering redox-sensitive reactions and ineffectively detoxifying the cell (Carri et al., 2015). Mutant *SOD1* antioxidant enzymes are linked to the increase in free radicals, cytotoxicity, and oxidative damage in serum, CSF, and urine samples of people with MND (D'Amico et al., 2013). However, the relationship between OS and mitochondrial dysfunction supports evidence to suggest they contribute to non-*SOD1* MND cases. **Figure 1.2** (Masrori and Van Damme, 2020) illustrates this relationship, alongside the downstream effects causing misfolded protein aggregates, impaired axonal transport, and protein disulphide isomerase enzyme dysfunction and upregulation (Therrien et al., 2016). Protein disulphide isomerase co-localises with *SOD1*, *TDP-43*, and *FUS* mutations, inactivating redox reactions and accumulating free radicals (Carri et al., 2015).

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Figure 1.2: The Pathogenicity and Mechanism of Action Associated with the Clustering of MND Genes

1. Mutations in *TBK-1*, *OPTN*, *SQSTM1*, *UBQLN2*, *C9orf72* and *VCP* affect protein degradation pathways and may contribute to *TDP-43* accumulation. **2.** Mutations in *TDP-43*, *FUS*, *MATR3*, *TIA1*, *hnRNPA1*, *hnRNA2B1* and *ATXN2* share interactions altering RNA processing and metabolism. **3.** Mutations in *TUBA4A*, *PFN1*, *KIF5A* and *DCTN1* alter cytoskeletal dynamics and axonal transport. These have downstream effects on **4.** microglial and astrocyte activation mediate neuroinflammatory responses, **5.** contributing towards mitochondrial dysfunction, **6.** neuronal excitotoxicity, **7.** impairing nucleoplasmatic transport, **8.** oligodendrocytes and myelin formation alongside **9.** mechanisms of DNA repair. *Adapted from (Masrori and Van Damme, 2020).*

1.1.6.5 Immune Involvement

Inflammation in MND is characterised by an activated microglial response (McCauley and Baloh, 2019). This response leads to neuroinflammation, accelerating disease pathogenesis by involving the reaction of non-neuronal cells outside of the CNS and contributing to upper and lower motor neuron degeneration (Lobsiger and Cleveland, 2007, Beers et al., 2008, Beers et al., 2006). Immune involvement is a well described pathological process in MND. This involves neuroinflammation and peripheral immune dysfunction. Similar to other neurodegenerative diseases, MND pathogenesis involves an inflammatory reaction in the immune cells of the CNS (Lobsiger and Cleveland, 2007). This neuroinflammation is the reaction of resident glial cells and other immune cells in response to viral load, trauma, or degeneration (Appel et al., 2011). Astrocytes and microglia represent the major population of CNS immune cells, although there is some macrophage (monocyte) infiltration. As part of the immune activation in the CNS, microglia become activated (McCauley and Baloh, 2019) and contribute towards motor neuron degeneration (Lobsiger and Cleveland, 2007, Beers et al., 2008, Beers et al., 2006). In addition to neuroinflammation, there is a peripheral immune response to degenerating motor neurons that involves the activation of immune cells and inflammatory molecules (Lyon et al., 2019).

1.1.7 Overview of neuroinflammation

Astrocytes and microglia represent the major population of glial cells present in the CNS. Microglia represent the resident macrophage-like cells of the CNS (Ginhoux and Prinz, 2015), and are the first responders to acute injury and inflammation (Bilbo and Stevens, 2017). Microglial activation is normally suppressed by astrocytes and healthy neighbouring neurons, secreting TGF- β , negative co-stimulatory molecule *CD200*, and inhibitory cytokine fractalkine *CX3CL1*. However, in response to acute inflammation, pathogen-associated molecular patterns (PAMPs) such as microbial DNA, RNA, lipopolysaccharides (LPS), and endogenous damage-associated molecular patterns (DAMPs) are activated. Aggregated proteins (*high-mobility group box 1*) and heat shock proteins (*HSP*) -60 and -70 (Béland et al., 2020) bind to pattern recognition receptors (Ashford et al., 2021), activating the microglia (Zhao et al., 2015). Upon activation, microglia function as phagocytes (Lyon et al., 2019), altering their resting ramified morphology to become ameboid and proliferate within the CNS. Activated microglia induce a multifaceted immune response, releasing pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF- α), interferon-gamma (IFN- γ), interleukin-1 beta (IL-1 β), IL-6, ROS, and nitrogen species (RNS) into circulation,

intended to prevent further damage by healing and repairing the surrounding CNS tissue described in **Figure 1.3** (Smith et al., 2012, Ashford et al., 2021).

The pro-inflammatory mediators released into systemic circulation from acute microglial activation influence cells in the periphery outside of the CNS, prolonging the central immune response and contributing towards neuronal damage (Huang et al., 2021). Accompanied with central microglial activation is the upregulation of their major histocompatibility complex (MHC) class 2 expression, playing a key role in the activation phenotype of microglial cells in the CNS and macrophages in the periphery (Conrad and Dittel, 2011). These phenotypes are classified into two subsets: M1 or M2.

The ‘classical,’ or M1 differentiated macrophages are activated by toll-like receptor signalling in response to DAMPs, which serve the pro-inflammatory function of further producing TNF- α , IFN- γ , and IL-1 β inflammatory factors, while other peripheral immune cells including natural killer (NK) cells, dendritic cells, and neutrophils are upregulated to clear cellular debris, promote repair, and resolve the injury (Brites and Vaz, 2014). Alternatively, ‘activated’ M2 differentiated macrophages are distinguished by their anti-inflammatory function, releasing cytokines IL-4, IL-13 and IL-10, transforming growth factor-beta (TGF- β), and vascular endothelial growth factor (VEGF) to aid in reducing inflammation and resolving the acute immune response (Philips and Robberecht, 2011, Brites and Vaz, 2014). However, progressive degeneration of the CNS in MND does not follow a simple pro-inflammatory M1 to anti-inflammatory M2 transition (Chiu et al., 2013), as activated M2 macrophages switch to the classical M1 phenotype over disease progression (Beers et al., 2011). This results in the release of pro-inflammatory factors TNF- α , IFN- γ , IL-1 β , IL-6, IL-12, IL-23, NOS, and MCP-1 by ‘activated’ M1 macrophages in the peripheral tissue, prolonging the activated microglial response (Béland et al., 2020).

1.1.7.1 Neuroinflammation in MND

During muscular atrophy, pro-inflammatory cytokines are released into peripheral circulation activating monocytes and lymphocytes (Lyon et al., 2019). Depending on the differentiated state of T-cells, the activation of other circulating immune cells is either dampened by T regulatory, or heightened by peripheral helper T cells (CD4⁺) Th-1 and/or Th-17 via positive feedback with activated microglia in the CNS (Melzer et al., 2009). Th-1 cells activate circulating immune cells, upregulating pro-immune M1 macrophages, IgG antibodies, and T-cell expansion. Alternatively, Th-2 cells dampen the inflammatory response, releasing anti-inflammatory IL-4 cytokines to resolve the immune response (Lyon et al., 2019).

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Figure 1.3: Central microglial phenotypes during acute neuroinflammation

A. Resting microglia in the CNS maintain homeostasis by secreting the neurotrophic factors BDNF, GFNF, and IGF-1. Healthy neurons surrounding the resting microglia secrete CD200 and CX3CL1, and astrocytes secrete TGF- β to provide further negative co-stimulation. **B.** Pathogens and injured, dying, or dead motor neurons release PAMPs/DAMPs, activating pattern recognition receptors to switch microglia from resting, to an 'activated' pro-inflammatory amyloid state. This produces pro-inflammatory factors like IL-1 β , IL-6, TNF- α , IFN- γ and ROS/RNS to clear cellular debris. **C.** Once the damage is resolved, surrounding neurons restore negative CD200 and CX3CL1 co-stimulation factors, shifting activated microglia to their ramified anti-inflammatory phenotype. These secrete anti-inflammatory cytokines like IL-4, IL-10, IL-13, growth factors, and CD163+ leading to the resolution of the acute central inflammatory response, returning to a resting state. *Adapted from* (Béland et al., 2020).

Derived from monocyte precursors, microglia and astrocytes serve to regulate and protect motor neuron homeostasis (Blackburn et al., 2009, Christoforidou et al., 2020). However, growing evidence suggests that, in addition to microglia, peripheral innate immune cells may also invade the CNS and are implicated in MND pathogenesis, contributing to a generalised hyper-immune response. It is hypothesised that peripheral monocytes, thought to be neuroprotective (Murdock et al., 2015), may invade the brain and spinal cord of MND patients to promote neurotoxicity and inflammation within the CNS (Zondler et al., 2016). Endothelial cells have shown to be affected causing progressive lesions in the blood brain barrier (BBB) from the loss of astrocytes that are located near blood vessels in the brain (Willis et al., 2004). Down regulation of tight junction proteins in SOD1^{G93A} mice weakened the integrity of the BBB leading to leaky capillaries (Zhong et al., 2008, Miyazaki et al., 2011), suggesting that disruptions to the BBB may lead to peripheral blood monocytes/macrophages crossing into the CNS, acting via positive feedback with activated microglia to further release pro-inflammatory cytokines and prolong neuroinflammation in MND (Garbuzova-Davis et al., 2019), described in **Figure 1.4**. Hence, both CNS and peripheral immune dysregulation occurs in MND contributing towards a prolonged, and generalised immune response.

Altered microglial activity has been associated with mutant *C9orf72*, dysregulating endosomal trafficking of autophagy and lysosomal proteins. Highly expressed in myeloid cells, mutant *C9orf72* elevates the Th-1 cell release of pro-inflammatory interleukins (Malaspina et al., 2015), hyper-activating the immune response and promoting neurotoxicity (Lall and Baloh, 2017). Heightened levels of inflammatory cytokines including IL-1 β , IL-6, and TNF- α have been described in MND patients and mice models (Han et al., 2016), increasing the activated microglial phagocytic potential, while producing neurotoxic ROS and RNS from the M1 microglial phenotype (Béland et al., 2020). IL-1 β secretion is influenced by microglial NLRP3 inflammasome activation, where inhibition may be a potential therapeutic approach to reduce neuroinflammation and MND progression seen in SOD1-^{G93A} mice (Deora et al., 2020). Other interleukins include IL-6, a potential contributor towards endothelial damage in MND (Wilkins et al., 2021); IL-17, a pro-inflammatory mediator responding to stress; and IL-33, a downstream mediator of MND progression.

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Figure 1.4: Central and peripheral neuroinflammation over MND progression

1. Over disease progression, denervation occurs at the NMJ causing muscular atrophy and the release of pro-inflammatory cytokines into circulation (TNF- α , IFN- γ , IL-1 β). **2.** Depending on whether Th-1, Th-2, or T regulatory cells are activated, **3.** Th-2 and T regulatory cells work favourably to reduce the activation of pro-inflammatory cytokines, while Th-1/Th-17 cells work via positive feedback to activate circulating M1 inflammatory macrophage (M) cells in the periphery. **4.** Degenerating motor neurons in the CNS activate microglia (MG). Disruptions to the blood brain barrier (BBB) over disease progression allow M1 macrophages to cross the BBB where **5.** they work via positive feedback with microglia (MG) to **6.** enhance neuroinflammation and **7.** release pro-inflammatory cytokines in the CNS, bridging both arms of the immune system. *Adapted from* (Lyon et al., 2019).

The association of TDP-43 mediated neuroinflammation with nuclear factor kappa-B (NF- κ B) related cytokines (Egawa et al., 2012, Zhao et al., 2015) promotes an upregulated type-1 IFN response (IFN-1), activating the microglial response (Wang et al., 2011). Within this IFN-1 pathway, cGAS/STING mechanisms are up-regulated (Decout et al., 2021). cGAS/STING stimulation has shown to reflect parts of the pro-inflammation and may be used as a marker of MND (Yu et al., 2020). Prolonged neuroinflammation in MND inducing an IFN-1 response via cGAS-STING pathways has shown to change the phenotype of microglia, switching to an amyloid structure that accelerates pro-inflammation and disease progression. The IFN-1 response was explored in STING^{-/-} mice, displaying an inhibition of the IFN-1 response, and an increase in microglial proliferation and activated amyloid morphology, indicating a dependency on IFN-1 receptors (Nazmi et al., 2019). IFNAR1 gene deficiency in SOD1^{G93A} mice has shown to slow MND progression, marking the cGAS-STING pathway as a mediator of activated microglia and pro-inflammation (Wang et al., 2011).

Immunofluorescent analyses of the grey and white matter of the motor cortex, medulla oblongata, and cervical/lumbar spinal cords showed that *TDP-43* pathology was involved with activated microglia and motor neuron damage (Brettschneider et al., 2012). Ubiquitin accumulation and axonopathy are involved with a heightened immune response in MND (Amor et al., 2014). The involvement of an aberrant immune response, whether it be a central microglial response (Appel et al., 2011, Boillee et al., 2006, Brettschneider et al., 2012), or a peripheral pro-inflammatory chemokine response (Kuhle et al., 2009), has been well documented. However, the dual role of immunity remains controversial, and neuroinflammation as the cause or outcome of MND is largely unknown (Murdock et al., 2015). Tissue resident macrophages are a heterogenous population of immune cells activated by IFN- γ (Davies et al., 2013). To avoid complicating an already heterogenous MND cohort, microglia and invaded cells from peripheral monocytes were discriminated in a study using CD169⁺, identified by (O'Neill et al., 2013), as a marker for mature dendritic cells and macrophages in MND patients (Zondler et al., 2016). It was found that the number of CD169⁺ cells of monocytic origin was significantly increased and the number of motor neurons was significantly decreased in lumbar spinal cord tissue of 10 people with MND, alongside an increased expression of CD169⁺ upon treatment with IFN- γ (Zondler et al., 2016). This reinforces the peripheral immune cell infiltration hypothesis, and highlights IFN- γ as a key peripheral immune cell mediator relevant to the discovery of neopterin as a marker of immune dysfunction, discussed in *Neuroinflammatory markers for MND*.

To gain further insight on the implications of peripheral macrophage activation in SOD1-^{G93A} mice models, Chiot *et al.*, (2020) evaluated whether macrophages based in the periphery are implicated in disease progression. From the advantage standpoint of accessibility, substitution of peripheral macrophages in SOD1-^{G93A} mice with non-mutant macrophages overexpressing wild type SOD1 through bone marrow transplantation demonstrated reduced microglial neurotoxicity, peripheral macrophage activation, and hence slowed MND progression, correlating with previous evidence highlighting peripheral monocyte toxicity (Zondler *et al.*, 2016). SOD1-^{G93A} was also deleted from glial cells, altering the onset and progression of the disease (Boillée *et al.* 2006, Wang *et al.* 2009, Yamanaka *et al.* 2008).

The neurotoxic origin of glial and macrophage cells was further explored in pre-clinical studies. SOD1-^{G93A} mouse studies exploring both macrophage activation phenotypes indicated an excessive IL-10 stimulation which was found to switch microglia to an amyloid phenotype at MND onset, following altered and dysregulated cytokine release at end-stage disease (Gravel *et al.*, 2016). Circulating immune cells were found to interact with complement factors to systematically promote neuroinflammation (Kjaeldgaard *et al.*, 2018). Knowing that peripheral macrophages and CNS derived microglia respond to any lesion of the nervous system, an increased activation of microglia is further implicated in the progression of MND (Gravel *et al.*, 2016), producing neurotoxic complement and neurotrophic factors (Barbeito *et al.*, 2010). These factors continue to play a significant role in the resident macrophage response, employing the adaptive immune response to accelerate disease progression. Complement activation pathways are known as the bridge between innate and adaptive immune responses, producing membrane-bound proteins to amplify the innate response to adaptive T-lymphocytes (Panda and Ding, 2015). This includes damaged motor neurons activating complement components via the release of stress signals, causing leukocytes to aggregate and bridge both arms of the immune system (Lo and Woodruff, 2020).

Complement-activated oligodendroglia are characterised by C3d and C4d deposition on myelinated fibres found near degenerating motor neurons. Post-mortem analyses on SOD1-^{G93A} mice found an elevation in CD4⁺ microglia and C4 complement factors (Kawamata *et al.*, 1992), alongside C1q and C4 fibres across disease progression (Ferraiuolo *et al.*, 2007). Other studies have reported an elevation in C3 and C4 complement factors in MND patient CSF samples, indicating modifications to cytoskeletal integrity contributing towards neuroinflammation (Ganesalingam *et al.*, 2011). Complement factors C1qB, C4, C4d, and complement factor B have all been related to MND progression, where Kawamata and

colleagues (1992) associated the complement system with the degeneration of motor neurons. This association was later reviewed by (Malaspina et al., 2015), observing the progressive and harmful effects of microglia and astrocytes, alongside the cytotoxic effects of the peripheral adaptive T-cell response influenced by complement activation (**Figure 1.5**). Complement factors C3a, C3b, and C5a are potent mediators of these proinflammatory pathways, where the conversion of complement factor C5, to C5a, to C5b facilitates the formation of C5b9, otherwise known as the membrane attack complex (MAC) (Lo and Woodruff, 2020). Complement factors C1q, C3b, and C5b-9 were later found to aggregate in close proximity to motor neuron terminals and peripheral Schwann cells (Bahia El Idrissi et al., 2016b), alongside the NMJ of pre-symptomatic human MND patients and SOD1-^{G93A} mice (Heurich et al., 2011). The complement molecules C5b9/MAC can induce cell lysis and apoptosis, upregulate cytokine production, while facilitating inflammasome activation and gene expression at the NMJ, coordinating the neurotoxic capabilities of the innate and adaptive immune systems (Lo and Woodruff, 2020). The extent of this pathological response has questioned complement C1a receptor 1 as a therapeutic target of inflammatory diseases like MND, where inhibitors have been explored in pre-clinical translational studies as therapeutic drug candidates targeting C5a and its receptors (Cui et al., 2022). These are yet to show promise in MND clinical trials.

Once these complement factors become activated, antibodies and auto-antibodies to unique antigens in the CNS become elevated (Malaspina et al., 2015), causing a shift in T-cell populations that promotes further inflammation (Pilli et al., 2017). Significant numbers of T-lymphocytes were observed augmenting across capillaries and venules into the parenchyma of peripheral post-mortem tissues, consistent with an autoimmune or slow virus theory of MND pathogenesis (Kawamata et al., 1992). The secondary adaptive immune response releases antibodies to neurons in the serum associated with CNS injury, elevating the extent of glutamate excitotoxicity (Henkel et al., 2009), neurofilament aggregation (Pilli et al., 2017), and altering the homeostasis of essential amino acids (Howland et al., 2002). It is evident that many mediators of pro-inflammation present in the CNS of people with MND due to a plethora of endogenous pathological events (Parakh et al., 2013) and changes in homeostasis from an immunological perspective (Amor et al., 2014, Barbeito et al., 2010). The extent of motor neuron damage endured in MND is sustained by a dysfunctional pro-inflammatory response directed by neighbouring non-neuronal cells, accelerating MND progression (Boillee et al., 2006).

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Figure 1.5: Immune response and inflammatory mediators of MND

Increased expression of complement receptors 3 & 4 within microglia **A.** increases the quantity of activated microglia in the brain and spinal cord (CNS) **B1.** Following this activation, mast cells, NK cells, macrophages and T lymphocytes infiltrate the CNS **B2.** increasing the expression of pro-inflammatory mediators, importantly a range of interleukins and $\text{TNF}\alpha$ / $\text{TNF}\alpha\text{R}$ promoting loss of motor and axonal function. Calcium homeostasis is lost in axons (nerves) travelling to the NMJ, severely impairing the mitochondrial respiratory chain, dysregulating ATP production, and impairing axonal transport **C.** Changes in expression at the NMJ reduce C4 **D.** causing an increase in cytokines, $\text{TNF-}\alpha$ **E.** and an infiltration of macrophages **F.** Antibodies against calcium channels are produced **G.** reducing nutrient and ATP supply to the NMJ causing destruction, axonal degeneration, and internalisation of acetylcholine receptors and denervation during disease development (Malaspina et al., 2015).

The inflammatory response in MND is complex, yet circulating cytokines may prove to be a predictive measure of the inflammatory response or disease prognosis and progression, acting as biomarkers of the disease in serum, CSF, and urine. This pro-inflammatory state indicates an activation of Th-1 and Th-17 lymphocytes and downstream release of IFN- γ and TNF- α , showing potential to identify immunologically homogenous patients to maximise the chances of therapeutic success and detection of benefit within phase 2 clinical trials (Malaspina et al., 2015). This review will continue to describe clinical aspects surrounding the disease, discussing how MND is diagnosed, the biomarkers involved and how they are normalised, alongside the methods of biomarker detection that are clinically available.

1.1.8 Diagnostic criteria

Clinical hallmarks are used to distinguish MND from other neurodegenerative disorders. Selective characteristics of MND include progressive motor weakness without sensory disturbance, late onset diagnoses, followed by rapid disease progression (Bäumer et al., 2014). However, the pathognomonic hallmark of MND is the combination of both upper and lower motor neuron involvement contributing to muscular atrophy and lateral sclerosis (Garg et al., 2017).

The revised El Escorial World Federation of Neurology (WFN) criteria is used by neurologists, combining pure UMN and LMN disease signs to provide diagnostic certainty (Brooks, 1994). Pure UMN or LMN diseases are typically less prevalent, slower in progression, and present on opposite ends of the MND spectrum (Garg et al., 2017). These criteria were revised in 1997 at Airlie House, USA, (Miller et al., 1999) to improve the sensitivity and determine earlier diagnoses important for patient enrolment into clinical trials (Traxinger et al., 2013). This reduced the median time from baseline to the time of diagnosis to 11 months (Traxinger et al., 2013). Since 1997, the original El Escorial criteria of MND diagnosis were revised (rEEC) in adjustment to stigma associated with the 'possible' category. The WFN Research Group on ALS/MND published their comments on the strengths and weaknesses of this diagnostic criteria (Agosta et al., 2015), leading to amendments allowing for the diagnosis of MND to be made if the former criteria of 'possible MND' are sufficed (Ludolph et al., 2015). This revision provides an opportunity to include restricted phenotypes in MND diagnosis and include these patients in clinical trials where the development of novel treatments and diagnosis biomarkers may reduce this time further.

The original El Escorial diagnostic criteria generated categories and definitions of MND that are based on the initial region of MND involvement. Patients are categorised into a bulbar,

cervical, thoracic, or lumbosacral site of disease onset based upon simultaneous UMN and LMN symptoms. People with MND are diagnosed using various iterations of the terms ‘suspected, possible, probable, probable laboratory-supported, and definite’ MND, however, these categories are poor independent predictors of prognosis and there are insufficient UMN signs in probable or definite MND cases (Traynor et al., 2000). December 2006 saw an evidence-based approach to the re-evaluation of the rEEC to consider how neurophysiology and the role of electrodiagnostic criteria could be employed to facilitate earlier MND diagnoses (Costa et al., 2012). The Awaji-Shima consensus changed the way clinical neurophysiological information was used, recommending the application of electrophysiological tests to the diagnosis of MND (de Carvalho et al., 2008).

Acknowledging these inherent limitations, the WFN, International Federation of Clinical Neurophysiology, the International Alliance of MND Associations, the ALS Association, and the Motor Neuron Disease Association came together to develop a potential, more effective criteria to reflect the clinical practice and diagnostic categories of MND. The Gold Coast Criteria (GCC) implemented a revised assessment of lower motor neuron impairment; recording the presence of UMN and LMN dysfunction in at least one body region, LMN dysfunction in at least two body regions, and the ability to differentiate atypical MND phenotypes (e.g., primary lateral sclerosis) (Vucic et al., 2021). Compared to previous criteria (Hannaford et al., 2021, Shen et al., 2021, Vucic et al., 2021), the GCC shows promise to remove the confusion caused by previous iterations of diagnostic category terms, replacing the site of symptom onset with a multi-axial classification based on the rate of MND progression (Turner, 2022, Vucic et al., 2021).

1.2 Biomarkers for MND

Biomarkers are described by the National Institute of Health (USA) as “measurable characteristics, indicative of biological, pathological, or pharmacodynamic responses to therapeutic interventions” (Working Group, 2001). Biological markers are of utmost importance to provide accurate and objective indications, quantifying biological processes to eradicate subjective judgement (Strimbu and Tavel, 2010). In order to provide accurate and objective indications, biomarkers should be fit for purpose (Goyal et al., 2020).

Biomarkers are classified into subsets of diagnostic, prognostic, pharmacodynamic, progression, and predictive markers. These can be physical (clinical scores), biofluid, neuroimaging, or electrophysiological markers. The only FDA approved biomarker for MND is the revised ALS functional rating scale (ALSFRS-R), a questionnaire-based clinical

scoring scale from 0-48. This consists of 12 questions around activities of daily living, where individual item scores are summed to produce a score between 48 (i.e., healthy individual) and 0 (i.e., permanent assisted ventilation, usually death follows). Powerful over time, this subjective score can introduce inter and intra-individual variability (Pfohl et al., 2018).

1.2.1 Diagnostic biomarkers

Given time and careful neurological and neurophysiological assessment, MND can be effectively diagnosed by a trained neurologist using signs, symptoms, electrophysiology, neuroimaging, and blood tests to rule out other diseases. However, once there are effective treatment options, diagnostic biomarkers would be useful. At present, neurologists use the revised El Escorial World Federation of Neurology criteria to assess a combination of pure UMN and LMN disease signs to provide diagnostic certainty (Brooks, 1994, Ludolph et al., 2015). Electrodiagnostic and neurophysiological techniques were introduced to create a more comprehensive diagnostic criteria, improving the opportunity for early MND treatment (Paganoni et al., 2014). Electrical impedance myography, structural magnetic resonance imaging, and reduced short-interval intracortical inhibition are techniques that potentially have diagnostic value to exclude mimics of MND (Verber et al., 2019) and warrant further investigation to facilitate earlier enrolment into clinical trials (Costa et al., 2012). The use of neurophysiological patient data in conjunction with the established rEEC (de Carvalho et al., 2008) and GCC mentioned above (Vucic et al., 2021), has not been widely implemented as of yet. Since there are no effective treatments, diagnostic markers are of less importance compared to aiding the development of treatment advancements and reducing heterogeneity in clinical trials by the discovery and validation of prognostic, pharmacodynamic, progression, and predictive biomarkers (Goyal et al., 2020). I will not be discussing diagnostic biomarkers further in this introduction.

1.2.2 Prognostic biomarkers

Prognostic biomarkers are baseline characteristics that will facilitate the categorisation of MND patients based on expected disease progression, survival, and trajectory (Benatar et al., 2016c). Prognostic biomarkers categorise patients by their individual risk for future disease outcomes or predict the progression of a specific aspect of the disease (Benatar et al., 2016). Prognostic markers are useful for reducing patient heterogeneity and stratifying patients with shared underlying biology at baseline; decreasing variability, sample size, and expenses for more efficient clinical trial designs (Huber et al., 2019, Benatar et al., 2020). An example of an FDA approved prognostic marker of MND is the Δ FRS, calculated as $(48 - \text{ALSFRS-R at baseline})/\text{months between symptom onset and baseline (first visit to$

neurologist). The Δ FRS provides an estimated rate of MND progression at baseline, that can predict survival outcomes (Kimura et al., 2006). Hence, the Δ FRS is prognostic for MND survival. Biomarkers of MND prognosis are useful for predicting survival and grouping patients with similar disease characteristics for the application of therapeutics in clinical trials (Malaspina et al., 2015).

1.2.3 Pharmacodynamic biomarkers

Pharmacodynamic biomarkers are indicators that demonstrate change with treatment, allowing the rate of efficacy to be measured over time (Schmidt-Richberg et al., 2016). Pharmacodynamic biomarkers are useful to show that a biological response has taken place in response to an experimental therapeutic (Shepherd et al., 2017), identifying ineffective treatments at an early time-point by identifying adequate drug delivery, target engagement, or biological activity in response to the experimental therapeutic. An approved pharmacodynamic biomarker is the ALSFRS-R, that is used in all clinical trials (Benatar et al., 2018, Miller et al., 2020, Goyal et al., 2020). The ALSFRS-R is the gold standard for determining clinical trial outcomes; however, it is limited in its sensitivity to determine treatment efficacy in phase 2 clinical trials and needs to be complemented by a panel of biomarkers (Benatar et al., 2024b). Serum NfL has been validated as a prognostic and potential pharmacodynamic biomarker (Benatar et al., 2020) that has been used to monitor clinical trial outcomes of experimental treatments (Benatar et al., 2024a) like tofersen, recently approved as a treatment in the USA for people carrying the SOD1 mutation (Miller et al., 2022), but is not yet approved by the TGA. Other potential pharmacodynamic markers of MND include CSF poly(GP) proteins (Gendron et al., 2017) and urinary p75^{ECD} (Shepherd et al., 2017). Urinary p75^{ECD} may change over disease progression, where any detected change in the slope of p75^{ECD} following a treatment intervention favors the ability to detect significant biological changes over time (Gendron et al., 2017, Benatar et al., 2020). Urinary p75^{ECD} has been implemented in multiple phase 2 clinical trials (Gold et al., 2019, Vucic et al., 2020b, Henderson et al., 2021, Koch et al., 2024, Paganoni et al., 2025), with future prospects of phase 3 clinical trials investigating experimental therapeutics in people with MND. Upon validation, this may offer an objective and time-saving method of designing randomized clinical trials, detecting therapeutic benefit, and assessing the efficacy of phase 2 therapeutics for phase 3 clinical trials (Goyal et al., 2020).

1.2.4 Progression biomarkers

Disease progression markers are a subset of pharmacodynamic markers that measure changes in disease characteristics over time, and can be used as pharmacodynamic markers (Benatar et al., 2016a). Biological fluids including serum, urine, and cerebrospinal fluid (CSF) are important sources from which to identify biological markers for the advancement of potential therapeutics (Huber et al., 2019). The only current FDA approved tool that is a pharmacodynamic marker and assesses disease progression is the monthly change in ALSFRS-R (Goyal et al., 2020), however, this tool is vastly underpowered in the context of MND heterogeneity (Kimura et al., 2006). Shephard *et al.*, (2017) discovered that urinary p75^{ECD} increased with disease progression, identifying the first biofluid progression biomarker of MND. Other potential biomarkers of disease progression are found outside of biofluids. Compound motor action potentials, motor unit number indices (Ibrahim et al., 2021), and short-interval intracortical inhibition (Vucic et al., 2006) are examples of neuroimaging and neurophysiological methods that have potential to be used to monitor disease progression. Measurement of progression data can improve the detection of benefit for outcome measures over multiple disease time-points, where true comparisons are needed to determine drug efficacy (Goyal et al., 2020).

1.2.5 Predictive biomarkers

Predictive biomarkers are used to predict the individuals most likely to respond to a therapeutic intervention. Prediction at present is based on genetics, and there have been very few other physiological or pathological predictive biomarkers discovered. Most useful when targeting a specific pathology, a predictive marker is a known gene mutation that predicts shorter survival after an MND diagnosis; and thus, identifying patients most likely to benefit from therapies targeting the particular genetic cause of disease (Miller et al., 2020). An example of this are individuals who have a familial A4V mutation in the *SOD1* gene (*SOD1*^{A4V}) which predicts an aggressive form of MND with a short survival (Bali et al., 2017). This has been proposed as a means of stratifying people with *SOD1*^{A4V} MND mutations for clinical trials targeting antisense therapies (Bali et al., 2017).

1.3 Current investigated biomarkers of MND

1.3.1 Non-biofluid markers of MND

The revised ALS Functional Rating Scale is a validated and approved questionnaire-based scale that measures the physical function of people with MND in activities of daily living (ADL). Calculated by interviewing the patient or their caregiver, the ALSFRS-R score

assesses 12 ADL, ranking the patient with a score from 0, through to 4, where 0 represents no function at all and 4 represents normal function. The ALSFRS-R incorporates questions that make an assessment on the quality of speech, fine motor skills, dyspnoea, orthopnoea, and the need for ventilatory support. This generates a total score range from 0, indicating survival by permanent assisted ventilation, or 48 for healthy functioning individuals (Cedarbaum et al., 1999).

The ALSFRS was originally validated against isometric muscle strength tests, milestones of disease progression, and other subjective rating scales including the Schwab and England ADL rating scale and the Clinical Global Impression of Change (CGIC) scale (Cedarbaum and Stambler, 1997). Since then, the estimated monthly change in ALSFRS-R score is an approved pharmacodynamic marker of MND (Benatar et al., 2018, Miller et al., 2020, Goyal et al., 2020), and the Δ FRS at the time of diagnosis (or baseline measure) predicts survival time i.e. prognosis (Kimura et al., 2006). Although the change in ALSFRS-R is clinically meaningful (Castrillo-Viguera et al., 2010), limitations indicate that early and late phases of MND can show rapid rates of decline (Gordon et al., 2010) and small plateaus/ reversals are common as the scale is curvilinear (Bedlack et al., 2016). Although the subjective score has proven to be underpowered in improving homogeneity (Goyal et al., 2020), the monthly change in ALSFRS-R is the only pharmacodynamic marker currently used in all clinical trials and the Δ FRS provides a prognostic indicator of survival (Kimura et al., 2006).

1.3.2 Neurophysiological biomarkers

Neurophysiological and electrophysiological biomarkers have been proposed as candidate prognostic and progression markers for people with MND. Clinical signs can be augmented by non-fluid based neurophysiological imaging and electrophysiological techniques, demonstrating physical and structural changes over time (Vucic et al., 2021).

Neurophysiological imaging biomarkers are part of a range of magnetic resonance imaging (MRI) and magnetic resonance spectroscopy markers examining the central nervous system. Neurophysiological tests of the CNS include structural MRIs, generating weighted images that serve as predictive and progression biomarkers longitudinally (Ferraro et al., 2017). Conventional MRI scans of the CNS have shown prognostic value for MND in the past (Chan et al., 1999), demonstrating extra-motor involvement and monitoring the progressive spread of protein aggregates (Lule et al., 2009). Advanced diffusion tensor imaging (DTI) was later introduced as a potential progression marker of UMN lesions (Sawalha et al., 2019), measuring anisotropic movement in damaged white matter tracts

(Bowser et al., 2011) and correlating with white matter changes along disease progression with the ALSFRS-R (Borsodi et al., 2017, Baek et al., 2020). Advanced DTI and anatomical imaging displaying muscle volume reduction represent the rate of disease progression, though remain under investigation (Baek et al., 2020). Other neurophysiological techniques like proton magnetic resonance spectroscopy and positron emission tomography are still under investigation as candidate progression markers may be useful for providing evidence of treatment target engagement over time in clinical trials (Maul et al., 2020).

Additionally, electrophysiological biomarker techniques objectively assess lower motor neuron function. The motor unit number estimation (MUNE) and motor unit number index (MUNIX) are measures of motor to muscle innervation identifying pre-clinical LMN loss (Neuwirth et al., 2016), showing correlation to decreased compound motor action potential (CMAP) amplitudes reflecting disease progression, the functionality of LMN (Dengler, 2002), and greater rate of decline vs. ALSFRS-R scores as a marker of MND progression (Ibrahim et al., 2021). Electrical impedance myography (EIM) is a sensitive measure correlated with disease progression and prognosis (Basaia et al., 2019), while transcranial magnetic stimulation is a measure of the reduced short-interval intracortical inhibition (SICI) of MND motor-evoked potentials (MEP) shown to reveal both inhibitory and excitatory effects of the motor cortex (Vucic and Kiernan, 2006). Measured as the stimulus intensity required to evoke target MEPs (Vucic et al., 2006), the intensity required to track a constant target MEP has shown to be reduced in limb and bulbar onset MND compared to healthy individuals due to the degeneration of inhibitory cortical interneurons (Vucic and Kiernan, 2006). SICI is useful as a biomarker of disease prognosis and progression as a measure of both UMN and LMN involvement (Vucic et al., 2006).

Neurophysiological and electrophysiological data has shown its utility in MND, though are costly and common causes of uncertainty (Davenport et al., 1996) protein and molecular-based biomarkers are economically advantageous for therapeutic clinical trials (Chen and Shang, 2015, Mitsumoto and Saito, 2018). Examined in CSF, blood, and urine, biomarkers proposed for MND will be discussed and reviewed, in addition to looking at the metabolite neopterin as a candidate biomarker for MND. Further research warrants the approval of other electrophysiological markers for MND.

1.3.3 Biofluid markers of MND

Biofluid markers are advantageous over pathological, electrophysiological, and neurophysiological imaging markers from an economical and biological perspective, with

lower expenses, better detectability, and easier quantification compared to the analysis of tissues (Meldolesi, 2021). Prognostic biomarker candidates important to reduce heterogeneity have been identified that may subgroup MND patients to how they might respond to a targeted therapy. Biofluid prognostic markers include the extracellular domain of the common neurotrophin receptor p75 (p75^{ECD}) in urine which has been shown to be a prognostic and progression marker (Shepherd et al., 2017) reflecting motor neuron degeneration in a phase 2 clinical trial (Gold et al., 2019). Serum NfL has been validated as a prognostic biomarker (Benatar et al., 2020, Benatar et al., 2024b) and used in clinical trials as a candidate pharmacodynamic biomarker (Miller et al., 2022, Benatar et al., 2024c, Benatar et al., 2024d). The ratio of urinary N-terminal titin fragments, a product of the myofibrillar component of skeletal muscle, has also been suggested as a candidate prognostic marker for MND (Yamada et al., 2021). Urinary neopterin has too been suggested as a biomarker of neuroinflammation (Westarp et al., 1993, Lunetta et al., 2020), possibly reflecting immune dysfunction and disease progression.

1.3.4 Serum Neurofilament Light

Neurofilaments are the structural components of the neuronal cytoskeleton, comprised of neurofilament light chains, neurofilament medium chains, neurofilament heavy chains (NfH), and internexin (Xu et al., 2016). Serum NfL has been validated as a prognostic and potential pharmacodynamic marker of MND (Benatar et al., 2020, Benatar et al., 2024b). Neuronal and axonal damage in MND releases these aggregate neurofilament light chains into the circulation, detectable in CSF and blood (Gnanapavan et al., 2013). Boylan and colleagues (2013) recorded a similar baseline increase in peripheral blood and CSF NfH levels using an ELISA, and western/immunoblotting techniques have been used quantify NfL and NfH chains, although with poor sensitivity.

Since the advent of a new detection method of the Single Molecule Array (SIMOA), the validation of NfL as a prognostic biomarker moved very fast. Serum NfL can be measured accurately in the femtomolar range using the SIMOA, which has allowed for previously undetectable quantities in serum to be accurately measured (Hendricks et al., 2019). The reproducibility of serum NfL detection by SIMOA is unmatched, with intra- and inter-assay coefficient of variation in MND serum specimens being less than 5% (Benatar et al., 2020). NfL was identified more recently in the CSF of people with MND using mass spectrometry with Tandem Mass Tag (TMT) labelling, identifying protein levels of neurofilaments (NEFM and NEFL) increased in both sporadic and genetic MND subtypes compared to controls (Trautwig et al., 2025) for the first time. Thouvenot *et al.* (2020) investigated the levels of

serum NfL chains to find significantly higher baseline serum NfL concentrations in MND patients, where a cox regression and multivariate analysis comparing serum NfL against known systemic prognostic factors provided strong evidence for the use of NfL and pNfH to determine survival outcomes (Thouvenot et al., 2020). The high specificity and sensitivity of NfL and pNfH may be valuable in the diagnosis of MND (Li et al., 2016), though levels in CSF have only been recommended as inclusion criteria for clinical trials (Gagliardi et al., 2019). People with slower disease progression have lower levels of serum NfL compared to those with a rapidly progressing disease (Meyer et al., 2024).

Studies have aimed to determine the presymptomatic levels of serum and CSF NfL. Increased levels of neurofilaments prior to the symptom onset and diagnosis of MND were observed (De Schaepdryver et al., 2019), while Benatar and colleagues (2019) reported an increase in neurofilaments 12-24 months before the onset of symptoms, and continued to increase for up to six months after disease onset (Benatar et al., 2019). Pre-symptomatic neurofilament measures were found to reflect the rate of disease progression and prognosis (Benatar et al., 2019). Since then, NfL levels have been used to identify clinical trial outcomes, implemented to evaluate the effectiveness of experimental treatments (Benatar et al., 2024a), including tofersen, a recently approved treatment for people with SOD1 mutations in the USA (Miller et al., 2022). While the quantification of serum NfL shows prognostic value (Benatar et al., 2020), urinary biomarkers have advantages in the ease of accessibility, with a safer, less costly, and more abundant sample collection process (Harpole et al., 2016).

1.3.5 Urinary p75^{ECD}

Human urine is a very accessible biofluid, facilitating protein and metabolite discovery, when compared to blood and CSF (Mischak et al., 2010). Previous research from our laboratory has demonstrated that the extracellular domain of the common neurotrophin receptor p75 (p75^{ECD}) represents a unique urinary biomarker of MND prognosis and progression (Shepherd et al., 2017). Shepherd *et al.*, (2017) discovered that p75^{ECD} was elevated at baseline and rises with disease progression, representing the first fluid-based progression marker of MND to be identified. This also demonstrated the potential of p75^{ECD} as a pharmacodynamic marker, where p75^{ECD} was employed in a Phase 2 MND clinical trial for the anti-retroviral therapy Triumeq (Gold et al., 2019). The primary outcome of the trial was safety and tolerability, and the observation that the level of urinary p75^{ECD} followed the effect of treatment has informed a larger Phase 3 placebo-controlled trial, that recently failed due to futility (unpublished). Urinary p75^{ECD} has been used in more recent clinical trials,

alongside serum NfL as a pharmacodynamic and progression biomarker for multiple treatment candidates in phase 2 clinical trials (Gold et al., 2019, Vucic et al., 2020b, Henderson et al., 2021, Koch et al., 2024, Paganoni et al., 2025), and has been discussed as a strong candidate response or monitoring biomarker (Benatar et al., 2024b).

1.3.6 Neuroinflammatory biomarkers for MND

Candidate neuroinflammatory markers including chitotriosidase-1 (CHIT-1), chitinase-3-like protein 1 (YKL-40) and monocyte chemoattractant protein-1 (MCP-1) have been investigated (Kuhle et al., 2009), where CHIT-1 and MCP-1 may be elevated in the CSF of MND subjects (Thompson and Turner, 2019). Released from microglia, CHIT-1 was found to be significantly elevated in the CSF of MND patients compared to healthy individuals (Varghese et al., 2013, Thompson et al., 2018, Steinacker et al., 2018, Varghese et al., 2020, Vu et al., 2020). The two studies examining CHIT-1 over disease progression saw only small fluctuations over time (Steinacker et al., 2018, Vu et al., 2020), and CHIT-1 was not always found to correlate to the ALSFRS-R (Thompson et al., 2018). While these studies were reported in CSF, the significance of CHIT-1 in serum and plasma are conflicting with only one study reporting significant levels in serum (Pagliardini et al., 2015). No difference has been reported in chitinase concentrations in the serum of people with MND compared to healthy individuals, but they may have strong value in the CSF (Rogers et al., 2023).

Since neuroinflammation is a part of MND pathology, there has been a significant interest in the development of therapeutics to inhibit inflammatory processes. MCP-1 is a Th-2 anti-inflammatory chemokine that has shown stability over disease progression in CSF (Huang et al., 2020, Guo et al., 2017), though linked to faster disease progression (Gille et al., 2019, Guo et al., 2017). MCP-1 was not found to be significantly increased in MND patients compared to healthy individuals when quantified in serum (Hu et al., 2017), warranting further investigation. Other pro-inflammatory cytokines like IL-6, IL-18, and TNF- α previously discussed as Th-1 phenotypes have been found upregulated in MND patient CSF (Malaspina et al., 2015), where IL-6 was particularly increased at late-stage disease (Lu et al., 2016)

C-reactive protein (CRP) is released as a universal marker of an activated innate immune response (Gewurz et al., 1982, Pepys and Hirschfield, 2003). Comprised of a pentamer chemical structure, CRP monomerises at sites of infection or inflammation, functioning as a phagocytic opsonin (Janeway and Medzhitov, 2002) and activating the complement factors discussed previously via the classical complement pathway (i.e., IgG and IgM antibody

isotypes) (Agrawal et al., 2001). CRP is released from multiple cell types, including hepatocytes (Pepys and Hirschfield, 2003), adipocytes (de Dios et al., 2018), macrophages (Devaraj et al., 2009), endothelium (Pasceri et al., 2000), and smooth muscle (Calabró et al., 2003), where several studies have documented its activation in MND. Blood CRP reportedly increased in a longitudinal cohort of people with MND as a measure of low-grade inflammation (Keizman et al., 2009), while serum CRP was found to correlate with serum creatinine, the ALSFRS-R, and decline in patients following the commencement of Riluzole (Sanjak et al., 2018). However, there is no current robust method for the reproducible detection of CRP available to suggest its utility as a predictive biomarker of MND disease progression to determine an outcome measure for neuroinflammatory-regulating therapeutics (Lunetta et al., 2017).

1.4 Neopterin as a measure of infectious and inflammatory diseases

Research into urinary metabolomics has identified multiple objective biological markers ranging from modified nucleosides (Godoy et al., 2020), intermediates from the kynurenine pathway (Tan and Guillemin, 2019), and pteridines (Burton and Ma, 2017). From these examples, pteridines and their derivatives have best exemplified the progress made in clinical investigations of urinary metabolomics to MND biomarker discovery through to the correlation of immunological dysfunction (Malaspina et al., 2015) and clinical outcomes (Goyal et al., 2020). Pteridines and their derivatives are functional intermediates involved in the metabolism of several hormones, vitamins, and cofactors, released as products of neuroinflammation (Kośliński et al., 2011). First isolated as insect pigments (Hopkins, 1895), it was later discovered that the pteridines xanthopterin, isoxanthopterin, and leucopterin contained the bi-cyclic nitrogenous ring pyrazino-(2,3 – d)-pyrimidine (Purmann, 1941). Huber *et al.* (1984) went on to identify neopterin as a precursor molecule of biopterin, an essential cofactor for neurotransmitter synthesis. Antigenic stimulation of mononuclear cells released neopterin into cell culture mediums from an *in vitro* immune response, where (Bitterlich et al., 1988) saw the release of neopterin from macrophages and monocytes stimulated by interferon-gamma (IFN- γ) released from activated Th-1 cells. Since then, the biological discovery of other pteridines, namely 5,6,7,8-tetrahydrobiopterin (BH₄) and neopterin, have been important for providing context and identifying a panel of molecular biomarkers of the immune response for disease detection, predicting disease outcomes, and monitoring therapeutic responses to neuroinflammatory diseases like cancer (Ma and Burton, 2013), bacterial infections (Molero-Luis et al., 2020), and MND (Westarpl et al., 1993, Lunetta et al., 2020).

Activation of the immune system has a systemic role in the pathogenesis of MND. Pteridines have been one of the largest classes of metabolites studied in urine as potential markers of immune system activation (Burton and Ma, 2017) encouraging their isolation and quantification in human specimens from a range of analytical methods. Sakurai and Goto (1967) were the first to isolate 25 mg of neopterin from 500 litres of human urine, while Wachter *et al.* (1979) later observed increased concentrations of urinary neopterin in patients with malignant tumours and viral diseases. These results were validated by future studies (Stea *et al.*, 1981, Fuchs *et al.*, 1988) suggesting urinary neopterin was increased as a product of the immune response against viral and malignant cells (Eisenhut, 2013).

1.4.1 Biosynthesis of neopterin and receptor expression

The three pterin-dependent hydrolases; phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase were identified by (Kaufman, 1974). The family of hydroxylase enzymes share a range of physical and catalytic properties important to the biosynthetic pathway of neopterin production (Kaufman, 1981). As a naturally occurring co-factor of the enzymatic hydroxylation of aromatic acids by a family of pterin-dependent mixed-function mono-oxygenases, tetrahydrobiopterin (BH₄) is an electron donor facilitating the hydroxylation reaction of phenylalanine to tyrosine in the liver; and of tyrosine to L-dopa and tryptophan to 5-hydroxyl-tryptophan in neuroendocrine tissue (Thöny *et al.*, 2000). Tetrahydrobiopterin facilitates the biosynthesis of dopamine, norepinephrine, and epinephrine from tyrosine, and of catecholamines and serotonin from tryptophan (Cook *et al.*, 2017).

Neopterin is a pyrazimo-pyrimidine derivative first discovered in 1963 (Hamerlinck, 1999). The chemical structure illustrated in **Figure 1.6** was identified as 2-amino-4-hydroxy-6-(D-erythro-1', 2', 3'-trihydroxypropyl)-pteridine. The larger pteridine structure is reduced to 7,8-Dihydroneopterin triphosphate (NH₂TP) from guanosine triphosphate (GTP) intermediates as a product of biosynthesis (Jones and Brown, 1967). Murr *et al.*, (2002) illustrated the biosynthesis of neopterin derivatives from GTP in **Figure 1.7**, where GTP cyclohydrolase-I cleaves the purine to synthesize NH₂TP (Murr *et al.*, 2002). This intermediate is converted to 7,8-dihydrobiopterin by 5,6,7,8-tetrahydrobiopterin (BH₄), functioning as an essential co-factor of nitric oxide synthases (Jones and Brown, 1967). The activation of GTP-cyclohydrolase-I is stimulated by IFN- γ , promoting a phosphorylation cascade via adrenergic—cyclic AMP mechanisms and further enhancing the activity of GTP-cyclohydrolase-I. Upon GTP-cyclohydrolase-I activation, BH₄ and neopterin derivatives are

produced in small amounts (Murr et al., 2002), however, the intracellular production of NH_2TP is accelerated. NH_2TP is a well-established precursor of BH_4 within the biosynthetic pathway of neopterin production. In the context of this hydroxylation reaction, NH_2TP is largely metabolised to BH_4 in the presence of 6-pyruvoyl-tetrahydropterin synthetase (Sakurai and Goto, 1967). However, human peripheral monocytes and macrophages, the primary location of urinary neopterin production from immunological stress (Huber et al., 1984, Kip et al., 2018), lack the enzyme 6-pyruvoyl-tetrahydropterin synthetase, causing the accumulation of neopterin.

1.4.1.1 Cellular source and induction signal of neopterin

Neopterin is produced by immune cells in the periphery and the CNS, as discussed above. Activated by central microglia and peripheral monocytes/ macrophages from $\text{IFN-}\gamma$ stimulation produce neopterin. The innate and adaptive immune system play equally important roles in the progression of MND and neuroinflammation caused by the activation of microglia and infiltration of monocytes and pyruvoyl-tetrahydropterin. The consequence of this absence results in the hydrolysis of NH_2TP by phosphatases, producing and excreting 7,8-dihydroneopterin (NH_2) and neopterin (Blau et al., 1989).

In MND mice studies, a leaky blood-brain barrier has shown to allow infiltration of CD8^+ and CD4^+ cells (Sta et al., 2011). This immune response to neuroinflammation results in macrophage/ monocyte activation outside of the CNS. Antibodies to neurons in the CNS injury promote glutamate excitotoxicity (Henkel et al., 2009), neurofilament aggregation (Pilli et al., 2017), and alter the homeostasis of essential amino acids (Howland et al., 2002). The release of the pro-inflammatory $\text{IFN-}\gamma$ and interleukin-1 acts on microglia, macrophages and lymphocytes involved in this immune response, modifying the behaviour of $\text{CD4}^+/\text{CD8}^+$ T-cells to reduce the level of pro-inflammatory cytokines (Van Den Eeckhout et al., 2021). Studies found that the depletion of CD4^+ regulatory cells accelerated disease progression, while increased activation of CD4^+ and CD25^+ cells prolonged of the life-span of on *SOD1-G93A* mutant mice models (Lasiene and Yamanaka, 2011). Neopterin production and tryptophan degradation are influenced by $\text{IFN-}\gamma$ acting on primary macrophages, peripheral blood mononuclear cells (PBMC), and THP-1 cells as a model for human monocytes (Weiss et al., 1999). Microglia and neurons respond to $\text{IFN-}\gamma$ like peripheral macrophages and are proposed as central sources of neopterin production (de Paula Martins et al., 2018). IL-12 is an adjuvant to the effects of $\text{IFN-}\gamma$, superinducing the production of neopterin, alongside IL-1 β , a T-cell growth factor, allowing T-cells to respond and stimulate IL-2 production (Kasahara et al., 1983).

Figure 1.6 has been removed due to Copyright restrictions

Figure 1.6: Chemical structure of neopterin

Neopterin (2-amino-4-hydroxy-6-(*D*-erythro-1',2',3' - trihydroxypropyl)-pteridine) is a small molecule with molecular weight of 253 Daltons (Murr et al., 2002).

Figure 1.7 has been removed due to Copyright restrictions

Figure 1.7: Interferon- γ stimulates neopterin biosynthesis within human monocyte derived macrophage and dendritic cells

Interferon- γ drives neopterin synthesis, stimulating an intracellular cascade activating guanosine triphosphate (GTP) by inducing GTP cyclohydrolase-I. 6-pyruvoyl tetrahydrobiopterin synthase is limited within human macrophages, causing neopterin derivatives to accumulate by inhibiting the production of 5,6,7,8-Tetrahydrobiopterin. *Adapted from (Murr et al., 2002).*

Th-1 lymphocytes are stimulated by IL-2 and upregulate IFN- γ production, while IL-4 and IL-10 are found to inhibit Th-1 type cytokine production and neopterin production in PBMC cells (Weiss et al., 1999). Illustrated in **Figure 1.8**, Th-1 cell-mediated immunity is regulated by endogenous cytokine production, imposing down-stream effects on IFN- γ concentrations and neopterin production in macrophage and monocytic immune cells (Plata-Nazar, 2011). Hoffmann *et al.* (2003) went on to demonstrate how interferon-alpha (IFN- α) and interferon-beta (IFN- β) at high concentrations elicited similar effects on neopterin production in monocyte derived macrophages. Bacterial pyrogens and toxins were identified as intracellular THP-1 cell stimuli, activating neopterin synthesis outside of the immune system in fibroblasts, vascular smooth muscle cells (VSMC) (Walter et al., 1998), and epithelial cells (Andert et al., 1992).

Preclinical data suggests that this protective response emerges as motor neurons and NMJs begin to degenerate (Liao et al., 2012). Previous evidence from animal models (Zhao et al., 2010) suggests that this response shifts from an anti-inflammatory to a pro-inflammatory response as motor neurons continue to degenerate and accumulate MND-related protein aggregates (Zhao et al., 2010, Liao et al., 2012). Microglia become pro-inflammatory, where several Th-1 cytokines (e.g., IL-6, IL-18) in CSF are also being investigated as markers of the pro-inflammatory state of the CNS (Molero-Luis et al., 2020), but there is sparse information on peripheral or systemic pro-inflammatory signals. The small molecule neopterin, released from macrophage and dendritic cells in the periphery (Berdowska and Zwirska-Korczala, 2001) in response to IFN- γ , microglia (Molero-Luis et al., 2013), and neurons in the CNS (de Paula Martins et al., 2018), is proposed as a candidate marker of this elevated pro-inflammatory state seen in people with MND. Urinary neopterin has been used to assess the Th-1 type immune response in pro-inflammatory conditions (Huber et al., 1984, Molero-Luis et al., 2020) and has previously been suggested as a biomarker of immunological dysfunction in MND (Westarpl et al., 1993, Lunetta et al., 2020) but has not been extensively studied.

BH₄ has been used like neopterin as a biological marker for a range of clinical disorders, including cardiovascular diseases (Bendall et al., 2014), kidney injury in diabetes (Deng et al., 2022), and neurotransmitter disorders (Opladen et al., 2020). Walter *et al.* (1998) was the first to discover a 600-fold increase of intra- and extracellular BH₄ following TNF- α and inflammatory cytokine stimulation, increasing downstream neopterin intermediates. Derived from guanosine triphosphate, BH₄ is an essential cofactor for amino acid hydroxylases involved in the biosynthesis of neurotransmitters, nitric oxide synthases regulating

vasodilation, and alkylglycerol monooxygenases regulating the chemical synthesis of ether lipids for chemical signalling (Opladen et al., 2020).

Around the same time, Hoffmann *et al.* (1998) studied the effects of proinflammatory tumor necrosis factors (TNFs) and interferons on macrophage-derived neopterin in cultured tissues. Since VSMC undergo apoptotic death in atherosclerotic lesions, the effects of neopterin, TNF- α , and IFN- γ were observed, leading to nitric oxide (NO) synthesis via upregulated nitric oxide synthase (iNOS) gene expression. Thus, neopterin, TNF- α , and IFN- γ were found to be regulators of apoptotic death in VSMC via NO synthesis (Walter et al., 1998, Hoffmann et al., 1998). This has not been replicated in MND, however, monocyte and dendritic immune cells upregulated in MND (Henkel et al., 2004) are factors known to activate the expression of iNOS genes. Studies *in vitro* have demonstrated the mechanisms of neopterin production, where the human myelomonocytic cell line THP-1 was found to degrade tryptophan and produce neopterin upon treatment with IFN- γ (Schoedon et al., 1986). Similarly, neopterin production was replicated through the treatment of THP-1 cells with tumour-associated antigen 90K, and studies *in vivo* saw a significant degradation of tryptophan in culture supernatants leading to the formation of kynurenine and neopterin production (Altindag et al., 1996).

IFN- γ is a central Th-1 cytokine and may drive systemic inflammation in MND. IFN- γ communicates with and regulates the actions of other pro-inflammatory cytokines, showing function as a master checkpoint regulator for the differentiation of cytokines and a heightened Th-1 response (Zha et al., 2017). From its biological function and clinical prevalence, urinary IFN- γ may be regarded as a candidate biological marker of systemic immune dysfunction, however, it presents many clinical disadvantages compared to urinary neopterin. IFN- γ binds to membrane-bound receptors on the surface of fibroblasts with high affinity, rapidly internalised at body temperature within 4-5 minutes of activation (Anderson et al., 1983). This characteristic requires IFN- γ to be quantified in serum, where the clinical measurement of IFN- γ in a soluble phase makes it difficult to relate to its biological effect (i.e., when receptor bound) (Esteve-Solé et al., 2018). In comparison to IFN- γ , neopterin is a biochemically stable metabolite in human urine, undergoes rapid renal clearance, and does not bind to receptors or have secondary biological effects (Berdowska and Zwirska-Korczala, 2001). This warrants urinary neopterin as a feasible candidate urinary biomarker for the immune dysfunction seen in MND, rather than IFN- γ (Stuart et al., 2020).

Figure 1.8 has been removed due to Copyright restrictions

Figure 1.8: Activated TH1-type cell-mediated immunity regulates IFN- γ release and neopterin production in macrophage and monocytic immune cells

Th-1 immune cells dendritic cells are stimulated by IL-12 produced by mature dendritic cells, driving the production of IL-2, tumour-necrosis factor beta, and interferon- γ (IFN- γ). IFN- γ stimulates monocyte-derived macrophages to produce reactive oxygen species (ROS) contributing to oxidative stress and neuroinflammation, indoleamine-2,3-dioxygenase (IDO) regulating tryptophan levels, and GTP-cyclohydrolase-I converting tryptophan to kynurenine and resulting in the formation of neopterin in humans. Excessive ROS activation from IFN- γ produces tumour necrosis factor- α (TNF- α), enhancing the sensitivity of macrophages to IFN- γ , further encouraging ROS activation, oxidative stress, inflammation, and neopterin production. *Adapted from* (Jenny et al., 2010).

1.4.2 Neopterin as a candidate prognostic, pharmacodynamic, progression, and predictive biomarker

It is evident that neopterin plays a role in neuroinflammatory diseases as a potential marker of pro-inflammation. Based on this biochemical evidence, upregulated neopterin biosynthesis is a product of the innate and adaptive immune responses. It has been suggested that astrocytes, microglia, and neurons respond to IFN- γ , and are candidates for neopterin production, like activated monocytes/ macrophages (de Paula Martins et al., 2018). Early and sensitive markers of immunological changes are important, by which the measurement of neopterin in human urine may be a non-invasive and objective measure of neuroinflammation (Jones and Brown, 1967).

Metabolites may be sampled non-invasively in an array of biospecimen types. These include exhaled breath, saliva, and urine, facilitating a greater variety of analytical techniques than is possible for protein biomarkers like p75^{ECD} (Taylor et al., 2021). Among these, urine confers several advantages to MND biomarker applications and is particularly well suited for metabolite analysis (Barr et al., 2005, James et al., 1988). Namely, urine can be sampled non-invasively and economically in large volumes suitable for long-term storage. Further, urine can be precipitated to remove proteins, immunological factors, cells, and other matrix components common to serum and tissue lysates that require substantial pretreatment of samples prior to analysis (Mischak et al., 2010). Neopterin is removed from the circulation by glomerular filtration (Fuchs et al., 1994). Rhesus monkeys received high intravenous doses of neopterin to observe excretion kinetics. Following a sharp increase in urinary neopterin, a long half-life of approximately 90 minutes indicates resistance to decay, and extended microglial and monocyte/ macrophage activity useful for detecting MND progression (Fuchs et al., 1994). Although urinary metabolomics have been studied in relation to immunological diseases for decades, it presents several challenges. Unlike genes or proteins, changes in metabolites can occur physiologically after exercise (Schraner et al., 2020), and it has been poorly explored whether urinary levels of neopterin become altered in the presence of MND (Westarpl et al., 1993, Lunetta et al., 2020).

1.4.3 History of neopterin detection in MND

Westarpl *et al.*, (1993) was the first to study the potential of neopterin and β_2 -microglobulin in cases of adult sporadic MND as markers of the disease. Based on the understanding that neopterin correlated with the immune activity of autoimmune disorders, Westarpl *et al.*, (1993) analysed neopterin levels in the serum and CSF of 8 adult sporadic MND patients. In this study, the concentrations of both neopterin and β_2 -microglobulin in CSF never

exceeded relative serum levels, alongside a failure to demonstrate an outright increase of neopterin and β_2 -microglobulin in the CSF in people with MND compared to healthy controls. Yoshida *et al.*, (1999) conducted a similar study to identify the levels of adenosine, regulating cerebral blood flow, and neopterin in the CSF of neurodegenerative patients. In contradiction to Westarpl's findings (1993), significantly higher levels of adenosine and neopterin were found in 12 MND patients compared to healthy controls, arguing the potential of neopterin as a sensitive marker of neuroinflammation, corresponding to the immunological component of MND as a potential marker of mononuclear cellular infiltration in the CSF.

Razumovitch *et al.* (2003) and Hoffmann *et al.* (2003) went on to identify crucial properties of neopterin relative to MND pathogenesis. Neopterin was found to exert physiological effects following host-defense reactions with ROS intermediates as a marker of OS. Neutrophils responded to the exposure of neopterin by generating oxygen, hydroxyl radicals, and NO by NADPH independent pathways (Razumovitch *et al.*, 2003), consistent with the progressive toxicity observed in MND pathogenesis. Neopterin was hypothesized to amplify the cytotoxic and neurotoxic potential of activated macrophages and dendritic cells, associating neopterin levels with MND progression and prognosis (Hoffmann *et al.*, 2003). Despite this compelling evidence, neopterin as a biochemical marker for MND has not been thoroughly explored. The authors argued that while activated macrophages were found infiltrating the spinal cord of MND patients, normal neopterin and β_2 -microglobulin levels contradicted the hypothesis of a persistent macrophage response in MND.

Süssmuth *et al.*, (2008) later reviewed these papers, recognising neopterin as a potential biochemical marker of OS mediated by astrocyte, microglia, and cytokine stimulation. Considering the work from Westarpl *et al.*, (1993) and Yoshida *et al.*, (1999), Süssmuth *et al.*, (2008) concluded these two studies had insufficient sample sizes to have any clinical correlation or statistical significance. Both studies failed to examine the presence of neopterin in the urine, highlighting a gap in the literature surrounding the potential of neopterin as a marker of neuroinflammation. Based on the knowledge that neopterin is a stable metabolite in the urine as a small and biochemically inert molecule (Berdowska and Zwirska-Korczala, 2001), further studies are needed to confirm whether neopterin is raised in MND compared to healthy controls to warrant the validation of neopterin as a marker of cell-mediated immunity in MND.

The elevation of neopterin in urine, serum, and CSF has since been recorded in other Th-1 cell or macrophage mediated inflammatory diseases (Lin, 1989), including advanced

Parkinson's Disease (Widner et al., 2002b), Human Immunodeficiency Virus-1 (HIV-1) (Heyes et al., 1992), and autoimmune disorders (Schoedon et al., 1987). The presence of urinary neopterin in other diseases rules out its potential as a diagnostic marker of MND, suggesting an application potentially suited as a predictive marker of immune dysfunction or prognosis. There has been a lack of description of predictive markers that could be useful in clinical trials that target the inflammatory pathology of MND, where neopterin as a characteristic marker of neuroinflammation may predict a response to anti-inflammatory treatments. Neopterin has been studied extensively as an objective marker of inflammation and an activated cellular immune response in a variety of conditions, however, the relationship between neopterin production and MND is poorly understood. This review presents multiple analytic methods for the determination of neopterin in urine to understand its role in MND as biomarker candidate of disease prognosis, progression, and predicting immune dysfunction.

1.4.4 Methods of detection: measurement of neopterin

Neopterin as an objective biomarker of neuroinflammation in MND has been previously explored by various methods. Neopterin has been detected in serum (Werner et al., 1987), cerebrospinal fluid (Yoshida et al., 1999), saliva (Kato et al., 1989), and ascites fluid (Zeimet et al., 1998). Methods for neopterin detection in urine include enzyme-linked immunosorbent assay (ELISA) (Westermann et al., 2000), and high-performance liquid chromatography (HPLC) with fluorescent and ultraviolet detection (Hausen et al., 1982, Fuchs et al., 1992, Lunetta et al., 2020). Recently, mass spectrometry (MS) with separation by HPLC has been the preferred method given its improved sensitivity in the identification of urinary metabolites (Jiménez Girón et al., 2012, Burton et al., 2016).

1.4.5 High performance liquid chromatography and fluorescent detection

Separating neopterin from other pteridines using HPLC has been widely used. The routine assessment of urinary neopterin requires a sensitive, repeatable, and robust method of detection and quantification. The HPLC separation method combined with fluorescent detection (HPLC-FD) was described for this purpose. HPLC-FD methods are used for its rapid separation and sensitive detection on reversed phase chromatography columns (Hausen et al., 1982). An isocratic separation gradient and fluorescent detection at a wavelength of 353 nm started the foundation of HPLC methods for the detection of urinary neopterin (Fuchs et al., 1992).

Small studies have examined the level of neopterin and other pteridines in the CSF (Molero-Luis et al., 2020), serum (Westarpl et al., 1993), and urine (Lunetta et al., 2020) using HPLC-FD. However, the only example of this in MND is the assessment of urinary neopterin in 81 patients by Lunetta *et al.*, (2020), exploring neopterin in serum as a candidate marker of neuroinflammation and MND prognosis. Lunetta *et al.*, (2020) detected urinary neopterin using non-specific FD at 355 nm, offering complete oxidation of urinary NH_2 to neopterin under acidic conditions (pH 3) by using dipotassium hydrogen phosphate. Pteridines can occur in three oxidation states with different levels of stability. The analysis of pteridines by FD often involves the oxidation of residual pteridine derivatives. Oxidized pteridines are more stable and fluorescent for this application (Burton et al., 2015). Due to these oxidation states, studies have used ion-pair, ion-exchange, or hydrophilic interaction liquid chromatography techniques to quantify neopterin using FD (Tomšíková et al., 2013).

The use of HPLC-FD for the separation and detection of urinary neopterin detection has been repeated across multiple studies. Current techniques of urinary pteridine separation by HPLC have been developed (Culzoni et al., 2011, Mancha de Llanos et al., 2011, Kośliński et al., 2014, Tomšíková et al., 2014, Burton et al., 2016, Lunetta et al., 2020). Advantages of the HPLC methods described for the routine assessment of urinary neopterin include its rapid, sensitive, and repeatable separation from other pteridines (Stuart et al., 2020). However, it would be advantageous to combine HPLC with mass spectrometry (HPLC-MS) for more specific detection by fragmenting pteridines to identify transition ions using multiple reaction monitoring (MRM) (Burton et al., 2016).

1.5 Mass spectrometry

Mass Spectrometry (MS) is a high mass accuracy quantitative technique used to study a broad range of analytes including lipids, sugars, proteins, and metabolites. The current generation of instruments can identify and quantify proteins and peptides (proteomics) (Muntel et al., 2015, Swensen et al., 2021, Dellar et al., 2024, Trautwig et al., 2025) and small molecules (metabolomics) (Xiao et al., 2012, Fischer et al., 2013). Quantification of new urinary biomarkers by MS is feasible due to its greatly reduced dynamic range and matrix effects (Mischak et al., 2010). MS is highly sensitive, specific, and reproducible platform for metabolomic analysis, coupled with liquid chromatography (LCMS) for the detection and quantification of urinary ion transitions from its high speed and resolution (Wang et al., 2016). The application of MS to MND research is useful for neopterin detection using multiple reaction monitoring (MRM), and for new protein discovery by differential

abundance analysis that has been completed in CSF (Dellar et al., 2024, Trautwig et al., 2025) but not urine, and validating these proteins using parallel reaction monitoring (PRM) (Ronsein et al., 2015).

The sensitive and specific detection of urinary pteridines has been explored using multiple MS platforms. High performance liquid chromatography-mass spectrometry (HPLC-MS) (Burton et al., 2013, Burton et al., 2016, Whiley et al., 2019, Stuart et al., 2020) has been recommended for its application in the pharmacodynamic monitoring of experimental immunosuppressant drugs and potential markers of immunosuppression (e.g., urinary neopterin) due to its high repeatability, sensitivity, specificity, and robustness (Atcheson et al., 2003). Pteridine profiling has been explored in urine using HPLC-MS platforms (Jiménez Girón et al., 2012, Burton et al., 2016), demonstrating the ability to fragment neopterin, identify its transition ions, and undergo quantification using MRM-MS in other diseases (Lee et al., 2019, Burton et al., 2016), but this has not been explored in people with MND.

HPLC-MS is a strong tool for measuring metabolites like neopterin with high specificity, sensitivity, and repeatability in urine. However, neopterin is a highly polar metabolite with a structural isomer called monapterin that may co-elute with neopterin (Burton et al., 2016). To minimize co-elution of two metabolites, analytical column compatibility should be carefully optimized. The Phenomenex Luna Phenyl-Hexyl analytical column was found to separate pteridines from other compounds with superior resolution under a flow rate of 0.15-0.40 mL/min, with a mobile phase consisting of 0-0.2% formic acid in water, acetonitrile, and methanol, compared to a LUNA amino column by (Allegri et al., 2012).

Despite this, HPLC-MS has shown to be more sensitive and specific than HPLC-FD for urinary neopterin separation and detection (Burton et al., 2013). This competitive sensitivity may be used to validate previous neopterin quantification from Lunetta *et al.*, (2020) in MND clinical trials targeting neuroinflammation. Burton and colleagues (2016) used high resolution accurate mass MS to quantitate urinary neopterin from other pteridine derivatives in aggressive and benign cancer patients. Giron *et al.*, (2012) identified urinary neopterin using a QTRAP triple quadrupole mass spectrometer. The method was validated in terms of its linearity and repeatability, and was the first to be applied to pteridine biomarker analysis in seven healthy adults (Jiménez Girón et al., 2012). The utility of a high throughput HPLC-MS assay to validate previous methods of neopterin detection (Burton et al., 2013, Burton et al., 2016) and fluorometric detection (Lunetta et al., 2020) is important for pteridine biomarker development, and validation of urinary neopterin in people with MND.

Orbitrap mass spectrometry can also be used as a screening tool to perform protein-based biomarker discovery studies in an un-targeted approach. Orbitrap mass spectrometers can detect positively charged peptides protonated on primary amine groups at the amino terminus, and on lysine or arginine residues located at the C-terminus, producing a minimum 2+ charge state (Streckel and Schlosser, 2019). These peptides can be ionized by a H-ESI source, generally limited to sequencing peptides of less than 40 amino acids in length (Coon et al., 2005). The ions are routed through a quadrupole, filtering out neutrals and generating an MS1 'precursor' spectrum representing the mass-to-charge (m/z) ratio of intact peptides. The orbitrap mass analyzer is fueled with selected precursor ions in mass range windows, applying high voltages and inert gas to break apart precursor ions that are measured by the detector to deduce the amino acid sequence of peptides for protein identification. This generates MS2 spectra, revealing fragment ions to identify the corresponding amino acid sequence. Proteome Discoverer (Orsburn, 2021) and Spectronaut (Bruderer et al., 2015) are software used to analyse these mass spectra, identifying peptides based on their mass and charge by comparing them to a database of known protein sequences. The peptide sequence coverage of each protein is determined by the fragment ions in the MS2 scan, allowing for the identification of protein groups in the urine compared to a spectral library. The intensity of the MS1 scan determines how much of those proteins were present, generating a relative quantification between people with MND and healthy individuals (Orsburn, 2021).

1.6 Biomarker correction factors

Urinary biomarkers are an easily accessible, non-invasive, and rich source of analytes; provided urinary dilution factors are rightly corrected for. To combat the lack of effective therapies, reliable and objective biological markers, alongside factors of normalisation (Mervant et al., 2021), have been extensively investigated to supplement newer therapies in clinical trials and transform disease outcomes (Benatar et al., 2016a). Advances aimed at improving biomarker analysis in MND have faced challenges regarding the exclusion of patients with physiologically low urinary concentrations (Alessio et al., 1985, Shephard et al., 2017). Like hormones, biomarkers must be adjusted to reflect an individual's hydration status and time since last urination (Miller et al., 2004). Urinary creatinine concentration, specific gravity, and osmolality are common methods for adjusting dilution. This can be done via a 24-hour urine sample or spot sample. Once validated, spot samples for an analyte are convenient for patient collection.

1.6.1 Urinary creatinine

Urinary creatinine is an endogenous measure of the glomerular filtration rate (Bennett and Porter, 1971). Using creatinine is an adjustment that involves dividing the analyte concentration (e.g., ng analyte per mL urine or μmol per litre of urine) by the creatinine concentration (e.g., mg/L or mol/L). Analyte results are then reported as weight of the analyte per milligram of creatinine (or $\mu\text{mol}/\text{mol}$ of creatinine). For example, urinary p75^{ECD} (Shepherd et al., 2017) and neopterin (Lunetta et al., 2020) in spot urines have been normalised with creatinine. However, very low or very high creatinine concentrations may overestimate or underestimate the abundance of a urinary analyte. These concentrations are outside of the limits of detection regarding any of the creatinine measurement assays. For this reason, there are WHO guidelines (Mikheev and Lowry, 1996) to indicate that spot urine samples below 0.3 mg/ml are unacceptably dilute specimens. In a subsequent National Health and Nutrition Examination Study survey of 22,245 people in USA (Barr Dana et al., 2005); 90-92% of the general population displayed a urinary creatinine concentration greater than 0.3 mg/ml overall and are considered adequate specimens based on WHO guidelines for occupational monitoring (Mikheev and Lowry, 1996). Hence, creatinine can be used for dilution correction so long as the urine specimens are within 0.3 and 3.0 mg/ml creatinine concentration.

Serum and plasma creatinine levels are associated with muscle mass (Patel et al., 2013), though they are not used as urinary dilution biomarkers. Since muscle mass declines in MND, it has been assumed that urinary creatinine would also decrease over disease progression. However, several studies have shown this is not the case. For example, Shepherd et al. (2017) reported that urinary creatinine levels remained stable over time in people with MND, despite progressive muscle loss over a longitudinal study, and similar findings were reported by Lunetta et al. (2020) and Yamada et al. (2021) in studies of urinary p75^{ECD}, neopterin, and N-terminal titin fragments, respectively. These findings indicate that urinary creatinine excretion is relatively independent of muscle atrophy in MND, supporting its reliability as a normalisation factor for urinary biomarker analysis. Nevertheless, plasma and serum creatinine does decrease with MND progression, and has been suggested as a prognostic biomarker for MND (Rafiq et al., 2016, van Eijk et al., 2018).

1.6.2 Urine specific gravity

Alternative clinical tests have been made available to account for urinary dilution or concentration when assessing urinary analytes. Urine specific gravity (USG) is bulk

specimen property proposed to have greater resistance to those factors influencing fluctuations in creatinine measurements (Queremel Milani and Jialal, 2022). Indicative of the kidney's capacity to concentrate or dilute urine, USG is defined as the ratio between urinary density and the density of an equal volume of pure distilled water (density = 1.000) at a constant temperature (Chadha et al., 2001). Primarily dependent on urinary mass ratio (Queremel Milani and Jialal, 2022), USG has been deemed a less reliable measure of quantifying the exact number of solute particles in urine compared to urinary osmolality (Imran et al., 2010) as a measure of the osmoles of solute per kilogram of urine (Shah and Mandiga, 2021). Hence, the practicality of USG is highlighted by its ability to rapidly estimate hydration status. For routine clinical application, several methods are available to measure USG, including hydrometer readings, harmonic oscillation, and dipstick reagent pads. However, USG is fundamentally correlated with the refractive index of urine (Weeth et al., 1969), and refractometry provides the most accurate means of USG quantification. This method allows precise identification of hydration abnormalities, where elevated USG indicates hypersthenuria (concentrated urine), and lower USG indicates hyposthenuria (dilute urine; Queremel Milani and Jialal, 2022).

Unlike urinary osmolality, USG has been found to be influenced by the abundance, weight, and size of the molecules found in urine and therefore regarded as an estimate of urinary solute concentration (Flasar, 2008), where this correlation is a reflection of the reduced absorption of small molecules and low molecular weight proteins filtered in infancy and early childhood (Leech and Penney, 1987). USG has been compared to the assessment of urine colour, bioelectrical impedance analysis, and the thirst perception scale (Perrier et al., 2017). Comparisons between USG and urinary osmolality considered USG subjective with too low sensitivity to determine hypohydration in an Olympic sport cohort (Fernández-Elías et al., 2014). The effect of temperature on USG has been well documented, proposing an inversely proportional relationship with urine density and temperature (e.g., high temperature, lower USG) affecting USG measurements in animals (Rishniw and Bicalho, 2015) and humans (Inman et al., 2013). Further, variations in subject diet and physical activity/muscle mass have shown to influence USG measurements (Wilson, 2021). Previous normalisation of pteridines by USG has marginally outperformed creatinine adjustments in 48 aggressive and benign breast cancer subjects (Burton et al., 2014), indicating that USG normalisation may offer improved performance for clinical biomarkers, particularly pteridines.

1.6.3 Osmolality

Urinary osmolality (Uosm) is a measure of the osmoles of solute per kilogram of urine and has been explored as a more accurate measure of the concentrating ability of the kidneys than creatinine and USG (Shah and Mandiga, 2021). Uosm is a valuable tool from a clinical perspective providing a quick and effective evaluation of patient hydration status, and their ability to produce and concentrate urine (Koumantakis and Wyndham, 1989).

Armstrong *et al.*, (1994) identified that urine colour, Uosm, and USG were not correlated with plasma osmolality, plasma sodium, or haematocrit. This suggests that Uosm may be a candidate normalisation factor for MND urine, unaffected by plasma concentrations. It has since been reported that Uosm increased alongside increased BMI categories using National Health and Nutrition Examination Survey data (Rosinger *et al.*, 2019), warranting further research into this variability in MND cohorts. Armstrong *et al.*, (2013) later re-evaluated these findings, validating the efficacy of the urine-to-plasma osmolality ratio as a marker of whole-body hydration status at a single time point. Around the same time, a similar trend was identified by exploring sensitive states of dehydration, associating euhydration with a plasma osmolality value of 280-290 mOsm/L (Popowski *et al.*, 2001, Oppliger *et al.*, 2005).

While urinary osmolality may be an attractive alternative to creatinine, its characteristics in the MND population, like USG, remain largely unknown. However, there have been many studies of Uosm in the general population. Yeh *et al.*, (2015) conducted a large-scale analysis of urine osmolality in the US population involving 10,769 individuals and compared these results to WHO guidelines and creatinine (Mikheev & Lowry, 1996). This study identified a healthy median value of 648 mOsm/kg for this demographic, between an interquartile range of 393 to 838 mOsm/kg (Yeh *et al.*, 2015). Based on creatinine and WHO guidelines, very dilute urine specimens (e.g., urinary creatinine <0.3 mg/mL) presented a median value of 176 (132-227) mOsm/kg. Normal urine (i.e., urinary creatinine between 0.3 mg/mL and 3 mg/mL) presented a median value of 666 (453-840) mOsm/kg, and very concentrated urine (e.g., urinary creatinine > 3 mg/mL) showed a median value of 990 (858-1076) mOsm/kg. This study explored the ranges of urinary osmolality from individuals at the upper and lower limits of WHO guidelines urinary hydration. However, it is not clear if there is an improvement in quantifying analytes in dilute urine using osmolality. As previously mentioned, measurement limits are the result of the detection method, and it is not clear if low osmolality (e.g., <176 mOsm/kg) is defined by measurement outside of instrument detection limits using a freeze-point osmometer.

1.6.4 Methods of urinary osmolality measurement

Clearly, osmometry is an important clinical tool for the investigation and normalisation of urinary biomarkers in MND subjects. The successful application of osmometry to MND urine requires an understanding of freezing-point thermodynamics, and the basis of solvent behaviour. Being the total solute concentration of an aqueous solution, osmolality is the total measure of solute particles in urine (Sweeney and Beuchat, 1993). Irrespective of molecular weight or ionic charge, osmometry assumes that the colligative, or concentrative, properties of urine can provide a measure of the solvent's chemical potential by changing in direct proportion of particles in solution (Flasar, 2008). Of these colligative properties, the freezing point and vapour pressure are lowered, while the boiling point and osmotic pressure are raised under examination by osmometer (Sweeney and Beuchat, 1993). Instruments are commercially available to examine urine by freezing point or vapour pressure osmometry, however, the freeze-point depression method can better determine the precise concentration of an aqueous solution with a coefficient of variation of 1%, compared to 2.5%, and can be used in the presence of solutes such as ethanol for various forms of urinary preparation (Lord, 1999).

Freeze-point depression is commonly used to determine U_{osm} , illustrated in **Figure 1.9**. This method shares the advantage of being time efficient and easy to conduct on small urine sample volumes (Lord, 1999), down to 20 μ L (Koumantakis and Wyndham, 1989). Freezing point osmometry is fundamentally appropriate for low viscosity aqueous solutions and is computed as $osmolality (mol/kg) = \Delta T / 1.86$ (magnitude of depression of freezing point below $0^{\circ}C$ / 1.86 (molar freezing point depression constant for pure water)). In solutions containing electrolytes, like urine, the change in this constant is irregular, however, high dilution of samples before measurement may produce unpredictable error (Sweeney and Beuchat, 1993). The alternative to freeze-point depression is osmolality measurement by vapour-pressure depression (Lord, 1999). Surpassing the surface of an aqueous solution, molecules of solvent are present in the gaseous phase. Through sealing the solution in a closed chamber, instruments have advanced to bring the liquid and vapour phases of the solvent into equilibrium with one another, raising the solvent vapour pressure to a stable value (Sweeney and Beuchat, 1993). The fundamental principles behind vapour-pressure depression osmolality presents some advantages over freeze-point depression methods for the analysis of tissue samples (e.g., quick-frozen brain tissue specimens), measuring osmolality with both high speed and reproducibility without the need for dilution, weigh-ins, and calculations of tissue-water content (Tornheim, 1980).

Figure 1.9 has been removed due to Copyright restrictions

Figure 1.9: Freeze-point thermodynamic properties of urine

The standard freezing curve of urine illustrates the temperature of a sample as it moves through the freezing cycle. **A.** The action of the osmometer begins to cool the urine. **B.** Urine sample is super-cooled below its freezing point, causing crystallisation of the urine. **C.** Freezing is initiated. **D.** The increase in energy (heat of fusion) is released, causing the urine temperature to rise. **E.** Urine sample reaches equilibrium temperature and plateaus for instrument measurement. *Adapted from* (Ming et al., 2020).

1.7 Concluding remarks

Motor Neuron Disease is highly heterogenous in terms of rate of progression, site of disease onset, and involvement of gene mutations. The ALSFRS-R is the only subjective questionnaire-based tool approved by the TGA for the assessment of therapies but has proven to be underpowered in handling heterogeneity in phase 2 clinical trials (Goyal et al., 2020). With no known cure, a significant lack of breakthrough therapies, and difficulties demonstrating the efficacy of new drug candidates in clinical trials using the ALSFRS-R, the ability to identify individuals that share a similar MND phenotype and stratify those individuals using biomarkers on anticipated disease prognosis, with the use of pharmacodynamic biomarkers to help demonstrate efficacy, is of utmost importance (Benatar et al., 2016a).

How can heterogeneity of patients enrolling for MND clinical trials be reduced? One way is to identify, validate, and implement prognostic, pharmacodynamic, and predictive biomarkers. Our laboratory has led the world in MND biomarker discovery in human urine (Shepherd et al., 2017), which is an easily accessible biofluid and rich source of analytes (Mischak et al., 2010). Predictive biomarkers of MND are not well described, including those measuring immunological dysfunction. Immune dysfunction is a characterizing feature of MND pathology, arising in response to oxidative stress, protein misfolding/aggregation, glutamate excitotoxicity, and other pathological processes (Malaspina et al., 2015). Since immune dysfunction plays a significant role in MND pathogenesis, it is important to establish new urinary markers that can predict and determine MND outcomes, subgroup patients based on disease trajectory, and group patients with similar disease characteristics and clinical phenotypes. It is therefore rational to discover, quantify, and validate biomarkers that can characterize neuroinflammation in people with MND. I hypothesize that neopterin will predict pro-inflammation in people with MND at baseline (Westarpl et al., 1993, Molero-Luis et al., 2020, Lunetta et al., 2020), and that other biomarkers will continue to be discovered using mass spectrometry that can stratify people with MND based on their immune status in clinical trials. During neuroinflammation, human monocyte-derived macrophage dendritic cells and glia can excrete neopterin in high concentrations in response to interferon-gamma in bodily fluids like urine, demonstrating the extent of immune system activation in response to the pathological events involved in MND (Huber et al., 1984). Outside of this mechanism, the breadth of proteins involved in detectable pathways of neuroinflammation are yet to be explored in MND urine. For treatments targeting neuroinflammation in phase 2 clinical trials, validating urinary neopterin has the potential to enrich homogenous populations for future

clinical trials. In addition, urine will be used to identify further potential biomarkers of neuroinflammation for use in MND trials to generate a panel of biomarkers in the future that can improve clinical trial outcomes, and progress candidate treatments into phase 3 clinical trials.

Urinary neopterin will be quantified by mass spectrometry to verify its involvement in the inflammatory response in people with MND, and urine samples will be explored to discover other protein candidates involved with inflammatory pathways (Skates et al., 2013). To do this, urinary neopterin will be targeted in the urine of people with MND and compared to healthy individuals and other conditions, and the urinary proteome will be explored in an untargeted analysis to screen urine specimens for changing proteins, and relate those proteins back to their respective pathways using gene ontology techniques (Lee et al., 2019). Outside of biomarker detection, urine osmolality as a renal dilution correction factor will be explored next to urinary creatinine as the gold standard to expand our inclusion criteria for biomarker quantification in people with MND. The aim of this investigation is to perform targeted and untargeted biomarker detection, quantification, and validation in people with MND to better determine survival outcomes from baseline, characterize changes in the immune response, and predict the people most likely to respond to candidate anti-inflammatory therapeutics to improve the outcome of clinical trials in the future.

HYPOTHESIS AND AIMS

It is hypothesised that urinary biomarkers related to immune dysfunction, and that are prognostic for MND, can be discovered, quantified, and verified in people with MND using mass spectrometry.

To address this hypothesis, the following aims are proposed:

1. To detect and quantify neopterin in the urine of people with MND using high performance liquid chromatography and mass spectrometry
2. To determine if urinary neopterin is a prognostic or predictive biomarker candidate of pro-inflammation in MND
3. To discover differentially expressed urinary proteins in people with MND
4. To relate candidate urinary proteins to pathways of immune dysfunction using gene ontology techniques
5. To verify a panel of candidate proteins in MND urine using parallel reaction monitoring and compare them to established indicators of prognosis

CHAPTER 2.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Human urine sampling and neurological data

People with MND and healthy individuals, all without untreated diabetes, other neurological disorders or untreated illness that affects kidney function, were recruited between September 2011 and March 2024. Written informed consent was obtained from all study participants following study approval from the Southern Adelaide Clinical Human Research Ethics Committee (#229.11), Macquarie Human Research Ethics Committee, or the University of Miami Institutional Review Board. MND patients were diagnosed by clinicians as having definitive MND per the revised El Escorial criteria (Brooks et al., 2000, Statland et al., 2015).

and their neurological status at first visit (baseline) was recorded. Neurological information included MND diagnosis date, date of symptom onset, type of symptom onset (limb or bulbar), ALSFRS-R, age at baseline, age at diagnosis, age at sample collection, sex (male/female), riluzole or untreated, and whether they had familial MND or not. Once consented, mid-flow spot urine samples were collected from persons with MND or healthy individuals. Samples were stored for no longer than 3 hours before centrifugation in a refrigerated centrifuge (Sigma-Aldrich; 6K15) at 2000g, 4°C for 5 min and followed by analysis using MultiStix 10 SG Reagent Strips (Siemens, Camberley, UK; # 2300), being aliquoted, and stored at -80°C. If MultiStix showed high glucose, high pH, or blood contamination, the sample was not used, and findings were reported to clinicians. Urine specimens were not treated with protease inhibitors. Collected urine was stored according to Urine & Kidney Proteome Project Standards (Yamamoto et al., 2008). Samples collected from Miami and Sydney were transported on dry ice (<7 days), coded to maintain anonymity, and stored at -80°C until analysis.

2.1.2 Standard solutions and buffers

Table 2.1: Neopterin and creatinine standards

Compound	Supplier
<i>D</i> -Neopterin	Novachem (#TRC-N390005)
<i>D</i> -Neopterin- ¹³ C ₅	Novachem (#TRC-N390007)
Creatinine	Novachem (#031-04592)

Table 2.2: List of buffers for experimental use

Buffer	Solutes	Experimental use	Supplier(s)
20x Phosphate Buffered Saline (PBS; pH = 7.4 at 1x)	0.036M KH_2PO_4 2.74M NaCl 0.054M KCl 0.2M Na_2HPO_4	Buffers	Chem Supply
Wash Buffer	PBS/ 0.1% tween20	p75 ^{ECD} ELISA	From 20xPBS and Tween-20 (Sigma-Aldrich, #P1379)
Mobile phase (A)	10% ACN and 0.1% FA in dH ₂ O	Thermo Altis QQQ	Thermo Fisher Scientific
Stationary phase (B)	0.1% FA in 100% MeOH	Thermo Altis QQQ	Thermo Fisher Scientific
Mobile phase (A)	0.1% FA in 80% ACN	Thermo Exploris 480 and Fusion Lumos	Thermo Fisher Scientific
Stationary phase (B)	0.1% FA in dH ₂ O	Thermo Exploris 480 and Fusion Lumos	Thermo Fisher Scientific
Sample and Standard Buffer	7% m/v MeOH in dH ₂ O	Buffers	Thermo Fisher Scientific
Running Buffer (10x)	125mM Tris, 1M glycine, 0.5% SDS In 500 mL dH ₂ O, 7.57g Tris/HCl, 37.5g Glycine, 25 mL 10% SDS	SDS-Page	Sigma-Aldrich
Peptide Resuspension Buffer	1% TFA/0.1% dodecyl maltoside in dH ₂ O	Buffers	Sigma-Aldrich
Mass Spectrometer Water	0.1% FA in dH ₂ O	Buffers	Thermo Fisher Scientific
Sodium Adduct Pre-treatment	0.1 mM Ammonium Oxalate (13 mg/L in 1:100 urine)	Buffers	Sigma-Aldrich, #221716
EZQ Destain Solution	7% acetic acid and 10% methanol in dH ₂ O.	Buffers	Sigma-Aldrich, Thermo Fisher Scientific

Table 2.3: Materials used for p75^{ECD} and neopterin ELISAs

Material	Details and catalogue numbers
Neopterin ELISA plates	IBL International, #RE59321
p75 ^{ECD} ELISA plates	96 well high binding hard plates (Corning, Grenier bio-one, #G655061)
Coating buffer	25mM Na ₂ CO ₃ , 25mM NaHCO ₃ . pH to 9.6 with HCl
Blocking buffer	33% Block Ace (BioRad, #BUF029)
Sample buffer	1% Block Ace (BioRad, #BUF029)
MLR1	Monoclonal anti-p75 ^{ECD} purified from MLR1 hybridoma supernatant (Rogers et al., 2006)
NGFR5	NGFR5 hybridoma cells grown from Dr. Mark Bothwell (Washington State University). Anti-p75 ^{ECD} purified from hybridoma supernatant (Thompson et al., 1989, Shephard et al., 2022)
EZ-Link™ Sulfo-NHS-LC-Biotin	Thermo Fisher Scientific Australia, #A39257
Biotinylated NGFR5	Per Thermo Fisher Scientific instructions, #A39257
Human p75 ^{ECD} standard	R&D systems, #AF367
Streptavidin horseradish peroxidase (HRP)	Code: 016-030-084, Size: 1.0mg, Jackson ImmunoResearch Laboratories. #800-367-5296
TMB Peroxidase EIA Substrate Kit Solution A/B	Bio-Rad Laboratories, USA, 900 mL/Bio-Rad Laboratories, USA, 100 mL #9701859
Stop solution	Sulphuric acid (2M; Univar #534)

2.1.3 General lab chemicals

General lab chemicals (NaCl, KCl, etc) were supplied from Chem supply or Sigma Aldrich, Australia, unless otherwise stated.

Table 2.4: Chemicals and reagents used to prepare the urinary proteome for mass spectrometry analysis

Chemicals or Reagents	Supplier and Catalogue Number
Acetonitrile, ACS, 99.5+% (1L)	ThermoFisher Scientific, Waltham, MA, USA, #036423.AP
Protein Assay Reagent A, 250 mL	BioRad Laboratories, CA, USA, #5000113
Protein Assay Reagent B, 1L	BioRad Laboratories, CA, USA, #5000114

Dithiothreitol, 5g	Fisher Bioreagents (ThermoFisher), Pittsburgh, Pennsylvania, USA, #R0861
Hydrochloric acid, 32%	Merck, Kilsyth, VIC, Australia, #7647-01-0
Isopropanol, 99+% for mass spectrometry (2.5L)	ThermoFisher Scientific, Waltham, MA, USA, #167880025
Methanol, LCMS grade, 99.8+% (2.5L)	ThermoFisher Scientific, Waltham, MA, USA, #047192-K2
MultiStix 10 SG Reagent Strips	(Siemens, Camberley, UK; #2300),
Ovalbumin from chicken egg	Invitrogen by ThermoFisher Scientific, Waltham, MA, USA, #R33200
Bovine Serum Albumin, heat shock fraction, pH 7, >98%, 100g	Sigma-Aldrich, St Louise, MO, US, #A7906-100G
Pierce™ Trypsin Protease enzyme, MS-grade	Pierce Biotechnology, MA, USA, #90058
Protein ladder (Dual colour protein Western Standards)	BioRad Laboratories, California, USA, #161-0377

Table 2.5: Equipment used to prepare the urinary proteome for mass spectrometry analysis

Equipment	Supplier
4-20% Mini-PROTEAN® TGX™ Precast Protein Gels, 10-well, 50 µL.	BioRad Laboratories, CA, USA, #4561094
Axygen 1.5 mL Maximum Recovery microcentrifuge tubes	Corning Axygen Scientific Inc, Union City, CA, USA
Beckman XPN-80 ultracentrifuge	Beckman Coulter Life Sciences
Bio-Rad Gel-Doc EZ Imager	Bio-Rad, Hercules, CA, USA
Bio-Rad Mini-Sub GT Electrophoresis apparatus	Bio-Rad, Hercules, CA, USA
ChemiDoc™ Touch Imaging System	Bio-Rad, Hercules, CA, USA
Christ Freeze Dryer – Alpha 2-4 LDplus	John Morris Scientific Pty Ltd, Osterode am Harz, Germany
Dry block heater	Ratek Laboratory Equipment, Boronia, VIC, Australia
Easy Breeze™ dryer	Hoefer Inc, Massachusetts, USA

EZQ™ Protein Quantitation assay	Thermo Scientific Revco, Waltham, MA, USA, #R33200
Medium Orbital Shaker, EOM5	Ratek, Boronia, VIC, Australia
Personal Vortex Mixer – VM1 (IC-VM1)	Ratek Laboratory Equipment, Boronia, VIC, Australia
Pierce™ High pH Reversed-Phase Peptide Fractionation Kit	ThermoFisher Scientific, Waltham, MA, USA, #84868
Plastic 2 mL Microcentrifuge low-binding tubes	Corning Axygen Scientific Inc, Union City, CA, USA
Pulled-tip nano HPLC trap and column (25 cm and 40cm, 3.0-micron, C-18 beads)	Pulled in-house, Proteomics Facility, Flinders University, SA, Australia
Luna® Phenyl-Hexyl HPLC Column (3.0 µm, 3.0×150 mm)	Phenomenex, Australia, #00F-4256-Y0
ReproSil-Pur C18 beads (3.0-micron)	Dr. Maisch, Ammerbuch, Germany, #r13.b9.
Sutter P-2000 laser tip puller	Sutter Instrument Company, California, USA
Soft 96-well plates	Costar 96-well protein assay plates, #2595
ThermoFisher S10 Ultrasonic Bath	ThermoFisher Scientific, Waltham, MA, USA
Thermo Dionex Ultimate 3000 nano uHPLC	Dionex, Sunnyvale, California, USA
Thermo Altis mass spectrometer	ThermoFisher Scientific, Thebarton, SA, Australia
Thermo Exploris 480 mass spectrometer	ThermoFisher Scientific, Thebarton, SA, Australia
Thermo Fusion Lumos mass spectrometer	ThermoFisher Scientific, Thebarton, SA, Australia
0.5 mL Amicon® Ultra 3k Da Spin Column	Sigma-Aldrich, #UFC500
Eppendorf 5424R Refrigerated Centrifuge	Eppendorf, Australia
Empore Octadecyl C18 47mm diameter extraction disk	Sigma-Aldrich, #66883-U

Table 2.6: List of software used throughout this investigation

Software	Supplier
Cytoscape (version 3.10.1)	http://cytoscape.org
GraphPad Prism 10	GraphPad Inc., La Jolla, CA, USA
ImageLab software	Bio-Rad, Hercules, CA, USA
InnateDB software version 5.4	http://www.innatedb.com
Skyline (version 24.1)	MacCoss Lab Software,
Spectronaut software v 19.0.2.210615.50606	Biognosys, Schlieren, Switzerland
Statistical Package for Social Sciences (SPSS)	IBM, Armonk, New York
The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 12.0	http://string-db.org
Protein Analysis Through Evolutionary Relationships (PANTHER) version 19.0	http://pantherdb.org , updated June 20, 2024

2.2 Methods

2.2.1 Ethics

This research was conducted with respect to the NHMRC National Statement on Ethical Conduct in Human Research (2007) and the Australian Code for the Responsible Conduct of Research. Written and informed consent was obtained from all study participants for the use of human urine in this project following ethical approval from the Southern Adelaide Clinical Human Research Ethics Committee (SAC HREC), #229.11. This is a conjunction of committees from the Southern Adelaide Local Health Network (SALHN) and Flinders University. This research also had approval through the University of Miami Human Subject Research Office.

2.2.2 Neopterin ELISA

The IBL International/Tecan competitive enzyme-linked immunoassay (RE59321) was used to measure urinary neopterin as per the manufacturer's instructions (Shepherd et al., 2022). Briefly, urine samples were diluted 1:100 in buffer, and samples, standards and controls were added to plates, followed by enzyme conjugate and neopterin antiserum. Following incubation, plates were washed, and neopterin quantity was visualized using a tetramethylbenzidine reaction, with optical density measured at 450 nm (PerkinElmer Victor-x4 Plate Reader (Waltham, MA, USA). Neopterin concentration ($\mu\text{mol/l}$) was determined using a four-parameter nonlinear inhibitor versus response standard curve using GraphPad Prism 10. Intra- and inter-assay reproducibility was determined by three urine samples in duplicate, across 3 plates, to determine the coefficient of variation (CV%). Urinary dilution was accounted for using mol/L creatinine per sample to report μmol neopterin/ mol creatinine.

2.3 Neopterin detection and quantification by liquid chromatography mass spectrometry

2.3.1 Liquid chromatography

The Luna® Phenyl-Hexyl HPLC Column (3.0 μm , 3.0×150 mm, Phenomenex, Australia) and a Thermo Fisher Dionex u3000 HPLC system (California, USA) comprised of a degasser, binary pump, autosampler, autosampler thermostat controller, and a column oven, were used for the separation of neopterin from other pteridines within human urine. Separation was carried out at 40°C with a flow rate of 0.200 mL/min using a mobile phase that had the composition of A: 10% acetonitrile in water containing 0.1% (v/v) formic acid, and stationary phase B: 0.1% (v/v) formic acid in methanol. The gradient used for neopterin separation was

adapted from (Burton et al., 2016), initialised with 20% buffer B, followed by a linear decrease to 7% over 5-minutes. Following neopterin elution at ~4.2 minutes, the Phenyl-Hexyl column was re-equilibrated with 20% buffer B for the remainder of the run. Run-to-run time was 9 minutes with a sample injection volume of 15 μ L and a standard injection volume of 5 μ L.

2.3.2 Mass spectrometry

A Thermo® Fisher Altis Triple Quadrupole (QQQ) Mass Spectrometer with positive-ion electrospray ionisation (H-ESI) was used to detect and quantify neopterin in urine samples. The QQQ-Altis MS was operated under targeted/optimised MS/MS mode for the characterisation and quantitative analysis of endogenous urinary neopterin and *D*-neopterin- $^{13}\text{C}_5$ neopterin. Multiple Reaction Monitoring (MRM) mode was used for the targeted detection and quantification of both neopterin derivatives, and full scan mode in Quadrupole 1 (Q1) was used for broad spectrum analysis of ions within the mass range of 150-500 m/z over the 9-minute HPLC gradient. Optimized operational parameters included an ion transfer tube temperature of 325°C, vapor temperature of 275°C, auxiliary gas flow of 35 L N_2/min , sheath gas flow of 7 L N_2/min , and a H-ESI voltage of +3,500 V. Neopterin was optimised for its precursor ion masses of 254.3 m/z for *D*-Neopterin and 259.3 m/z for *D*-Neopterin- $^{13}\text{C}_5$ in MRM mode. Nitrogen gas was used in the Q2 collision cell to produce four fragment ions, three confirming ions of 196.13 m/z , 211.13 m/z , and 236.13 m/z , and one quantification ion, 206.11 m/z . Optimal peak resolution was achieved with a dwell time of 98.6 milliseconds in MRM mode, resulting in a total of 15 measurements taken across a 60-second wide peak, and 0.25 cycles per peak. The full method is described in **Appendix 8.1**.

2.3.3 Standard curve preparation

D-Neopterin (Novachem) was prepared in methanol to generate a stock solution of 10 $\mu\text{g/mL}$ upon complete dissolution by sonication and vortexing. Calibration standards were prepared from the stock solution by serial dilutions in ultra-pure water, generating a 12-point standard curve from 250 ng/mL to 0.24 ng/mL.

2.3.4 *D*-Neopterin- $^{13}\text{C}_5$ internal standard preparation

D-Neopterin- $^{13}\text{C}_5$ (Novachem) was prepared in methanol to generate a stock solution of 2 $\mu\text{g/mL}$ upon complete dissolution by sonication and vortexing. *D*-Neopterin- $^{13}\text{C}_5$ was added to human urine and the 12-point calibration curve to generate a final injection concentration of 14 ng/mL. This was used to generate a standard curve ratio for sample interpolation and neopterin quantification.

2.3.5 Urine sample preparation

Urine samples were stored in a biobank freezer at a temperature of -80°C . Urine samples were collected and fully thawed to room temperature, followed by centrifugation at 8,000g for 5 minutes at 4°C . The supernatant was transferred for three testing replicates, stored over ice, and protected from ultraviolet light. Two 10-fold dilutions were made in methanol containing *D*-Neopterin- $^{13}\text{C}_5$ to generate a final 100-fold dilution in the presence of *D*-Neopterin- $^{13}\text{C}_5$.

2.3.6 Neopterin peak integration and quantification

Thermo Scientific TraceFinder version 5.1 SP1 and FreeStyle version 1.8 SP1 were used to automate peak detection and integration for the quantification of neopterin derivatives. FreeStyle was used by default to generate a workspace for raw data files (.RAW) containing the data from a single acquisition run. MRM transitions and calibration curve parameters were defined in TraceFinder for targeted peak integration from FreeStyle raw files, used to perform peak integration from the area under the curve (AUC), quantification, and report generation for the concentration of neopterin in human urine. TraceFinder reports were exported into Microsoft Excel. The total area of *D*-neopterin was divided by *D*-neopterin- $^{13}\text{C}_5$ for each acquisition, and the ratio between neopterin/ $^{13}\text{C}_5$ neopterin was graphed and fitted with a linear trendline labelled with an equation and r^2 value ($r^2 > 0.99$). The ratio of neopterin/ $^{13}\text{C}_5$ neopterin was divided by the slope of the curve to interpolate the concentration of neopterin in ng/mL. The average interpolation of neopterin in duplicates was normalised to $\mu\text{mol/L}$ of creatinine to generate a concentration of urinary neopterin in μmol neopterin/mol creatinine.

2.4 Osmolality quantification

Urine osmolality quantification was performed using the Osmo1 Single-Sample Micro-Osmometer (Advanced Instruments). The instrument was operated as per the manufacturer's instructions. Before the assessment of human urine, 300 and 800 mOsm/kg H_2O Renol ® Urine Osmolality Controls (#3LA085) were used for instrument calibration. Supercooling and freeze-induction of 1:3 diluted urine samples was performed using the provided 20 μL syringe. Urine testing was performed in triplicates, providing an osmolality output in mOsm/kg H_2O .

2.5 Protocol for p75^{ECD} detection:

Urinary p75^{ECD} was quantified using a sandwich ELISA as indicated in (Shepherd et al., 2022), and materials as per **Table 2.3**. Briefly, 96-well high binding hard assay plates were coated with 100 μ L/well of 8.8 μ L/mL capture antibody (monoclonal, mouse anti-human p75^{ECD} MLR1) in coating buffer, and Biotinylated anti-p75^{ECD} NGFR5 detection antibody (hybridoma, monoclonal) was prepared to 2 μ g/mL and used for p75^{ECD} detection. BlockAce was used as blocking and sample buffer, and human recombinant p75^{ECD} was used to generate the standard curve (1500 pg/mL – 50 pg/mL). The enzyme reaction was achieved using streptavidin-HRP diluted to 1.0 μ g/mL, and colouric measurement was performed after adding TMB colour substrate and stop solution at 450 nm on a Molecular Devices Spectramax Plate Reader (BioStrategy, Australia). Values were reported as ng p75^{ECD}/mL urine, and urinary creatinine was used to account for urinary dilution, with the final value presented as ng p75^{ECD}/mg creatinine.

2.5.1 Protocol for urinary creatinine detection

The Creatinine Detection Kit based upon the Jaffe reaction was used to detect creatinine in human urine as per manufacturer's instructions (ENZO #ADI-907-030A). The Perkin Elmer Victor X4 performed creatinine quantification at an absorbance of 490 nm, determining the concentration of urinary creatinine in mg/dL using a standard curve. Creatinine concentrations were converted from mg/dL to mg/mL for p75^{ECD} normalisation, and mol/L for neopterin normalisation.

2.6 Urinary protein detection and quantification

The concentration of protein quantified in undiluted urine was determined by SA Pathology (SALHN; courtesy of Brad Rumbelow) using a turbidimetric benzathonium chloride method on a Cobas 8000 instrument (Roche Diagnostics, Rotkreuz, Switzerland).

2.6.1 Diafiltration

Diafiltration was refined from (Shepherd et al., 2014), using a 3 kDa Millipore Ultra 0.5mL spin column (Sigma-Aldrich #UFC500) to separate macromolecules greater than 3 kDa in size (i.e., proteins) from salts and electrolytes (i.e., NaCl, K⁺, Cl⁻, etc.) metabolites (i.e., urea, creatinine, ketones, glucose, etc.), and other low molecular weight compounds less than 3 kDa dissolved in the urinary matrix. Urine was thawed on ice and spun in an Eppendorf centrifuge at 8,000g for 5 minutes at 4°C to remove cellular debris and the supernatant was transferred to 0.5 mL 3 kDa spin columns. Urine was centrifuged at 10,000g at 4°C to

produce a concentration factor of 20-50 times its original concentration (i.e., 0.05 mg/mL to 1 mg/mL). 1x filtered sterile PBS (fPBS) at pH 7.3 was used in ten buffer exchange steps. On the final exchange, centrifugation was performed until the remaining concentrated and filtered volume was between 40-60 μ L. The final volume was recorded to the 0.5 μ L level, and the concentration factor was calculated. Recovered aliquots were labelled and stored at -80°C for downstream protein estimation, normalisation, and digestion. This procedure is illustrated in **Figure 2.1**.

2.6.2 Chloroform and methanol precipitation

Precipitation of proteins using chloroform and methanol was used as an alternative to concentrate and purify proteins from urine specimens to remove interfering salts, detergents, or lipids. Adapted from (Saito et al., 2019a), neat urine was thawed on ice and spun at 21,000g for 10 minutes at 25°C to remove cellular debris. In 1:20 urine, 1M Tris-HCl pH 8.0 buffer was added to each sample, followed by 1 part urine to 1 part 100% methanol (MeOH), and finally, chloroform in a 1:4 urine to chloroform ratio to make up a total volume of 1.5 mL. This was centrifuged at 12,000g for 15 minutes at 25°C. The upper phase was removed using low vacuum suction and 100% MeOH was re-added to each sample and mixed by gently inverting the tubes and placement on a thermomixer at 1000 rpm for 5 minutes. Specimens were centrifuged again at 12,000g for 15 minutes at 25°C, and the supernatant was discarded using low vacuum suction. The pellet was air dried on low for 2 minutes with lids open. The resulting pellets were resuspended in 50 μ L of 8M urea/50mM Tris-HCl at pH 8.0. For a final time, the samples were vortexed for 5 minutes, centrifuged at 12,000g for 5 minutes, and placed on a thermomixer overnight at 1000 rpm protected from light. Samples were stored at -80 °C for downstream protein estimation, normalisation, and digestion.

2.6.3 Protein quantification

Total protein concentrations in patient urine samples were determined using either a colourimetric Bio-Rad assay, or a fluorescence based EZQ™ Protein Quantitation assay (Thermo Scientific Revco, Waltham, MA, USA, as per remanufacturers instruction). Urine was spun at 10,000g at 4°C to produce a concentration factor of 20-50 times its original concentration, the flow-through was discarded. Once concentrated, 1x fPBS at pH 7.3 was used in ten filtration steps, exchanging any remaining salts, metabolites, and solvents to isolate the urinary proteome. On the final exchange, the remaining volume was concentrated by centrifugation, extracted in aliquots free of urinary constituents <3 kDa in size, and stored at -80°C for downstream analysis.

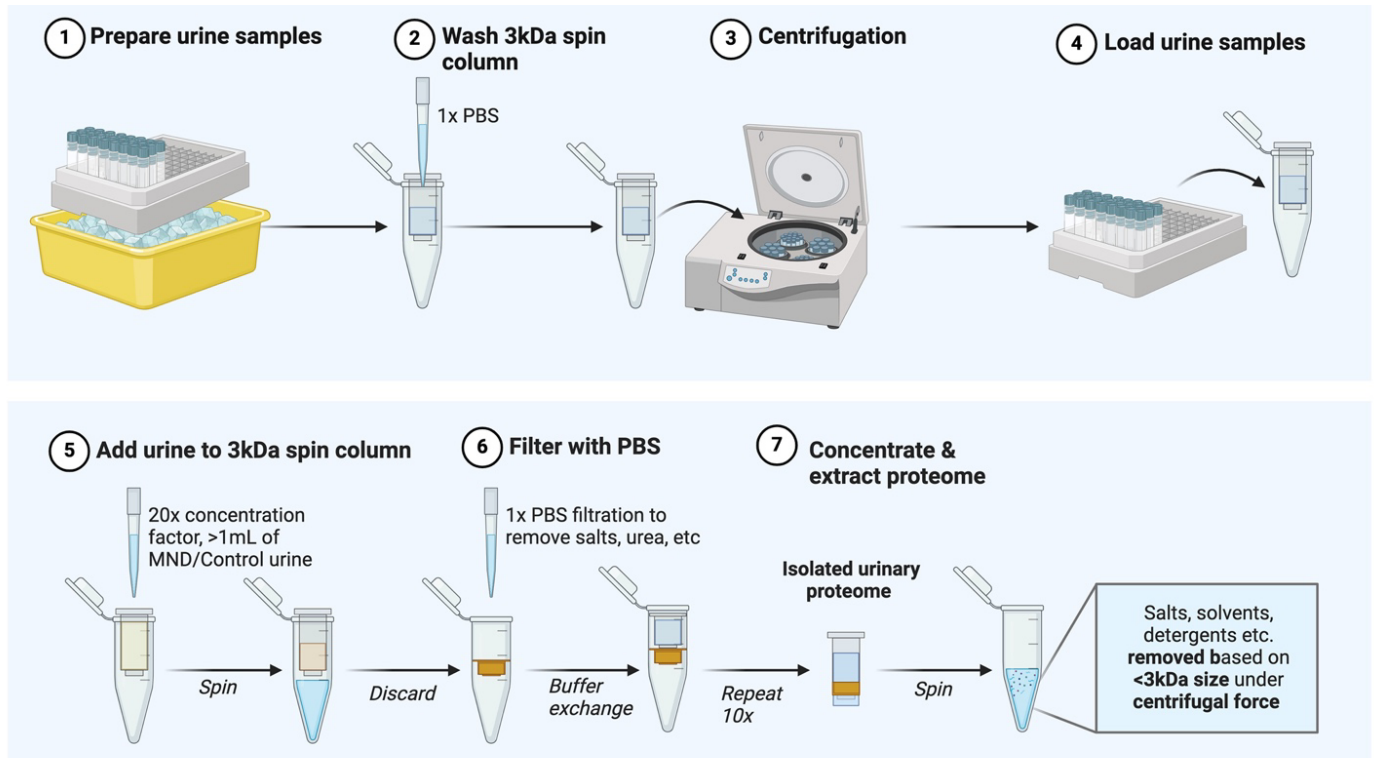


Figure 2.1: Schematic illustration of diafiltration workflow

Urinary proteins were thawed on ice (1) and prepared for in-solution filtration and concentration using 0.5 mL 3 kDa centrifugal spin columns conditioned with 1x fPBS (2, 3). Neat urine was transferred into 3 kDa spin columns in 0.5 mL aliquots (4), and centrifuged at 10,000g at 4°C to produce a concentration factor of 20-50 times its original concentration (5), the flow-through was discarded. Once concentrated, 1x fPBS at pH 7.3 was used in ten filtration steps, exchanging any remaining salts, metabolites, and solvents to isolate the urinary proteome (6). On the final exchange, the remaining volume was concentrated by centrifugation, extracted in aliquots free of urinary constituents <3 kDa in size (7), and stored at -80°C for downstream analysis. *Created with Biorender.*

2.6.4 BioRad protein quantification assay

The BioRad protein assay was performed as per manufacturer's instructions (BioRad #500-0116). For protein samples prepared by the diafiltration method, samples and standards were made in 1x PBS. For samples prepared by chloroform and methanol precipitation method, samples were diluted 1:2 to achieve 4M urea/25mM Tris-HCl at pH 8.0 and withstand the limit of 4M urea detection. Concentrated urine samples were diluted twice (i.e., 1:2 and 1:3) to fit within a standard curve of 1.4 mg/mL to 0.2 mg/mL of Bovine Albumin Serum (BSA, Sigma) protein standard. 5 μ L of standards and samples were pipetted into a Costar 96-well protein assay plate in duplicates. 25 μ L of reagent A was added to each well, and 200 μ L of reagent B was added. The plate was read at 750nm in a PerkinElmer Victor-x4 Plate Reader (Waltham, MA, USA) and protein concentration in urine samples were interpolated.

2.6.5 EZQ protein quantification assay

The Thermo Fisher EZQ Protein Quantification Assay was performed as per manufacturer's instructions (Thermo Scientific Revco, Waltham, MA, USA). Six serial dilutions of 0.02 mg/mL to 2.0 mg/mL from the 2 mg/mL Ovalbumin protein stock (BioRad) were prepared to generate a standard curve for protein quantification in urine. 1 μ L of standards and protein samples were spotted onto EZQ assay film paper in triplicates. The film paper was transferred to a container, and the spots were left to air dry. To fix the proteins to the film paper and remove any contaminants, 50 mL of methanol was added and incubated on a rotator tray at 50 rpm for 5 minutes. Following protein fixation, the assay paper was dried low heat using an Easy Breeze Gel Dryer (Hoefer Inc, Massachusetts, USA), and 40 mL of EZQ Protein Quantification Reagent was added. The assay container was incubated on the rotator for 30 minutes at room temperature to stain the proteins with proprietary fluorescent dye. The assay paper was rinsed three times in two-minute intervals using 40 mL EZQ Destain solution. The ChemiDoc Touch Imaging System (BioRad) was used for fluorescent detection of the protein spots, and the data was analysed using ImageLab Software (BioRad). The standard curve was generated, and protein concentrations were extrapolated in mg/mL.

2.6.6 SDS-PAGE polyacrylamide gel electrophoresis

SDS-PAGE 1D gel electrophoresis was used for protein normalisation. 10-lane, 50 μ L well, 4-20% Stain-Free Mini-PROTEAN precast gels (BioRad) were used to load 10 μ g of protein in varying volumes. dH₂O and 10 μ g of urinary protein was used to dilute SDS sample buffer

made in Dithiothreitol (DTT) by 1:4 in a total volume of 30 μL . Samples were heated at 95°C for 5 minutes on a heating block and loaded into their positions on the gel. One 10 μg positive-control urine was used for normalisation, and 5 μL Precision Plus Protein Dual Colour Standard (BioRad) was used as a molecular weight marker. Using the Mini-Protean II Cell Equipment (BioRad) at 170 V, proteins were separated for approximately 45 minutes until the loading dye reached the base of the gel. The gel was removed and imaged using the ChemiDoc Touch Imaging System (BioRad) using the stain free gel protocol, activating the cross-links for five minutes, and imaging the gel with optimal auto exposure (10.3 seconds). Using ImageLab, the saturation of protein bands revealed in each specimen were defined by their lanes and normalised to the 10 μg positive-control. This ensured even loading onto the mass spectrometer during analysis.

2.6.7 Sample preparation for mass spectrometry

Following protein estimation and normalisation, the appropriate volume for each sample to achieve 10 μg of protein was transferred to maximum recovery tubes (Axygen). DTT was added to a final concentration of 10 mM, and urine samples were incubated at 56°C for 30 minutes. Following this incubation period, iodoacetamide (IAA) was added in a final concentration of 20 mM, and samples were incubated at room temperature for 30 minutes while protected from light. Reduced and alkylated peptides underwent tryptic digestion in the presence of 100 mM Ammonium Bicarbonate. The Trypsin enzyme (Pierce Biotechnology, MA, USA) was added at a final concentration of 100 ng/ μL in a 200 μL final volume. Samples were incubated at 37°C overnight. The digest was halted by acidification (pH<4) with 10 μL 0.1% formic acid the next day. Digested peptides were reduced to dryness in a Christ vacuum concentrator (Alpha 2-4 LDplus, Osterode am Harz, Germany) and stored at -80°C. For peptide resuspension, 1% trifluoroacetic acid and 0.1% dodecyl maltoside buffer was used in a dilution ratio of 3 μL : 1 μg of urinary peptides. This complied with the sample loop, preventing column pressures from exceeding 600 bar.

2.7 Generating a spectral library

Spectral library generation was required to identify quantifiable proteins in MND urine. To generate the most comprehensive spectral library, an 'offline' high-pH reversed-phase fractionation technique was performed. This generated eight fractions analysed by Data Dependent Acquisition (DDA) mode. Reversed-phase fractionation was used to decrease the complexity of peptides in each fraction, increasing the identification of low abundance proteins below the limit of detection.

2.7.1 Constructing of C18 Stage-Tips

Stage tips with carbon-18 (C18) chemistry were made for high pH reversed-phase fractionation of peptides. C18 stage-tips were made from 1,000 μL and 200 μL maximum recovery pipette tips. Using punch utensils made by Biomedical Engineering (BME, Flinders University) six extra-large (i.e., for 1 mL tips) or large (i.e., for 200 μL tips) C18 discs were punched from an Empore Octadecyl C18 47mm diameter extraction disk (Sigma-Aldrich, #66883-U) and pressed into the bottom of the tip. Stage tips were stored at RT until use.

2.7.2 Conditioning of C18 Stage-Tips

The stage tip was placed into a new 1.5 mL maximum recovery tube and conditioned before use. 300 μL of acetonitrile was used to 'wet' the stage tip by centrifugation at 3000 rpm for 1 minute. The flowthrough was discarded. This step was repeated twice. The stage tip was equilibrated with 300 μL TFA solution by centrifugation at 3000 rpm for 1 minute. The flowthrough was discarded. Equilibration was repeated twice.

2.7.3 Offline High pH Reversed-Phase Fractionation

The high pH reversed phase fractionation technique was performed for its run-to-run reproducibility. This technique was performed under alkaline pH conditions to maximise the separation of negatively charged peptides. The Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Scientific) was adapted on urine specimens. 1 μg of urinary peptides from each specimen was pooled (total 65 μg) into a 1.5 mL maximum recovery tube and diluted to 650 μL in dH_2O . The fractionation kit was hybridized with C18 stage tips for the flow through of peptides across eight fractions using a gradient of high-to-low pH separation from 5% to 50% acetonitrile and 0.1% Triethylamine (**Table 2.7**), creating fractions of peptides ordered by hydrophobicity. 650 μL of 65 μg urinary peptides were loaded into the pre-conditioned C18 stage tip. Centrifugation was performed at 1000g for 2 minutes, and the eluant was retained as the 'flow through fraction.' The stage tip was placed into a new 1.5 mL maximum recovery tube and washed with 300 μL dH_2O . Centrifugation was performed at 1000g for 2 minutes. The 'wash through' fraction was discarded. The stage tip was placed into new tubes labelled fractions 1 to 8, and 300 μL of the first fraction solution (5% ACN in 0.1% TEA) was centrifuged at 1000g for 2 minutes. This step was repeated for 8 fraction solutions. Each 300 μL fraction was evaporated to dryness using a Christ Vacuum Concentrator (Christ, Osterode am Harz, Germany). Each fraction was re-suspended in 6 μL of 0.1% formic acid, sonicated for 1 minute, and transferred into mass spectrometry vials for analysis.

Table 2.7: High pH step-elution solution gradient for library generation by offline high pH reversed-phase fractionation technique

For 1 Sample					
Fraction #	Acetonitrile %	Acetonitrile Volume (μL)	Triethylamine (0.1%) Volume (μL)	Total (μL)	Volume per Sample (μL)
1	5	17	317	333	300
2	7.5	25	308	333	300
3	10	33	300	333	300
4	12.5	42	292	333	300
5	15	50	283	333	300
6	17.5	58	275	333	300
7	20	67	267	333	300
8	50	167	167	333	300

2.8 Mass spectrometry methods

2.8.1 Data Dependent Acquisition (DDA)

To achieve the objectives of protein discovery and quantification, understanding the distinctions between data-dependent acquisition (DDA) and data-independent acquisition (DIA) modes is important. Mass spectrometers operate in DDA mode primarily for protein identification. In DDA mode, precursor ions are selected based on their intensity or abundance for fragmentation and subsequent analysis (Bekker-Jensen et al., 2020). Initially, an MS1 scan is performed to capture all ions entering the instrument at that point in time. The instrument isolates the most abundance peptides and fills the collision cell with those peptides only using a quadrupole which filters out all masses, except for the target mass. This is followed by MS2 fragmentation of the most intense multiply charged ions (2^+ to 6^+ charge states) in the collision cell (Olsen and Mann, 2004). Advanced quadrupole technology adjusts the voltages to act as a mass filter upstream of the collision cell. For example, if the MS1 scan identified four multiply charged ions, the instrument selectively accumulates the most intense ions into the collision cell. Inside the collision cell, peptide ions are accelerated using RF and DC voltages, colliding with inert nitrogen gas. These collisions increase the vibration within the peptides, and the peptides break between the C=O and N-H atoms of peptide bonds, generating b^+ and y^+ fragment ions. The b^+ ions originate from the N-terminus, while the y^+ ions are derived from the C-terminus (Pappireddi et al., 2019). Fragment ions are then transferred to into the Orbitrap mass analyzer where their mass to charge (m/z) ratios are determined. The instrument sequentially isolates and fragments the next most abundant peptides until a predefined number (e.g., top 20 peptides) has been analyzed. Once completed, another MS1 scan is initiated to restart the process. While simplified here, this method is termed data dependent as MS2 scans depend solely on ions identified in MS1 (Kreimer et al., 2016). This method was effective for identifying high-abundance urinary proteins to generate a spectral library and is described in the final workflow of **Figure 2.2**.

2.8.2 Thermo Exploris® 480 mass spectrometry DDA library acquisition

All 8 fractions were analysed on a Thermo Exploris 480 Mass Spectrometer (Thermo Fisher Scientific) coupled with a Thermo Dionex nano u3000 HPLC (Dionex, Sunnyvale, California, USA). The instrument was operated in positive ion mode utilizing 60,000 MS1 resolution, 30,000 MS2 resolution, allocated charge states of 2^+ to 6^+ , with an automatic gain control (AGC) target set to 40,000 and an intensity threshold of 50,000 Hz. Twenty MS2 scans were performed following each MS1 scan, and the maximum injection time was set to automatic.

Internal mass calibration was enabled and performed using EASY-IC. The expected peak width was 30 seconds wide and dynamic exclusion was set to 15 seconds after a single hit was found, with high and low mass tolerances of 4 ppm. 1 µg of peptides from each sample were loaded onto an in-house pulled-tip trap column (75 µm x 2cm, 3.0 µm C18 beads with 100Å pore size) and eluted onto another in-house pulled-tip nano HPLC column created from 75 µm inner diameter fused silica capillary packed with 3.0 µm ReproSil-Pur C18 beads (Dr. Maisch, Ammerbuch, Germany) to an exact length of 25 cm or 40 cm in length. The column was heated to 40°C using a Sonation PRSO-V2 column heater (Sonation Lab Solutions, Biberach, Germany) and the spray tip was pulled using a Sutter P-2000 laser tip puller (Sutter Instrument Company, California, USA). The eluted flow rate for loading samples onto the trap was 3 µL/minute and the flow rate for the gradient pump was set to 300 nL/minute. Both the trap and column were equilibrated in stationary phase (A) containing 0.1% FA in dH₂O and eluted with mobile phase (B) containing 0.1% FA in 80% ACN using a linear gradient from 2% to 30% over 107 minutes. The total run time with column re-equilibration was 140 minutes. The full method can be found in **Appendix 8.2**.

2.8.3 Proteome Discoverer and DDA data analysis

Proteome Discoverer (version 2.4.1.15, Thermo Scientific) was used to analyse (.RAW) data from DDA mode. Proteome Discoverer compares the mass of the intact peptide and the masses of the fragment ions with a known database containing these masses for the organism (homosapiens) for protein identification. Proteome Discoverer then constructs a list of peptides and forms a list of proteins that are present in the sample. This generated a spectral library from DDA fractions, and optimized DIA mode m/z window allocations dependent on the number of high confidence peptide specific matches (PSMs). For peptide identification, the protein database 'Uniprot-Human-Reviewed-Sep2023.fasta' using the integrated SEQUEST search engine was used. Library fractions were reviewed of their high-confidence protein groups to ensure mass tolerances were within 0.02 Da (20 ppm). Courtesy of James Chataway, spectral data was exported from Proteome Discoverer into a script generated to optimize DIA mode m/z windows using DDA data from the number of PSMs. The average peptide mass from the run time in seconds between 1,000 and 9,000 (i.e., 15 – 150-minute HPLC gradient) was imported into an MGF java script. This compared retention time to m/z, distributing the most abundant PSMs across 45 variable m/z windows. Windows ranging from 350.5-409 m/z to 1,083.5-1,199 m/z were generated for DIA mode. DIA windows can be found in **Appendix 8.3**.

2.8.4 Data Independent Acquisition (DIA)

Data Independent Acquisition (DIA) was used for protein quantification. In DIA mode the instrument performs an MS1 scan, but rather than isolate each peptide for MS2 fragmentation, it injects pools of peptides into the collision cell. These are called m/z windows. The procedure involves a single MS1 scan to detect precursor ions within a predefined mass range (e.g., 353-411 m/z). MS1 mass ranges were set to 45 variable m/z windows derived from Proteome Discover and MGF analysis with a 1 m/z overlap (i.e., 353-411 m/z and 411-424 m/z). Subsequent MS2 scans utilized sequential windows, 20 m/z wide, to fragment peptides across a broader range (200 to 1,200 m/z). For each MS1 scan, 40 MS2 scans were conducted to fragment the identified precursor ions. This MS1/MS2 scanning cycle was repeated throughout the entire gradient length of the analysis. The name data independent acquisition comes from the MS2 scan, performed independently of the MS1 scan. This comprehensive fragmentation approach increased the number of peptides identified and improved the accuracy of their peak elution shapes (i.e., more points per peak) by providing multiple fragmentation events (~10) per peptide. This method enabled comprehensive coverage of mass ranges between 353 m/z and 1,196 m/z within the urinary proteome and was fundamental for the relative quantification of protein groups between people with MND and controls. The full method can be found in **Appendix 8.4**.

2.7.8 Spectronaut for DDA and DIA data analysis

Spectronaut (v19.0.2.210615.50606, Biognosys, Schlieren, Switzerland) was used to analyse all MS1 and MS2 spectral data. This found an application in analyzing DDA fraction data and DIA sample data for the generation of a comprehensive spectral library, and differential analysis of proteins in MND and control urine. Spectronaut used the Pulsar search engine and the 'Uniprot-Human-Reviewed-Sep2023' database build the spectral library. The b^+ and y^+ fragment ions from MS2 scans were correlated with their precursor peptides, determining the sequence and identity of unknown peptides discovered in human urine. Spectronaut integrated the chromatographic peaks, producing a relative quantity for each peptide and protein group. Label free quantification data was used in DIA mode for relative protein quantification. Urinary proteins were analysed in Spectronaut using the sample-specific spectral library with altered analysis settings. Spectronaut identified protein groups that were differentially changed by a Log2Fold-change > 0.6 and a q value < 0.05, which translated to a greater than or equal to, or less than or equal to 50%-fold change between protein groups found in each condition. This was exported into a custom report file for further analysis using MatLab and Microsoft Excel.

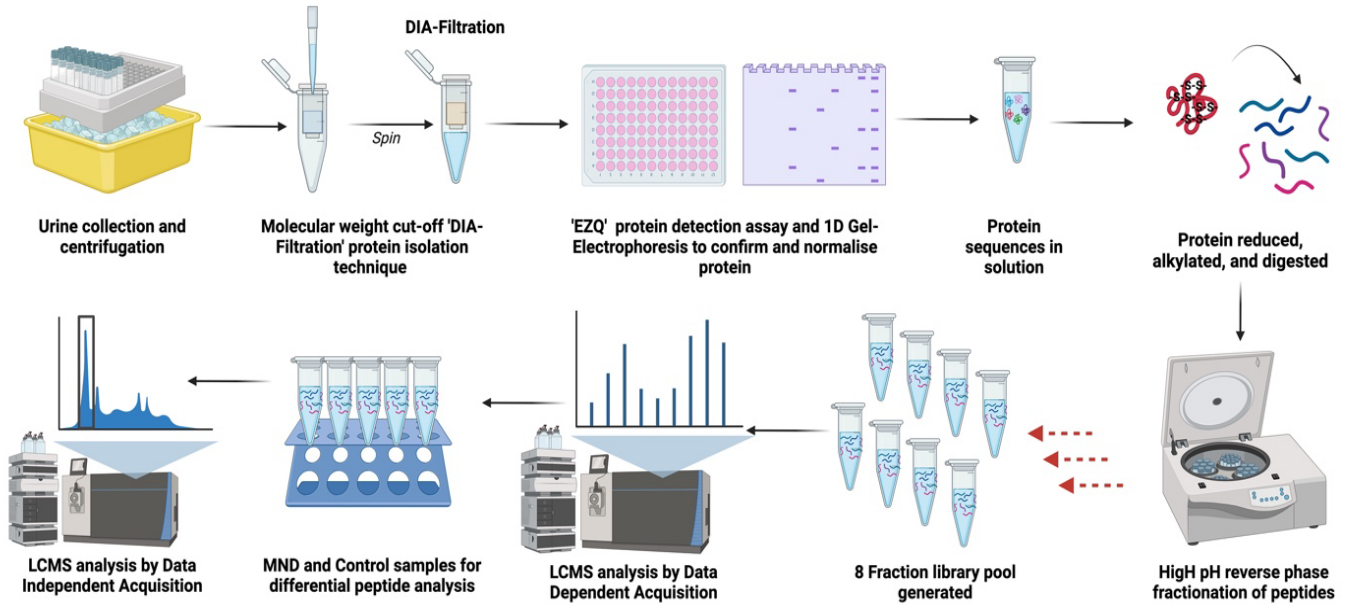


Figure 2.2: Schematic of in-solution protein clean-up, digestion, and LCMS analysis

In summary, urinary proteins were papered and underwent protein concentration and filtration using the diafiltration technique (1), followed by protein estimation by EZQ assay and normalisation by 1D gel-electrophoresis (2). Post-cleanup and normalisation, 10 μg of urinary peptides (3) were subject to reduction by DTT, alkylation by IAA, and digestion by Trypsin enzyme in-solution (4). The library of pooled peptides post-digest was subject to high pH reverse-phase fractionation (5), producing 8x fractions from 65 μg of urinary peptides (6) which were analysed by LCMS in DDA mode (7). 1 μg of remaining peptides from 70 people with MND and healthy individuals were individually analysed by LCMS using DIA mode (8), identifying differentially expressed proteins in people with MND. *Created with Biorender.*

2.9 Statistics and gene ontology pathway analyses

2.9.1 MatLab for Spectronaut analysis (prior to PANTHER GO analysis)

MatLab R2023a (Update 2, 9.14.0.2254940) was used in collaboration with Yuri Ogawa to generate a script for performing statistical analyses on the data output from Spectronaut. The custom report was re-organized to include patient data, and the quantity of each protein group was normalised to urinary creatinine and osmolality. The MatLab script identified unique protein accessions and row numbers for each protein, generating an output file containing the following data; unique protein accessions with matched descriptions, average normalised quantity for each protein in people with MND and healthy individuals, fold-differences between averaged normalised quantities, and Welch's T-test (unpaired two-tailed T-test, not assuming equal standard deviations) to provide *p* values for each protein accession, indicating the statistical difference between both conditions. This MatLab script provided statistical results for further pathway analysis and gene ontology.

2.9.2 Pathway analysis for gene ontology terms

Pathway analyses were used to understand the cause-and-effect relationships between differentially expressed proteins and the biological processes that regulate cellular and molecular pathways in MND (i.e., neuro inflammation, muscular degeneration, etc.). This was used to identify pathways in biological processes, molecular functions, and cellular components that were enriched in people with MND compared to healthy individuals.

2.9.3 Rationale for using the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system.

Protein interactions and their expression profiles with biological processes, molecular functions, and cellular components could be analysed using different platforms. Routinely used examples include Ingenuity Pathway Analysis (IPA), the Database for Annotation, Visualisation, and Integrated Discovery (DAVID), Innate Database (InnateDB), and Protein Analysis Through Evolutionary Relationships (PANTHER). For this study, PANTHER (version 19.0) was used (publicly available at <http://pantherdb.org>). Updated on June 20, 2024, PANTHER was used to classify proteins and their genes to identify differentially expressed proteins and their processes in MND. This analysis explored a total of 144 total genomes containing 2,017,190 genes, indexed with 2,200 terms for biological processes, 537 terms for cellular components, and 611 terms for molecular functions.

PANTHER inferred the biological roles of proteins based on evolutionary data. This predictive capability was advantageous for annotating novel or poorly characterized genes

compared to other classification systems. Further, PANTHER integrated well with other databases and resources, using cross-referencing to increase the robustness and confidence of the output. Compared to other classification systems, PANTHER compared specific GO pathways to a reference Homosapien database, describing the entirety of genes available inside that pathway and highlighting the number of genes discovered from the data (i.e., from 40 genes, 16 genes discovered). PANTHER's interface allowed for sorting on the expected false discovery rate (FDR), fold-enrichment, p value, and hierarchy of genes discovered within GO processes to interpret functional pathways. Based on this rationale, PANTHER was used for the biological pathway analysis and statistical over-representation of the data generated by this study.

2.9.4 Gene Ontology analysis using PANTHER

Significant Uniprot IDs from the list of differentially expressed proteins were converted to gene IDs using SYNGO (publicly available at <https://www.syngoportal.org/convert>). Gene IDs were uploaded into PANTHER. The list type was selected as 'ID List' and Homosapiens was selected as the organism database. The functional classification viewed in gene list analysis was submitted to explore gene IDs and their associated pathways. Statistical enrichment and over-representation tests (release 20240226) were selected to identify the functional classifications of significant genes in people with MND. The data was submitted and run through the PANTHER GO Ontology database DOI: 10.5281/zenodo.10536401 released 2024-01-17, comparing the analysed list (i.e., gene ID input) to the Homosapien reference organism. The annotation data was cycled between GO biological, molecular, and cellular processes, where 'Fisher's Extract' and 'Calculate FDR' were selected to adjust for multiple comparisons. The data was sorted by fold enrichment and exported into tables for further interpretation analysis.

2.9.5 STRING gene clustering

The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was used to visualize and annotate gene clusters. STRING (version 12.0, publicly available at <http://string-db.org>) constructed networks of interactions for the gene ID input generated by PANTHER. This network plotted protein-to-protein interactions from significant differentially expressed proteins discovered in people with MND compared to controls. Significant gene IDs were submitted into a STRING search, selecting a reference Homosapien database and a high confidence score of 0.70. From an initial list of genes and their descriptions, the list was mapped generating a network of 'nodes' representing genes, and 'edges' representing protein-to-protein interactions with high confidence. STRING performed network clustering

and functional enrichment analyses, identifying clusters of genes that share similar biological functions based on their interaction patterns. Within the network, 'MCL clustering' was selected to find natural clusters based on the stochastic flow of the data. The database was exported, and the network was imported to Cytoscape 3.10.1 for visualization.

2.9.6 Cytoscape 3.10.1 for STRING network visualization

Cytoscape (version 3.10.1, publicly available for download at <http://cytoscape.org>) was used to integrate models of interaction networks and pathway datasets from STRING. Nodes (genes) and edges (interactions) were plotted as a 'STRING network' and 'yFiles Layout Algorithms v1.1.4' was installed to visualize the data in clustered layouts. For cluster detection and functional enrichment, ClusterMaker2 (v2.3.4) and EnrichmentMap (v3.3.6) were installed to group genes based on network topology and visualize biological pathways that were over-represented in each cluster. The STRING network was distributed into a clustered network. This was organized into an Enrichment Map with a node cutoff (p value) set to 0.05, and an edge cutoff (similarity) set to 0.8. Both networks were explored using the 'Node, Edge, Network, and Enrichment' tables to navigate through key results. The data was exported to demonstrate key gene clusters, gene interactions, and functional relationships in MND.

2.9.7 Remaining data analysis

Microsoft Excel and GraphPad Prism 10 were used to conduct statistical analyses. Comparisons of neopterin in MND urine to the urine of healthy individuals, and p75^{ECD} in MND to the urine of healthy individuals was completed using unpaired T-tests in GraphPad Prism 10. The comparison of neopterin and p75^{ECD} in the urine of people with MND to the urine of healthy individuals was calculated using unpaired T-tests. This data was presented as mean $\pm$ SEM with a $p < 0.05$ level of significance. Comparisons were made using a Pearson's correlation coefficient to determine if urinary neopterin is significantly correlated with urinary p75^{ECD}, and the ALSFRS-R. Analysis was conducted on the time from baseline urine sample collection to time of patient death using Kaplan-Meier Survival Tests to determine prognostication. Standard curves from the urinary p75^{ECD} ELISAs and neopterin LCMS assays were compared using r^2 values of linear regression and signal to noise (S/N) ratios.

3.0 Parallel Reaction Monitoring (PRM) technique

To validate the presence of ten protein groups in the urine of people with MND and healthy individuals from the differential analysis study, a combination of Proteome Discoverer and

Skyline software were used for peptide identification and validation. Proteome Discoverer (version 2.4.1.15, Thermo Scientific) was used to match the spectra from human urine against a human protein database to identify potential peptide candidates. For each of the 10 protein groups, two unique peptides were selected based on their MS1 precursor ion signals, their number of peptide specific matches (PSMs), and intensity of up to four known fragment ions (y^+ ions) to ensure that these peptides were distinct to their respective proteins and not shared with other proteins in the database (i.e., different charge states, same precursor mass). The corresponding transition ions from the MS2 spectra for each peptide were validate using Skyline version 24.1.0.199. Skyline was used to verify the fragmentation pattern of the selected unique peptides, ranking their consistency and intensity for quantification. By targeting the MS2 transition ions and confirming the presence of both precursor and fragment ions in the mass spectrometry data, Skyline ensured that each protein group was accurately identified by its MS1 spectra compared to a comprehensive spectral library (DDA data), and validated by their fragmentation pattern, performing validation with high quantitative accuracy and precision by measuring peptides with more points per peak.

3.1 PRM data acquisition

Samples were separated with a Thermo Scientific Vanquish NEO uHPLC system using an in-house 25 cm C18 column (particle size 2 μ m, 100 Å, 75 μ m inner diameter, Dr. Maisch). For LC-MS/MS analysis, samples were loaded at 0.7 μ L/min with 100% mobile phase A (99.9% H₂O, 0.5% FA) with a pressure limit of 400 bar. The loading volume was set to automatic. Peptides were eluted with a stepped gradient of 5% to 32% mobile phase B (80% ACN, 0.1% FA) in 80-minutes. The flow rate was set to 300 nL/min.

Samples were measured as replicates on an Orbitrap Exploris 480 mass spectrometer with an API providing hybrid-PRM/DIA capabilities (Thermo Fisher Scientific). DIA MS1 full scans were acquired on the mass spectrometer with an Orbitrap resolution of 60,000 m/z . The normalised AGC target was set to 600% with a maximum injection time set to 200 ms within scheduled windows, and 75 ms for the DIA MS1 full scan. DIA MS2 scans were performed over a scan range of 400-1200 m/z with a resolution of 60,000 m/z . For an AGC target value set to 600% with a maximum injection time of 150 ms, the Q1 isolation window was set between 0.7 and 1.4 Da depending on the peptide precursor mass. The precursor mass-to-charge (m/z) value, charge state, and retention time windows for each peptide were provided in *Chapter 6*, and the following parameters were optimised: automatic gain control (AGC) target, maximum injection time, cycle time, retention time scheduled window. In addition to

the scheduled isolation list, no heavy or light peptides were provided for this study. The full method is described in **Appendix 8.5**.

3.2 PRM data analysis

For DDA analysis, optimisation files with an 80-minute gradient were searched using Proteome Discover version 2.4.1.15 (Thermo Scientific). Files were searched using a result filtered for high-confidence peptides, with a target FDR for peptide specific matches (PSMs) set to 0.01. Proteome Discoverer was used to identify the number of peptide groups per candidate protein, displaying any modifications (i.e., carbamidomethyl, deamidation, oxidation) in parts of the amino acid sequence and providing the number of peptide specific matches per unique peptide. This was used to generate a library for further analysis. MS-PRM data analysis from PRM acquisitions was performed in Skyline version 24.1.0.199. The number of target unique peptides was specified and time aligned with their retention time in FreeStyle and Proteome Discoverer, and the peptide quantification process was performed using the MS2 scan and up to 3 transition ions (i.e., y^+ ions ranked on intensity).

CHAPTER 3.

URINARY NEOPTERIN DETECTION AND QUANTIFICATION IN MOTOR NEURON DISEASE

Co-first authored on **Urinary neopterin: A novel biomarker of disease progression in amyotrophic lateral sclerosis**. S. R. Shepheard, V. Karnaros, B. Benyamin, D. W. Schultz, M. Dubowsky, J. Wu, et al. Eur J Neurol 2022 Vol. 29 Issue 4 Pages 990-999. Accession Number: 34967083 PMCID: PMC8901536 DOI: 10.1111/ene.15237. The percentage contribution of Stephanie Shepheard and myself was equal for *section 3.2* of this chapter, equally contributing towards the study design, data curation, data analysis, data presentation, and scientific writing.

3.1 Introduction

Only two treatments are available for people with sporadic MND, including riluzole (Miller et al., 2012), and edaravone (Yoshino, 2019). Incorporating biomarkers that reflect underlying disease pathology will be useful to reduce heterogeneity for people with MND such that the efficacy of treatments in clinical trials can be detected (Kiernan et al., 2021). At present, the ALSFRS-R is the only approved clinical measure of MND progression used in clinical trials and has limited power in phase 2 clinical trials (Benatar et al., 2016b). More than one biomarker that describes MND heterogeneity is needed as MND pathology does not reflect a single disease mechanism. These are biomarkers with prognostic (i.e., the ability to predict future survival), progression and pharmacodynamic (i.e., change over time and show response after a therapeutic intervention), and predictive (i.e., identify individuals who are more likely to experience an effect from a drug) utility to enable stratification based on objective biological mechanisms and survival outcomes (Benatar et al., 2016b, Goyal et al., 2020, Group, 2001).

The lack of an effective treatment for MND renders biomarker discovery of utmost importance. Previous research in our laboratory has found the extracellular domain of the neurotrophin receptor urinary p75 (p75^{ECD}) as a candidate biomarker of prognosis and progression in people with MND that reflects neurodegeneration (Shepherd et al., 2017) and serum neurofilament light (NfL) is a validated prognostic biomarker that reflects axonal degeneration (Benatar et al., 2020) that has some use in clinical trials (Benatar et al., 2024d). Biomarkers of immune dysfunction are of importance to MND as a pathological feature of disease. Immune dysfunction arises in response to oxidative stress, protein misfolding/aggregation, and glutamate toxicity (Malaspina et al., 2015). Although inflammation has been well characterised with MND pathology, very few inflammatory biomarkers have been explored, and none have been validated. The most promising, Chitotriosidase-1 (CHIT-1) (Thompson et al., 2018) and monocyte chemoattractant protein-1 (MCP-1) have been investigated (Guo et al., 2017). CHIT-1 is released from microglia, and was found to be significantly elevated in the CSF of people with MND compared to controls (Gille et al., 2019, Thompson et al., 2018, Steinacker et al., 2018, Vu et al., 2020, Thompson and Turner, 2019), but not always correlated to ALSFRS-R. MCP-1 levels in CSF appear to be related to faster progression (Huang et al., 2020) but a meta-analysis of eight studies found that serum MCP-1 was not significantly increased in MND compared to controls (Hu et al., 2017). Other pro-inflammatory cytokines including IL-6, IL-18 and TNF-

α may be candidate markers of the Th-1 response (Malaspina et al., 2015) but they have not been described in MND urine.

One possible inflammatory biomarker of MND is neopterin, a small metabolite with a molecular weight of 254 Da derived from guanosine triphosphate (GTP) belonging to a family of pteridines (Huber et al., 1984). Induced in response to interferon-gamma (IFN- γ), neopterin is released from pro-inflammatory macrophages, dendritic cells (Berdowska and Zwirska-Korczala, 2001), and microglia (Molero-Luis et al., 2013). Neopterin is biochemically inert and is excreted by the kidneys, making urinary detection possible (Berdowska and Zwirska-Korczala, 2001). Small cross-sectional studies of neopterin in MND have been inconclusive in serum (Westermann et al., 2000) and CSF (Yoshida et al., 1999). Urinary neopterin was examined in MND urine using a non-specific high-performance liquid chromatography (HPLC) separation technique coupled with fluorescent detection at 355 nm that reported a significant difference to controls (Lunetta et al., 2020), and urinary neopterin has been identified in people with breast cancer using liquid chromatography coupled with mass spectrometry (LCMS) by a multiple reaction monitoring (MRM) technique (Burton et al., 2016). The aim of this study was to characterise urinary neopterin in people with MND using a commercial IBL competition ELISA, and to refine urinary neopterin detection by optimising an adapted LCMS method of neopterin quantitation (Burton et al., 2016) to determine if urinary neopterin can predict pro-inflammation in people with MND.

The following methods and results quantified urinary neopterin by an IBL competition ELISA to characterise pro-inflammation in people with MND, that was published in (Shepherd et al., 2022). Neopterin measurement was refined using liquid chromatography mass spectrometry in MND urine at baseline. MRM was used to target neopterin by accurate mass analysis, comparing techniques to determine the cross-platform sensitivity, specificity, and robustness of neopterin detection in people with MND. To account for hydration status in spot-urine, neopterin was normalised to urinary creatinine as done previously (Shepherd et al., 2017). Biomarkers that can predict the immune response need to be identified to improve stratification and evaluate anti-inflammatory drug candidates in phase 2 clinical trials. This chapter describes the work-up and characterisation of urinary neopterin using LCMS to validate its response as a candidate biomarker of pro-inflammation in people with MND.

3.2 Neopterin detection by ELISA

3.2.1 IBL competition ELISA

The following section (*section 3.2.1-3.2.4*) includes results that have been published as part of a collaborative study (Shepherd et al., 2022). To identify whether urinary neopterin was detectable in MND urine and identify its prognostic utility, urinary neopterin was quantified in 46 people with MND compared 21 healthy individuals using a competitive/inhibition ELISA (see *section 2.2.2*).

Urinary neopterin was detected by a commercial competitive/inhibition ELISA. Simply, an unknown quantity of antigen in human urine competes with the fixed amount of enzyme labelled antigen for antibody-binding sites (rabbit-anti-neopterin) in the neopterin ELISA. Endogenous neopterin displaces streptavidin HRP-biotinylated neopterin from goat-anti-rabbit-neopterin IgG (polyclonal). Unbound antigen was removed, and after developing the reaction with TMB, the intensity of the colour developed was inversely proportional to the amount of antigen in the sample. The quantification of neopterin from human urine samples was determined from direct interpolation of a standard curve, with an example reported in **Figure 3.1**. The absorbance (450nm) of the standards shown on **Figure 3.1** (y-axis, linear) was plotted against concentration (x-axis, logarithmic) using a 4-parameter logistics model in Prism 9. This allowed the concentration of human urine samples to be interpolated using this model. To account for urinary dilution, creatinine was measured to determine the quantification of neopterin in urine expressed as μmol neopterin/mol creatinine from the molecular weight of neopterin (253.21 g/mol) and creatinine (113.1 g/mol).

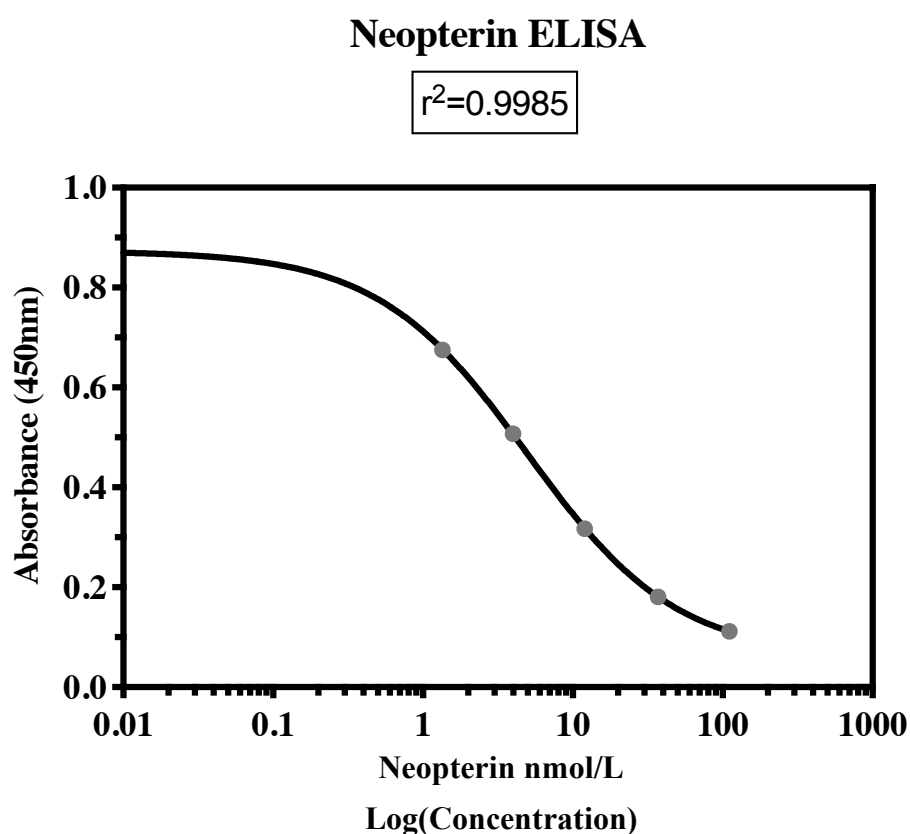


Figure 3.1: Neopterin ELISA standard curve

Competitive or inhibition Enzyme immunoassay (ELISA) used for the quantitative determination of neopterin in human urine. The absorbance (450 nm) results (y-axis, linear) were plotted against their concentration in nmol/L (x-axis, logarithmic) using a 4-parameter logistics model. The concentration of the unknown sample was determined using non-linear regression tests: [inhibitor] vs. response (variable slope, four parameters), Prism 10.

3.2.2 Urinary neopterin quantification in MND urine by competition ELISA

To identify whether urinary neopterin was detectable in MND urine and identify its prognostic utility, urinary neopterin was quantified using the competitive/inhibition ELISA in 21 healthy individuals and 46 people with MND at baseline. The clinical characteristics of this cohort are described in **Table 3.1**. The average ($\pm$ SD) age of people with MND was 66 ± 11.7 years old, and in healthy individuals was 60.6 ± 6.9 years. The average time from disease onset to diagnosis was 10.4 ± 8.1 months, where 64% presented with limb onset and 72% of people with MND were male. The average baseline Δ FRS of 0.68 ± 0.5 indicated a mixture of slow and fast progressors. Genetic mutations were identified in two people with MND, one with a C9orf72 repeat expansion, and the other with an E101G (E100G) mutation in the SOD1 gene. The remaining 44 people with MND were sporadic patients with no documented family history, and from those individuals, longitudinal data was available from 29 individuals (**Table 3.1**).

Baseline urinary neopterin was significantly increased in 46 people with MND (181.7 ± 78.5 μ mol neopterin/mol creatinine, $p=0.0002$, Welch's T-test) compared to 21 healthy individuals (120.4 ± 60.8 μ mol neopterin/mol creatinine) in **Figure 3.2A**. When combined with previously published results on urinary p75^{ECD} from 22 people with MND (Shepherd et al., 2017) shown in red, with data from an additional 24 people with MND (6.0 ± 2.7 ng p75^{ECD}/mg creatinine), urinary p75^{ECD} was higher in people with MND compared to healthy individuals (3.2 ± 1.0 ng p75^{ECD}/mg creatinine, $p<0.0001$, Welch's T-test) in **Figure 3.2B**. From the 46 people with MND, a correlation was found between baseline levels of urinary neopterin and urinary p75^{ECD} (Pearson's correlation, $r=0.38$, $p=0.009$) in **Figure 3.2C**. ROC analysis using the area under the curve (AUC) indicated that neopterin distinguished between MND and controls 74% of the time ($p=0.002$, 95% CI: 0.61-0.87) with 56% sensitivity, compared to the ability of p75^{ECD} to distinguish MND from controls 87% of the time ($p<0.0001$, 95% CI: 0.78-0.96) with 76% sensitivity in **Figure 3.2D**.

Table 3.1: Baseline cohort of study participants for neopterin detected by ELISA

All disease characteristics and clinical information for people with MND and healthy individuals at baseline were reported. Abbreviations: ALSFRS-R: revised amyotrophic lateral sclerosis functional rating scale. Δ FRS: estimated rate of progression (change in ALSFRS-R per month from symptom onset to baseline). Range: 25th–75th percentile.

Table 3.1 has been removed due to Copyright restrictions

Figure 3.2 has been removed due to Copyright restrictions

Figure 3.2: Urinary neopterin quantified in people with MND compared to healthy individuals

Urinary neopterin in (**A**, $p=0.0017$, 2-way T-test) and $p75^{ECD}$ (**B**, $p<0.0001$, 2-way T-test) levels were higher in people with MND ($n=46$) compared to healthy individuals ($n=21$) at baseline, with previously published $p75^{ECD}$ data shown in red. Neopterin levels were significantly correlated with $p75^{ECD}$ in (**C**) in the same participants ($n=46$) at baseline ($r=0.38$, $p=0.009$, Pearson's correlation). In (**D**), receiver operator curve (ROC) analysis of urinary neopterin and $p75^{ECD}$ demonstrated the difference between people with MND and healthy individuals, with an area under the curve of 0.74 ($p=0.0024$, 74%) and 0.87 ($p<0.0001$, 87%) respectively (Wilson-Brown method, Prism 10). Statistical analysis was performed in Prism 10.

Both urinary neopterin ($r=-0.36$, $p=0.01$) in **Figure 3.3A**, and p75^{ECD} ($r=-0.36$, $p=0.01$) in **Figure 3.3B** were significantly correlated with the ALSFRS-R at baseline. In 21 healthy individuals, there was no correlation between the concentration of neopterin and age (median: 61.3 years, range: 48-72, **Figure 3.3A**), or p75^{ECD} and age in **Figure 3.3C**. However, in the 46 people with MND at baseline (median: 66.7 years, range: 42-85), age was significantly correlated with neopterin ($r=0.5$, $p=0.0001$) and p75^{ECD} ($r=0.37$, $p=0.01$). Exclusion criteria were used to control for age between 48 and 72 years, such that healthy individuals ($n=21$) and people with MND ($n=26$) were age-matched (median: 63.5 years, range: 48.6-71.5 years) in **Figure 3.3B** and **Figure 3.3D**, showing no correlation. Baseline urinary neopterin in 26 people with MND remained significantly increased (167.1 ± 66.36 μmol neopterin/mol creatinine, $p=0.01$) compared to 21 healthy individuals (120.4 ± 60.8 μmol neopterin/mol creatinine). Urinary p75^{ECD} similarly remained significantly increased in 26 people with MND at baseline (5.49 ± 2.52 ng p75^{ECD}/mg creatinine, $p<0.0001$) compared to 21 healthy individuals (3.2 ± 1.0 ng p75^{ECD}/mg creatinine).

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Figure 3.3: Urinary neopterin and p75^{ECD} related to clinical characteristics

Urinary neopterin in (A) was significantly inversely correlated with ALSFRS-R scores at baseline in people with MND ($n=46$, $r=-0.36$, $p=0.013$, Pearson's correlation). Urinary p75^{ECD} was similarly correlated with ALSFRS-R scores in people with MND ($n=46$, $r=-0.36$, $p=0.013$) at baseline in (B). There was no correlation between urinary neopterin and age (C, $r=-0.10$, $p=0.4$) or between urinary p75^{ECD} and age (E) $r=-0.17$, $p=0.45$) in 21 healthy individuals aged 49-72 years. Further, there was no correlation between urinary neopterin and age (D) $r=0.02$, $p=0.93$) or between urinary p75^{ECD} and age (F) $r=0.002$, $p=0.99$) in 26 people with MND aged between 48-72 years from the total cohort ($n=46$). Statistics were performed in Prism 9.

3.2.3 Urinary neopterin as a candidate prognostic biomarker

Baseline urinary neopterin and p75^{ECD} were investigated for their utility as potential predictors of prognosis in MND. Survival analyses were performed on 46 people with MND, 44 of whom passed away over the course of this study. In a univariate Cox-Regression model analysis, the age of people with MND at diagnosis, gender, and site of onset were not associated with survival which was consistent with previously published data (Shepherd et al., 2017). Higher values of urinary p75^{ECD} (hazard ratio [HR] 1.13, 95% CI: 1.02-1.25, $p=0.019$) and faster estimated progression rate (Δ FRS) were univariate predictors of shorter survival (HR 3.66, 95% CI: 1.9-6.85, $p<0.0001$). Higher neopterin levels at baseline, whilst statistically significant, did not meaningfully predict survival in a univariate analysis (HR 1.01, 95% CI: 1.001-1.01, $p=0.01$). Univariate prognostication of urinary neopterin (**Figure 3.4A**) and p75^{ECD} (**Figure 3.4B**) was illustrated by Kaplan-Meier survival curves. Those people with MND that had lower than the median neopterin (161 μ mol neopterin/mol creatinine) survived 19.8 months compared to 10.7 months for those with neopterin above the median value ($p=0.046$, **Figure 3.4A**). Longer median survival from baseline was observed in people with MND from urinary p75^{ECD} levels below the median (5.33 ng p75^{ECD}/mg creatinine), surviving 20.5 months compared to 8.7 months ($p=0.005$, **Figure 3.4B**; Wilcoxon test, SPSS, and Prism 9). Multivariate Cox-Regression model analysis indicated that only the Δ FRS predicted survival (HR 3.61, 95% CI: 1.62-8.05, $p=0.0002$) whereas urinary neopterin and p75^{ECD}, gender, site of onset, and baseline age did not contribute prognostic value over the Δ FRS. This indicates that neither neopterin, nor p75^{ECD}, could add prognostic value beyond what can be determined by the Δ FRS in people with MND.

3.2.4 Urinary neopterin as a candidate progression biomarker

The utility of urinary neopterin as a biomarker of MND progression was explored in 29 people with MND who provided longitudinal clinical data. All 29 people provided more than one sample post-baseline (median: 3 visits, range: 2-6 visits; **Table 3.1**). The median duration between the first and second sample was 3.9 months (range: 2.0-14.7 months). 14 individuals were included from previously published results on urinary p75^{ECD} (Shepherd et al., 2017), however, 8 individuals provided additional samples collected at later time points. To report the consistency of urinary neopterin and p75^{ECD} as biomarkers of disease progression, changes in these biomarkers were examined using the time from symptom onset, time from baseline, and time from diagnosis. The dates of diagnosis and baseline were reliably obtained from clinical notes (Shepherd et al., 2017) and were chosen for our

primary analyses in **Figure 3.5B, 3.5C, 3.5D, and 3.5F**. The urinary concentration of neopterin increased from symptom onset each month by an average of 4.5 μmol neopterin/mol creatinine (95% CI: 2.2-6.8, $p<0.0001$; **Figure 3.5A**), from diagnosis each month by an average of 6.8 μmol neopterin/mol creatinine (95% CI: 4.6-9.1, $p<0.0001$; **Figure 3.5B**), and from baseline by an average of 6.8 μmol neopterin/mol creatinine (95% CI: 4.1-9.5, $p<0.0001$; **Figure 3.5C**). Urinary p75^{ECD} increased by an average of 0.15 ng p75^{ECD} /mg creatinine per month symptom onset (95% CI: 0.1-0.2, $p<0.0001$; **Figure 3.5D**), and by 0.19 ng p75^{ECD} /mg creatinine per month from diagnosis (95% CI: 0.14-0.23, $p<0.0001$; **Figure 3.5E**), which was the same as our previously published results (Shepherd et al., 2017), and by 0.19 ng p75^{ECD} /mg creatinine from baseline (95% CI: 0.14-0.24, $p<0.0001$; **Figure 3.5F**).

From all 29 longitudinal samples, the slope of the ALSFRS-R (ΔFRS) declined by an average of -0.61 points/month from diagnosis (95% CI: -0.49 to -0.73, $p<0.0001$) and by -0.66 points/month (95% CI: -0.54 to -0.79, $p<0.0001$) from baseline. Urinary neopterin increased significantly each month ($p<0.0001$) and was associated with a monthly decrease in ALSFRS-R. When the data was split across the median ΔFRS at baseline (-0.49 ALSFRS-R points/month) to differentiate between faster (>0.49 points/month; $n=16$) and slower (<0.49 , $n=13$) progressors. Neopterin increased by 4.7 μmol neopterin/mol creatinine per month (95% CI: 1.4-8.0, $p<0.0001$) from diagnosis in the slower progressors. In the faster progressors, neopterin increased by 16.6 μmol neopterin/mol creatinine per month (95% CI: 13-20, $p<0.0001$, red lines in **Figure 3.5B**). The difference between groups was significant ($p=0.0005$). p75^{ECD} increased by 0.17 ng p75^{ECD} /mg creatinine per month from diagnosis (95% CI: 0.12-0.22, $p<0.0001$) in slower progressors, and by 0.36 ng p75^{ECD} /mg creatinine per month (95% CI: 0.26-0.45, $p<0.0001$) in faster progressors (red lines in **Figure 3.5E**). This difference was likewise significant ($p=0.0002$).

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Figure 3.4: Urinary neopterin and p75^{ECD} as candidate biomarkers of disease prognosis

Kaplan-Meier survival estimates of 46 people with MND, from which 44 had passed away during this study. 22 people with MND who had baseline urinary neopterin in (**A**) above (dashed line) or below (solid line) the median of 161.1 μmol neopterin/mol creatinine, or with urinary p75^{ECD} in (**B**) above (dashed line) or below (solid line) the median of 5.33 ng p75^{ECD}/mg creatinine) were plotted. This demonstrated a longer median survival in those with lower compared to higher urinary neopterin ($p=0.046$; 10.7 vs 19.8 months) and lower p75^{ECD} levels ($p=0.005$; 8.7 months vs 20.5 months). Kaplan-Meier analysis (Wilcoxon test) in Prism 9.

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Figure 3.5: Urinary neopterin as a biomarker candidate of disease progression

Individual longitudinal trajectories of neopterin (**A-C**) and p75^{ECD} (**D-F**) in 29 people with MND. Neopterin was increased from symptom onset at a rate of 4.5 μmol neopterin/mol creatinine per month in (**A**, 95% CI 2.2-6.8, $p < 0.0001$), from time of diagnosis in (**B**) at a rate of 6.8 μmol neopterin/mol creatinine per month (95% CI 4.6-9.1, $p < 0.0001$), and from baseline (**C**) at a rate of 6.8 μmol neopterin/mol creatinine per month (95% CI 4.6-9.5, $p < 0.0001$). p75^{ECD} also increased from time of symptom onset (**D**) 0.15 ng p75^{ECD}/mg creatinine per month, 95% CI 0.1-0.2, $p < 0.0001$), from diagnosis (**E**) 0.19 ng p75^{ECD} /mg creatinine per month, 95% CI 0.14-0.23, $p < 0.0001$), and baseline (**F**) 0.19 ng p75^{ECD} /mg creatinine per month, 95% CI 0.14-0.24, $p < 0.0001$). Each participant was sampled at least twice. Fast progressors with a $\Delta\text{FRS} > 0.49$ are highlighted in red.

3.3 Neopterin detection by liquid chromatography mass spectrometry

Having confirmed the presence of urinary neopterin in people with MND by ELISA (Shepherd et al., 2022), (Shepherd et al., 2022), the following aim of this study was to analytically validate urinary neopterin concentrations in people with MND using liquid chromatography-mass spectrometry (LCMS). To achieve this, a multiple reaction monitoring (MRM) method that produced high-throughput quantification of urinary neopterin from small sample volumes, with high specificity, sensitivity, and repeatability was generated. To analytically validate neopterin as a candidate biomarker of pro-inflammation in MND, an LCMS method was adapted from (Burton et al., 2016) to quantify urinary neopterin in people with MND by an LCMS and MRM technique.

3.3.1 High performance-liquid chromatography and mass spectrometer optimisation

LCMS was used to validate urinary neopterin detection in MND (Shepherd et al., 2022). The Thermo Altis Triple Quadrupole (QQQ) mass spectrometer was optimised, tuning each quadrupole (Q1, Q2, Q3) to ensure the best possible detection and fragmentation of the pteridine compound *D*-Neopterin, and its internal standard, *D*-Neopterin-¹³C₅. Key parameters which were optimised included precursor ion selection (Q1), collision energy gas flow (Q2), and product ion detection (Q3). The precursor ion masses of *D*-Neopterin and *D*-Neopterin-¹³C₅ were 254.3 m/z and 259.3 m/z, respectively. The four product ions detected for *D*-Neopterin were (1) 190.13, (2) 196.13, (3) 206.11, and (4) 236.13 m/z; **Table 3.2**, with the collision energy required to split each fragment ion being (1) 20.92 volts (V), (2) 19.28 V, (3) 18.4 V, and (4) 15.19 V. The four product ions detected for *D*-Neopterin-¹³C₅ were (1) 194.13, (2) 197.13, (3) 210.13, and (4) 241.13 m/z, with the collision energy required to split each fragment ion being (1) 21.01 V, (2) 19.32 V, (3) 18.31 V, and (4) 15.28 V; **Table 3.3**. Confirmation ions used for neopterin quantification in all further analyses were highlighted in bold, with *D*-Neopterin quantified by the **206.11 m/z** ion, and *D*-Neopterin-¹³C₅ quantified by the **210.13 m/z** ion. A flowrate of 200 µL/minute was applied through the Phenomenex Phenyl-Hexyl HPLC column, and operational parameters of the mass spectrometer were optimised to an ion transfer tube temperature of 325°C, vapour temperature of 275°C, auxiliary gas flow of 35 L N₂/min, sheath gas flow of 7 L N₂/min, and a H-ESI voltage of +3,500 V. The chromatographic peak width was optimised to 6 seconds wide, using a cycle time of 15 points-per-peak, Q1 and Q3 resolutions of 0.7 Da, and CID gas set to 1.5 mTorr reported in **Table 3.4**.

Table 3.2: Neopterin and creatinine compound optimisation

Using a Thermo Scientific Altis Triple Quadrupole mass spectrometer, the compounds *D*-Neopterin, *D*-Neopterin-¹³C₅, and creatinine were optimised by syringe injection for their detection in human urine. Confirming/quantification ions highlighted in bold.

Compound	Precursor mass (m/z)	Product ion 1 (m/z)	Product ion 2 (m/z)	Product ion 3 (m/z)	Product ion 4 (m/z)
<i>D</i> -Neopterin	254.3	190.13	196.13	206.11	236.13
<i>D</i> -Neopterin- ¹³ C ₅	259.3	194.13	197.13	210.13	241.13
Creatinine	116.3	44.13	72.13	79.13	86.13

Table 3.3: Multiple reaction monitoring (MRM) method parameters

MRM parameters including compound optimisation, precursor and product ions, collision and radiofrequency voltages, and dwell time for optimum chromatography

Compound	Start time (min)	End time (min)	Precursor mass (m/z)	Product ion mass (m/z)	Collision energy (V)	RF Lens (V)	Min dwell time (ms)
<i>D</i> -Neopterin	0	9	254	190.13	20.92	69	65.344
<i>D</i> -Neopterin	0	9	254	196.13	19.28	69	65.344
<i>D</i> -Neopterin	0	9	254	206.11	18.4	69	65.344
<i>D</i> -Neopterin	0	9	254	236.13	15.19	69	65.344
<i>D</i> -Neopterin- ¹³ C ₅	0	9	259	194.13	21.01	73	65.344
<i>D</i> -Neopterin- ¹³ C ₅	0	9	259	197.13	19.32	73	65.344
<i>D</i> -Neopterin- ¹³ C ₅	0	9	259	210.13	18.31	73	65.344
<i>D</i> -Neopterin- ¹³ C ₅	0	9	259	241.13	15.28	73	65.344
Creatinine	0	9	116	44.13	18.48	45	65.344
Creatinine	0	9	116	72.13	17.34	45	65.344
Creatinine	0	9	116	79.13	17.64	45	65.344
Creatinine	0	9	116	86.13	12.67	45	65.344

Table 3.4: Thermo Scientific Altis Triple Quadrupole instrument settings from Xcalibur

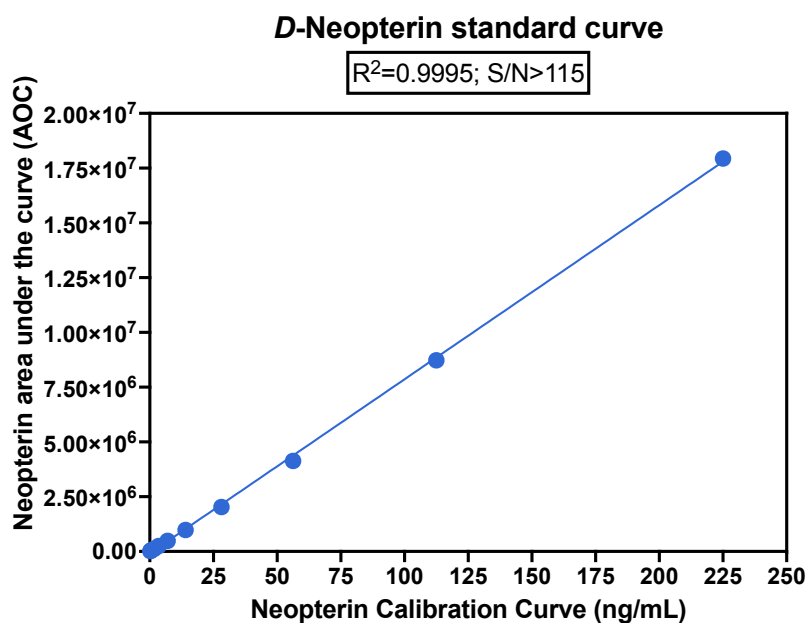
Parameter	Value/Description
Flow rate	200 µL/minute
Ion transfer tube temperature	325°C
Vapour temperature	275°C
Auxiliary gas flow	35 L N ₂ /minute
sheath gas flow	7 L N ₂ /min
H-ESI voltage	+3,500 V
Chromatographic peak width	6 seconds wide
points-per-peak	15 points
Q1 and Q3 resolution	0.7 Da
CID gas	1.5 mTorr

3.3.2 Neopterin standard detection and optimisation using LCMS

Under optimised LCMS and MRM conditions, a calibration curve for D-Neopterin was constructed to demonstrate its linearity under an appropriate analytical range. The calibration curve was produced from *D*-Neopterin standard by monitoring MRM transitions from **Table 3.2**. 1 µg/mL of *D*-Neopterin standard was used to generate a linear 12-point standard curve between 225 ng/mL and 220 pg/mL in **Figure 3.6A**, where $r^2=0.9995$ and the S/N ratio was >115, based off the lowest 225 pg/mL standard in the Thermo FreeStyle software. *D*-Neopterin- $^{13}\text{C}_5$, structural isomer of *D*-neopterin, was used as an internal standard and incorporated into the methodology by creating a standard curve ratio between *D*-neopterin and *D*-Neopterin- $^{13}\text{C}_5$. Having identical chemical structure, *D*-Neopterin- $^{13}\text{C}_5$ was spiked into the 12-point standard curve to co-elute with *D*-neopterin and generate two chromatograms with the same retention time. The area of these two chromatograms was paired with their concentration ratio ($C_{\text{Neopterin}}/C_{^{13}\text{C}_5}$), with a 1:1 concentration ratio at 14 ng/mL, constructing a 12-point interpolated calibration plot ratio of *D*-neopterin and *D*-neopterin- $^{13}\text{C}_5$ between 0.01 and 20 (**Figure 3.6B**; $r^2=0.9997$, linear regression, prism 10).

Repeat analysis of *D*-neopterin demonstrated excellent standard curve reproducibility, sensitivity, and linearity, however, a significant variation of 30.3% change in calibration curve sensitivity was generated over a period of 10-months (**Figure 3.7A**, 10 standard curves, $r^2>0.999$, 30.3% change) due to inconsistencies in ionisation efficiency and instrument sensitivity. To address this, the same 10 calibration curves from **Figure 3.7A** constructed *D*/ $^{13}\text{C}_5$ -neopterin ratio standard curves from an internal standard spike in, which resulted in a decrease in variation from 30.3% to 6.1% in **Figure 3.7B** (10 ratio standard curves, $r^2>0.999$, 6.1% change). This is evidence in practice of how internal standard, *D*-Neopterin- $^{13}\text{C}_5$, was implemented into each assay to improve the sensitivity, repeatability, and robustness of *D*-neopterin detection and quantification in human urine. Human urine and *D*-neopterin standard curves were spiked with internal standard *D*-Neopterin- $^{13}\text{C}_5$ from here-in, and urinary neopterin quantification was performed by the ratio standard curve to account for changes in ionisation efficiency, instrument performance, and analytical column performance.

A



B

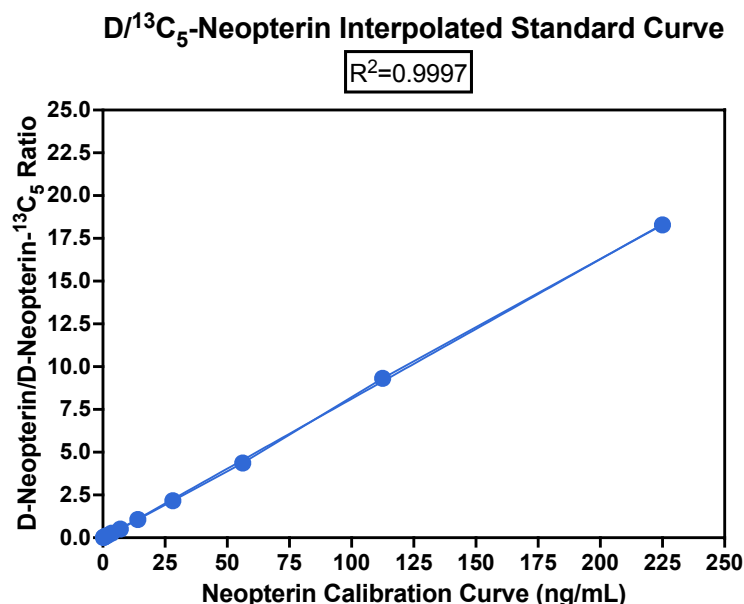


Figure 3.6: Neopterin standard curves by LCMS

Liquid chromatography-mass spectrometry was used for the quantitative determination of a 12-point *D*-neopterin calibration curve in human urine. In **(A)**, the area under the curve (AUC) results (y-axis, linear) was plotted against the concentration of *D*-neopterin standard (x-axis, linear) using a linear regression model (Prism 10). The 12-point standard curve ranged from a concentration of 225 pg/mL to 225 ng/mL with an average r^2 value of >0.99 and a S/N ratio >115. Structural isomer *D*-Neopterin-¹³C₅ was spiked as an internal standard, producing a 12-point linear (r^2 >0.99) ratio between ($C_{\text{Neopterin}}/C_{^{13}\text{C}_5}$) shown in **(B)** for the quantification of endogenous neopterin in human urine.

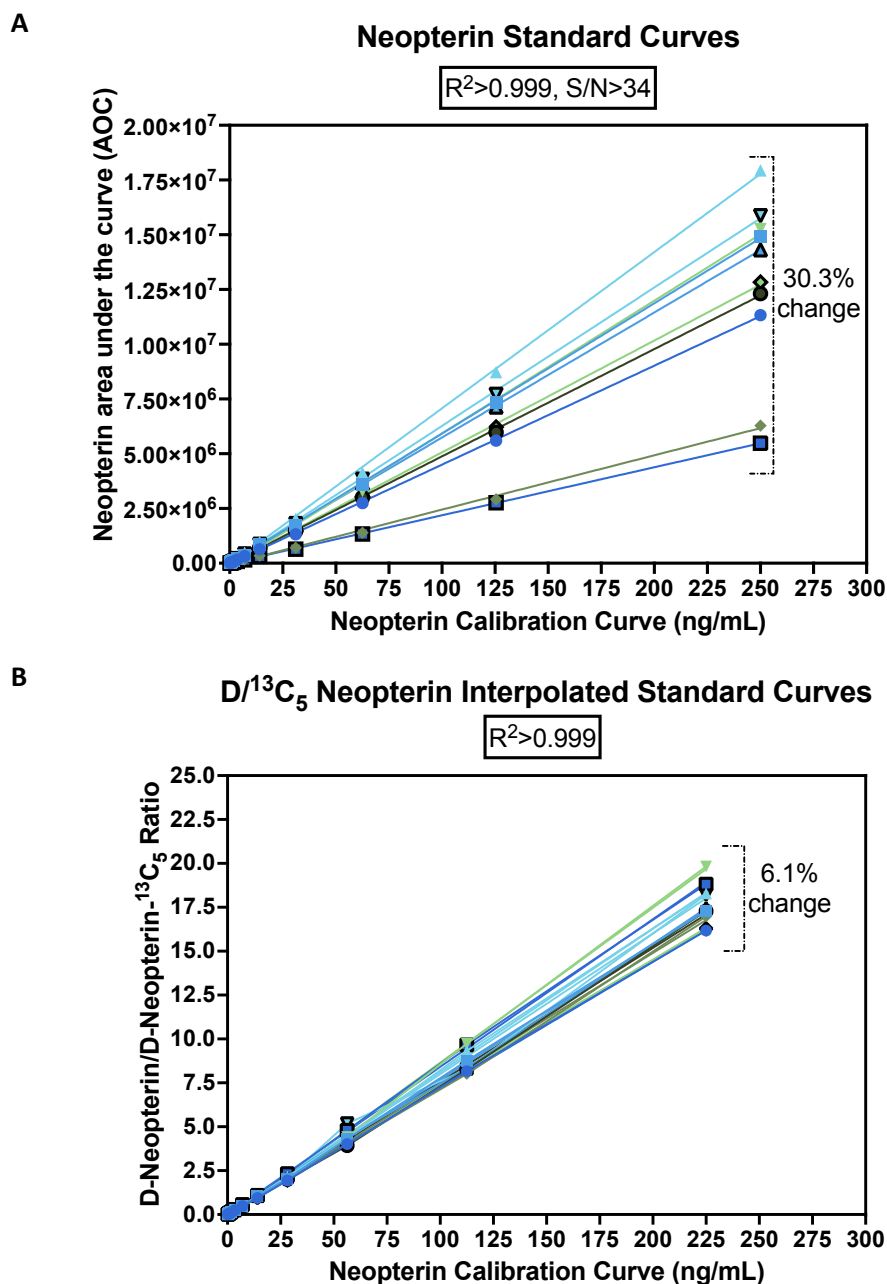


Figure 3.7: Variability of multiple neopterin standard curves

D-neopterin standard curves from multiple testing assays were shown in (A) to display excellent repeatability and linearity ($r^2 > 0.999$, simple linear regression, Prism 10), however, up to 30.3% change was noted in instrument sensitivity between 10 standard curves. However, internal standard D-neopterin-¹³C₅ in (B) demonstrated the same linearity ($r^2 > 0.999$, simple linear regression, Prism 10) in the ratio between ($C_{\text{Neopterin}}/C_{^{13}\text{C}_5}$), changing only 6.1% between 10 standard curves used for the quantification of endogenous neopterin in human urine.

3.3.3 Neopterin detection in human urine

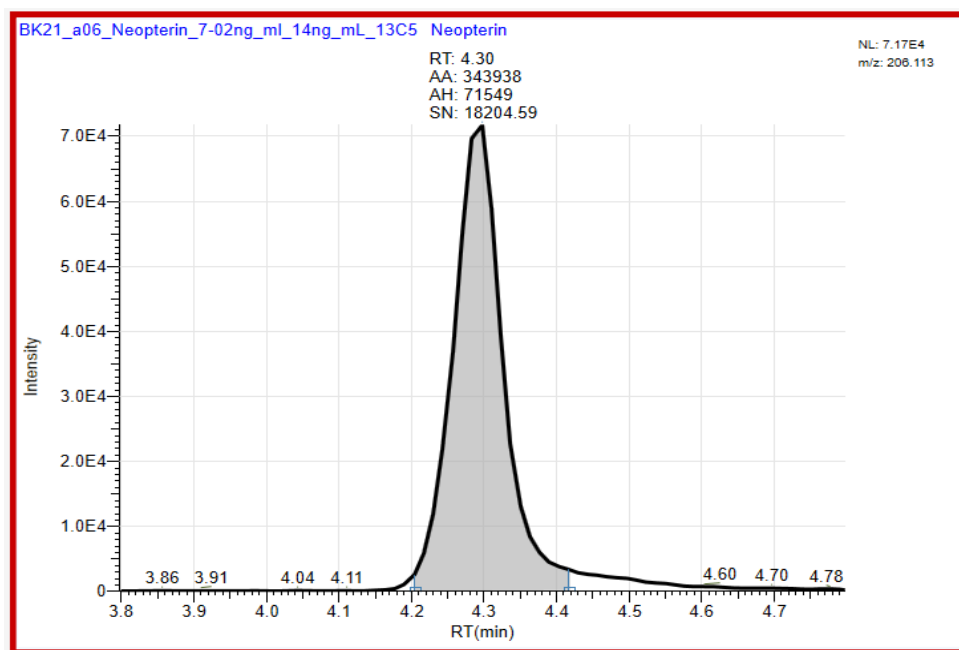
To analyse the effectiveness of neopterin detection in human urine, the *D*-neopterin calibration curve was performed in 100-fold urine and compared to a standard curve produced in water and methanol (H₂O/MeOH) to determine the likes of ion suppression and urinary matrix effects. Four *D*-neopterin product ions were reliably quantified from the standard curve in 100-fold urine and H₂O/MeOH from a precursor ion of 253.21 m/z. Over the 9-minute HPLC gradient duration, one distinct peak was observed for *D*-neopterin in H₂O/MeOH, with a retention time of ~4.30 minutes eluting as previously described in **Table 3.2**, reported in **Figure 3.8A**. The total ion current (TIC) described the overall signal intensity of each extracted ion chromatogram (XIC) eluting at the same retention time. However, three distinct chromatographic peaks were observed in 100-fold urine, labelled at ~4.20, ~4.30, and ~4.50-minute retention times, building toward the idea that endogenous neopterin and other compounds with the precursor mass of 253.21 Da, were eluting at three time points across the 9-minute gradient, reported in **Figure 3.8B** using TraceFinder 5.1 for the high-throughput analyses of *D*-neopterin detection and quantification in standard curves and MND urine.

To confirm the chromatographic peak representing endogenous *D*-neopterin from other analytes with similar specificity (i.e., structural isomers *D*-monapterin or *L*-monapterin), a base peak analysis was performed in **Figure 3.9A, B, and C**, to identify the linear regression coefficient of all four *D*-neopterin fragment ions (or XICs) under the selected analytical range for each chromatographic peak at each retention time. The AUC from each chromatogram with retention times of ~4.20 and ~4.50-minutes were analysed in **Figure 3.9A** and **Figure 3.9B** respectively. The AUC of these each fragment ion and did not correlate with the spiked linear regression curve, indicating that the analytes eluting at ~4.20 and ~4.50 minutes were not *D*-neopterin. **Figure 3.9C** identified linear regression coefficients above $r^2=0.99$ for all four *D*-neopterin fragment ions (simple linear regression, $r^2>0.99$) at retention time ~4.30 minutes. No ion suppression up to 1 µg/mL was observed in **Figure 3.9D** to suggest any spill-over of neopterin from one chromatographic peak to another. This approach, with the co-elution of *D*-neopterin-¹³C₅ at ~4.30 minutes (**Figure 3.10A-B**) validated the quantification of endogenous neopterin in human urine at 4.30 minutes, with **Figure 3.9C** showing confirming product ion 206.11 m/z to have the highest sensitivity to *D*-neopterin standard with a S/N ratio value of 63 in FreeStyle and used as confirming ion in **Figure 3.10A**.

Endogenous urinary neopterin was quantified using the confirmation 206.11 m/z ion, and internal standard *D*-Neopterin-¹³C₅ was quantified using the 210.11 m/z ion as confirmation for all quantitative assays. The remaining 4-point *D*-neopterin calibration curve from 250 pg/mL to 1 µg/mL was compared in 100-fold diluted human urine to H₂O/MeOH (**Figure 3.8D**; $r^2 > 0.99$, linear regression, Prism 10) to validate in practice that the desired calibration range did not undergo ion suppression well past the analytical range of endogenous neopterin in MND urine. In the presence of 100-fold human urine, *D*-neopterin sensitivity from 250 pg/mL to 1 µg/mL did not undergo ion suppression compared to H₂O/MeOH buffer, with almost-identical sensitivities of *D*-neopterin standard up to 1 µg/mL in H₂O/MeOH (1.08E^{-8}) compared to 100-fold urine (1.06E^{-8}).

3.3.4 Benefits of LCMS detection compared to IBL competition ELISA

So far, neopterin detection by LCMS has provided some advantages in comparison to detection and quantification inhibition ELISA. The benefits of detecting urinary *D*-neopterin by LCMS include the generation of a calibration curve with excellent linearity (**Figure 3.6A-D**, $r^2 > 0.99$) and repeatability (**Figure 3.7A-D**), compared to a logarithmic scale of the ELISA standard curve (**Figure 3.1**). The IBL competition ELISA is an inhibition assay that uses a logarithmic and inverse response curve, making urinary neopterin difficult to quantify accurately with increasing risk of misinterpretation and poor sensitivity at both ends of the inverse response curve. The LCMS method benefits from a linear standard curve, reliably quantifying urinary neopterin over a broad dynamic range. The LCMS assay has a lower limit of detection (LLoD) with a minimum detection value of 225 pg/mL, compared to the ELISA of 340 pg/mL, and a maximum value of detection of 225 ng/mL, compared to a value of 28 ng/mL by ELISA. The LCMS assay has a far greater signal to noise ratio reported multiple times across **Figures 3.6-10**, with the implementation of internal standard *D*-neopterin-¹³C₅ that has shown to co-elute at ~4.30 minutes with *D*-neopterin in **Figure 3.10A-B**, with higher confidence in chromatographic separation of other analytes or structural isomers at retention times ~4.20 and ~4.50 minutes by the LCMS technique. These variables provide higher confidence in *D*-neopterin detection by LCMS and MRM with absolute mass accuracy, in comparison to the IBL competition ELISA.



B

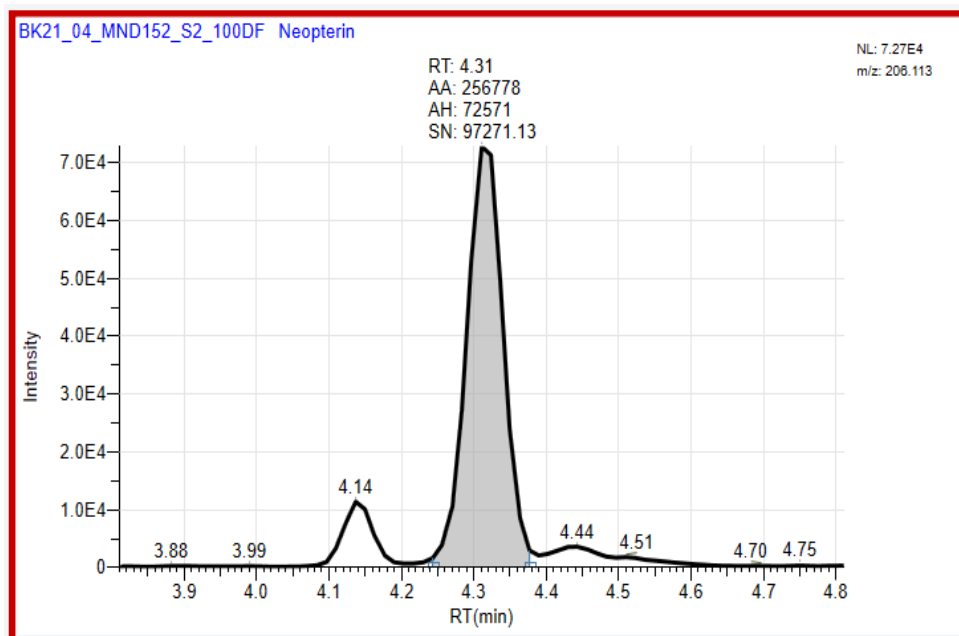


Figure 3.8: Urinary neopterin detection and quantification in standard buffer and MND urine using TraceFinder 5.1

Urinary neopterin was detected and quantified by standard curve generation in water and methanol solution ($\text{H}_2\text{O}/\text{MeOH}$), and in MND urine. When *D*-neopterin was detected in $\text{H}_2\text{O}/\text{MeOH}$ standard curve solution and quantified (7.17E^4) in (A), one observable peak was reported in TraceFinder 5.1 at a retention time of ~ 4.30 minutes. (B) When endogenous *D*-neopterin was detected in MND urine at a similar concentration (7.27E^4), three observable peaks were reported at ~ 4.14 minutes, ~ 4.31 minutes, and ~ 4.44 minutes, suggesting the co-elution of analytes of the same mass and charge in MND urine. LCMS chromatogram integration was performed by TraceFinder 5.1.

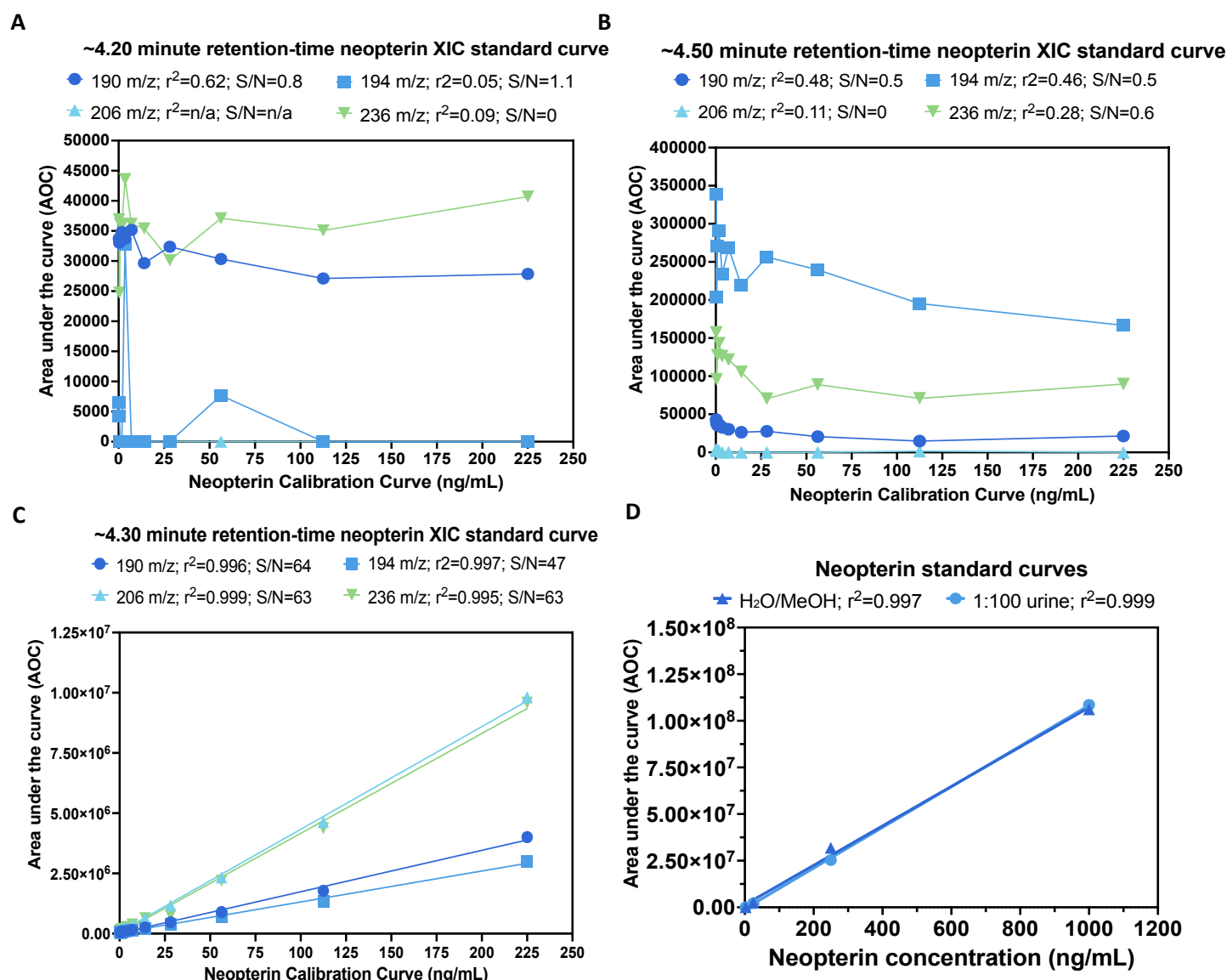
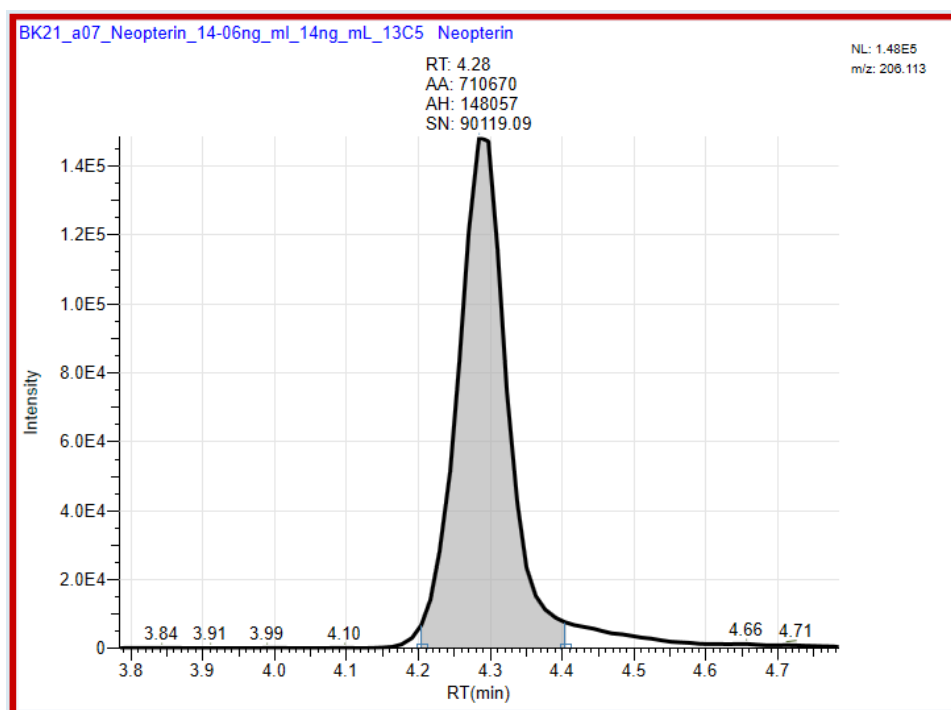


Figure 3.9: Base peak analysis of neopterin standard curve product ions

When *D*-neopterin was detected in human urine, neopterin eluted at three retention times: ~4.14 minutes (**A**), ~4.44 minutes (**B**), and ~4.30 minutes (**C**). To identify the retention time that corresponded with neopterin elution, all four *D*-neopterin product ions from **Table 2** were monitored over the 12-point calibration range for their linearity. No linearity was observed in (**A**) with $r^2 < 0.62$, or in (**B**) with $r^2 < 0.48$, however (**C**) observed complete linearity at ~4.31 minutes retention time, with $r^2 > 0.99$ for each product ion. Product ion 206.11 m/z in (**C**) generated the highest signal, used for *D*-neopterin confirmation by TraceFinder 5.1. (**D**) A 4-point neopterin standard curve from 250 pg/mL to 1 μ g/mL was generated confirming no ion suppression or loss of signal specificity from *D*-neopterin standard curves spiked into 1:100 human urine or H₂O/MeOH up to a concentration of 1 mg/mL, 4-times higher than the 225 ng/mL reported in the quantitative 12-point standard curve. Linear regression analysis performed in Prism 10.

A



B

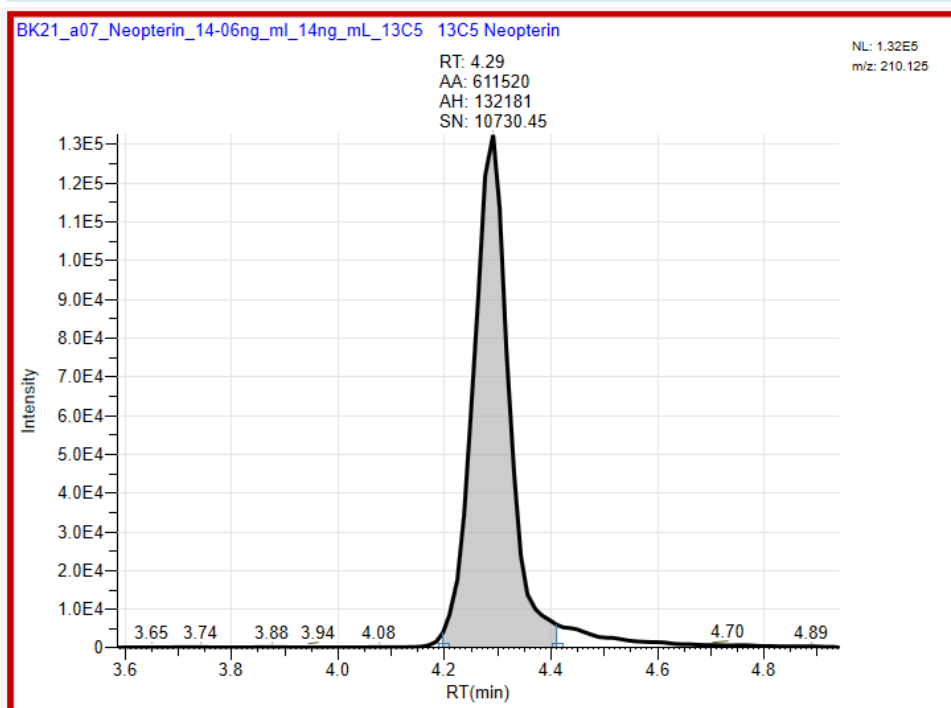


Figure 3.10: *D*-neopterin and *D*-neopterin- $^{13}\text{C}_5$ detection and quantification using TraceFinder 5.1

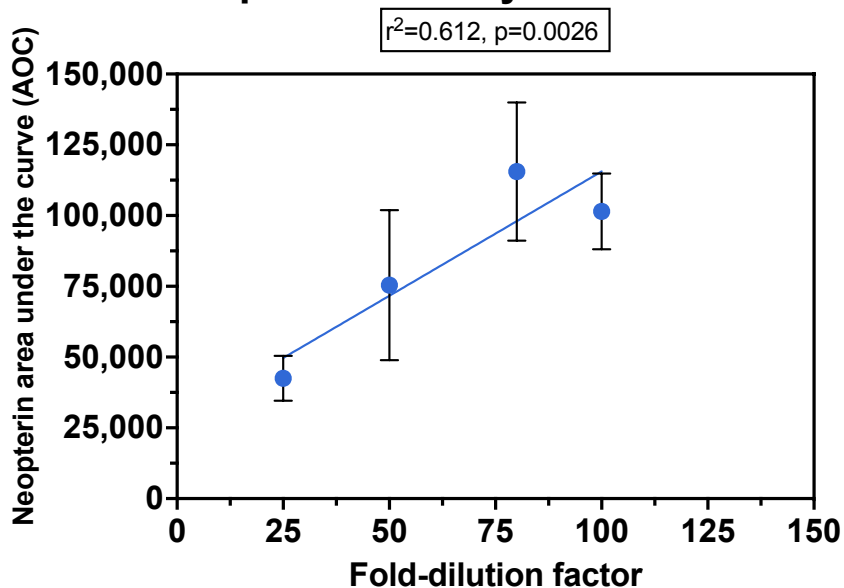
D-neopterin and *D*-neopterin- $^{13}\text{C}_5$ were detected and quantified by standard curve generation in water and methanol solution ($\text{H}_2\text{O}/\text{MeOH}$). In (A), *D*-neopterin at a concentration of 14 ng/mL (intensity 1.48E^5) eluted at a retention time of ~4.28 minutes. In (B), *D*-neopterin- $^{13}\text{C}_5$ at a concentration of 14 ng/mL (intensity 1.32E^5) co-eluted with *D*-neopterin at ~4.29 minutes. The co-elution of both compounds confirms the elution of endogenous neopterin at ~4.30 minutes, providing confidence for down-stream endogenous neopterin quantification assays. LCMS chromatogram integration was performed by TraceFinder 5.1.

3.3.5 Optimisation of urinary sample treatment

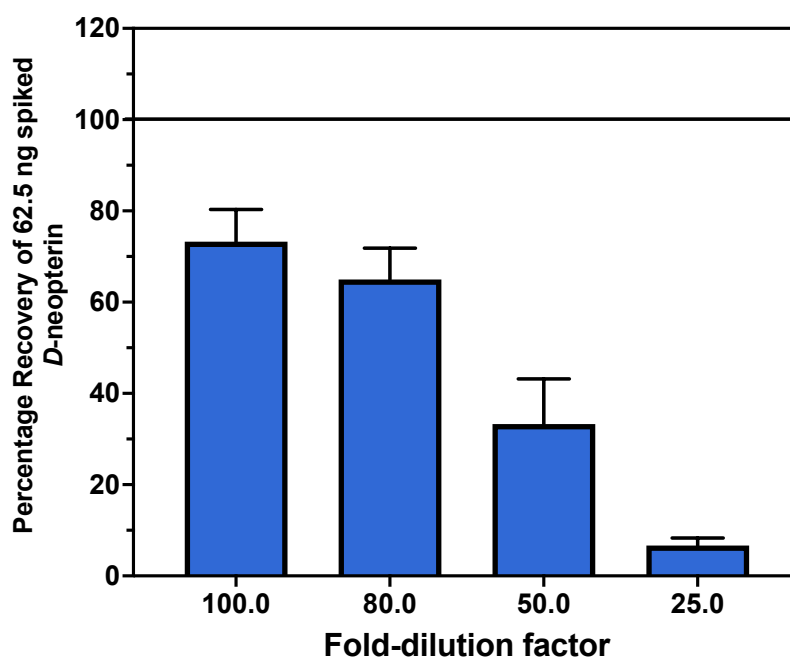
The Altis QQQ mass spectrometer, MRM technique, and TraceFinder 5.1 data analysis tool were optimised for the detection of two neopterin compounds in linear standard curves and MND urine. The next step in study design was to determine an optimal treatment protocol for urine samples from people with MND and healthy individuals for sensitive and repeatable neopterin detection and quantification. Pteridine analysis was validated through performing spiked recoveries of *D*-neopterin in serially diluted urine specimen MND173 to minimise matrix interference concerns. Since urine is a complex matrix, potential interferences with equivalent precursor and transition ions were identified in **Figure 3.11A**, with lower fold-dilution factors resulting in significant ion suppression in human urine caused by poor ionisation efficiency at the H-ESI source, and ion saturation at the detector (Q3). There was a statistically significant linear relationship between MND173 urine dilution factors and *D*-neopterin LCMS signal ($p=0.0026$). Moderate linearity was reported ($r^2=0.612$) that may have been influenced by matrix effects, ion suppression, or variability in the sample preparation. Higher fold-dilution factors up to 100-fold diluted urine reduced interfering matrix effects and ion saturation, improving the efficacy of *D*-neopterin detection and longevity of the Phenyl-Hexyl analytical column.

Urine specimen MND173 was spiked with 62.5 ng of *D*-neopterin, and a percentage recovery experiment was performed. At higher urine concentrations the recovery of 62.5 ng *D*-neopterin was decreased in **Figure 3.11B**. From a 25-fold dilution factor, only $6.7\% \pm 1.7\%$ of *D*-neopterin was recovered in human urine, indicating severe ion depression due to urinary matrix components interfering with detection. Recovery improved with increased fold-dilution factors, recovering $33.3\% \pm 9.9\%$ in 50-fold diluted urine, $65\% \pm 6.8\%$ in 80-fold diluted urine, and peaking at $73\% \pm 7.1\%$ recovery in 100-fold diluted urine. As a result, remaining urine samples were diluted 100-fold minimise matrix ion suppression, meet the desired range for the 12-point standard curve, and improve analytical column longevity. Standard isotope labelled internal standard *D*-neopterin- $^{13}\text{C}_5$ was introduced *after* this experiment to co-ionise and co-elute with *D*-neopterin, aiming to correct for ion suppression and improve absolute quantification.

A

Neopterin linearity in human urine

B

Human urine neopterin recovery**Figure 3.11: Recovery of *D*-neopterin in serially diluted human urine**

Urine specimen MND173 was serially diluted from 25-fold to 100-fold dilutions and observed for its linearity. In (A), significant linearity was reported on three replicates of serially diluted human urine ($p=0.0026$, $r^2=0.612$). Higher fold-dilution factors up to 100-fold reduced matrix ion suppression in *D*-neopterin detection. (B) observed the percentage recovery of spiked 62.5 ng *D*-neopterin into MND173, reporting a recovery of $6.7\% \pm 1.7\%$ for 25-fold, $33.3\% \pm 9.9\%$ in 50-fold, $65\% \pm 6.8\%$ in 80-fold, and $73\% \pm 7.1\%$ recovery in 100-fold diluted urine. 100-fold dilution factor was used to maximise compound discovery and minimize interfering matrix ion suppression. Percentage recovery values were calculated in Prism 10 and plotted as mean $\pm$ SD.

3.3.6 Stability of neopterin in urine

Three human urine samples were tested across multiple technical (intra-assay) and biological (inter-assay) replicates as experimental controls to measure the intra- and inter-assay variability for urinary *D*-neopterin quantification by LCMS. These three urine samples were described as a positive control, high-neopterin control, and low-neopterin control. Described in **Table 3.5**, the variability of neopterin measurements in these samples was tested using the coefficient of variance (CV, ratio of the standard deviation to the mean). The CV was lower for replicates on individual assays (i.e., intra-assay) performed in duplicate, with the highest average CV reported at 4.8% for MND162, the low-neopterin control, between 6 inter-assay repeat measurements. CV's were higher between biological replicates on different days (inter-assay), reporting a highest average CV of 10.9% for MND173, the positive control, over the course of 31 repeat biological measurements.

In comparison, the precision of the IBL competition ELISA reported an intra-assay range of 4.0% to 10.2% on average in **Table 3.6** across 6 people with MND tested in duplicates on each assay plate, again with high, medium, and low concentrations of urinary neopterin. The intra-assay range was reported with an average of 3.1%, and the inter-assay range was reported with an average of 16.2% across 3 biological replicates on different days, with an acceptable score of less than 15% as per the manufacturer's instructions. Using IBL's guidelines of an intra-assay range of 5.3% – 11.2% and inter-assay range of 9.3% – 14.4%, the variability of human urine samples by LCMS was within an acceptable range, with consistent LCMS performance over time.

The precision and stability of inter-assay neopterin detection in urine samples by LCMS from **Table 3.5** were plotted in **Figure 3.12**, demonstrating a CV of 10.9% over 31 biological replicates for MND173, 7.2% for MND171 as a high-neopterin control, and 8.3% for MND162 as a low-neopterin control over 6 biological replicates, respectively.

Table 3.5: Neopterin variability in human urine tested by LCMS

Three human urine samples were quantified for neopterin in duplicate (intra-assay) over multiple repeat assays (inter-assay) with varying concentrations of endogenous urinary neopterin.

Sample number	Mean neopterin (ng/mL)	Mean intra-assay variability	Mean inter-assay variability	Repeats
MND162	0.43	4.8%	7.2%	6
MND171	1.5	1.7%	8.3%	6
MND173	0.79	2.5%	10.9%	31

Table 3.6: Neopterin variability in human urine between IBL competition ELISA and LCMS assay

Six human urine samples were quantified for neopterin in duplicate (intra-assay) over three repeat assays (inter-assay) by both IBL competition ELISA and LCMS assays with varying concentrations.

Sample number	Mean neopterin (μmol/mol) ELISA	Mean intra-assay variability ELISA	Mean inter-assay variability. ELISA	Mean neopterin (μmol/L) LCMS	Mean intra-assay variability LCMS	Mean inter-assay variability. LCMS	Repeats
MND90	76.709	4.0%	16.2%	71.11	3.5%	11.1%	3
MND10	67.531	7.4%	3.1%	56.40	2.9%	6.12%	3
MND34	172.34	8.8%	12.1%	119.07	2.9%	15.9%	3
MND91	188.23	6.5%	10.8%	148.84	6.6%	12.96%	3
MND126	312.57	10.2%	14.0%	297.12	0.7%	3.28%	3
MND78	305.88	6.0%	10.7%	330.95	2.2%	12.28%	3

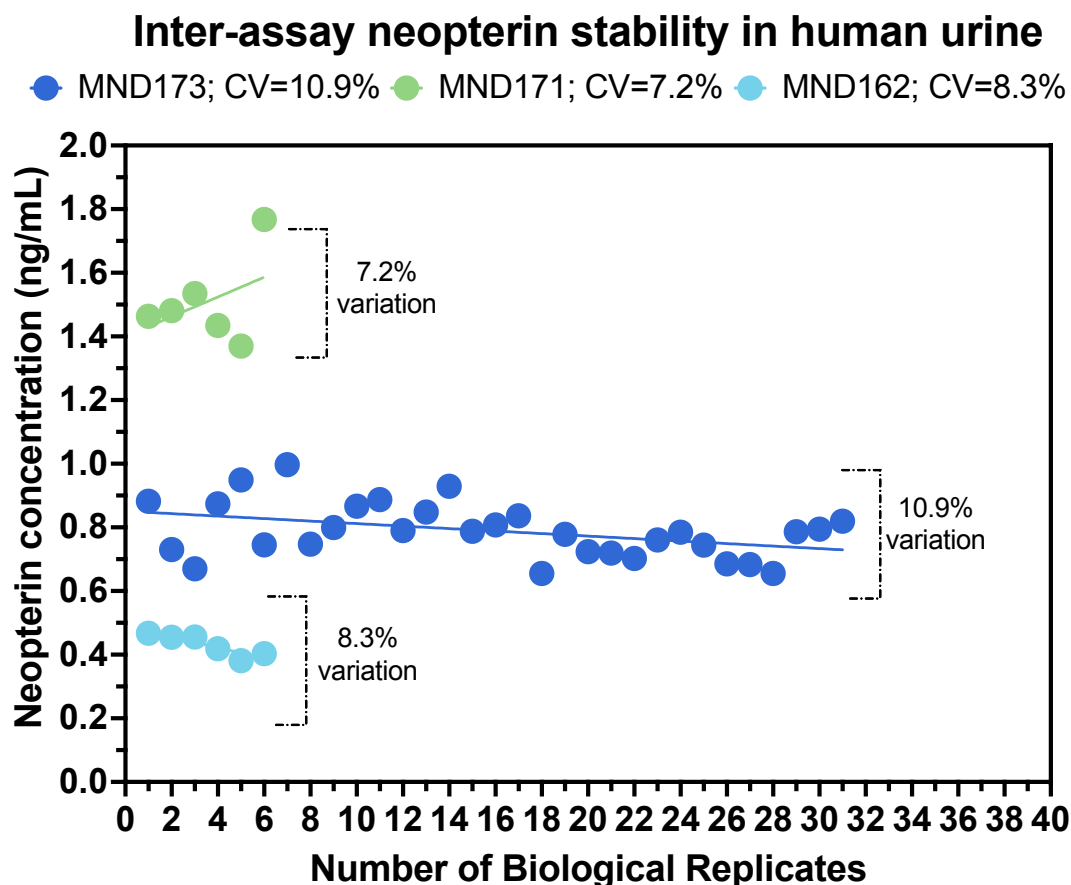


Figure 3.12: Inter-assay stability of endogenous urinary neopterin detected by LCMS over multiple assays

MND173, positive assay control, MND171, high-neopterin control, and MND162, low-neopterin control were repeated over multiple biological assay replicates. Positive assay control sample, MND173, was tested across 31 biological replicates. MND171 and MND162 were tested across 6 biological replicates as high and low-neopterin controls, respectively. MND173 reported an inter-assay CV of 10.9% over 31 repeat assay measurements using LCMS, and MND171 reported a variance of 7.2%, and MND162 reporting a variance of 8.3% over 6 repeat assay measurements. Descriptive statistics were performed in Prism 10.

3.3.7 Variability in neopterin measurement

Day-to-day variability and instrument performance was assessed of this LCMS method for quantifying *D*-neopterin in human urine samples repeatedly. The intra-assay variability within a single run, performed in duplicate, was low. The coefficient of variation of intra-assay variability was reported in **Table 3.5** and **Table 3.6**, with a CV as low as 0.7% (MND126, **Table 3.6**), or as high as MND91 (6.6%, **Table 3.6**). This indicated high precision within runs. In contrast, the inter-assay variability across different days was higher, with a CV as low as 3.28% (MND126, **Table 3.5**), or as high as 15.9% (MND34, **Table 3.6**). This was reflective of the day-to-day fluctuation in instrument performance. This variation was possibly due to factors like source or quadrupole contamination, analytical column contamination, or electrospray ionisation stability. To explore this variability, standard calibration curves were reported in **Figure 3.13** before and after routine instrument servicing. This included cleaning, tuning, calibrating, or replacing contaminated components. **Figure 3.13** reported a 21.5% increase in signal sensitivity post-maintenance, with the signal-to-noise ratio improving markedly from 75 to 258 reflecting a substantial improvement in instrument sensitivity and reduction of noise. Despite this shift in instrument signal sensitivity, both pre- and post-maintenance calibration curves remained highly linear ($r^2=0.999$), indicating that all quantification of *D*-neopterin in human urine remained valid. Endogenous neopterin quantification in urine was performed with isotope-labelled *D*-neopterin- $^{13}\text{C}_5$, normalising the signal of *D*-neopterin to the signal of labelled *D*-neopterin- $^{13}\text{C}_5$. To account for instrument performance losses, sample preparation losses, and matrix effects over time, the ratio of internal standard remained consistent allowing for accurate quantification of *D*-neopterin in human urine throughout this investigation.

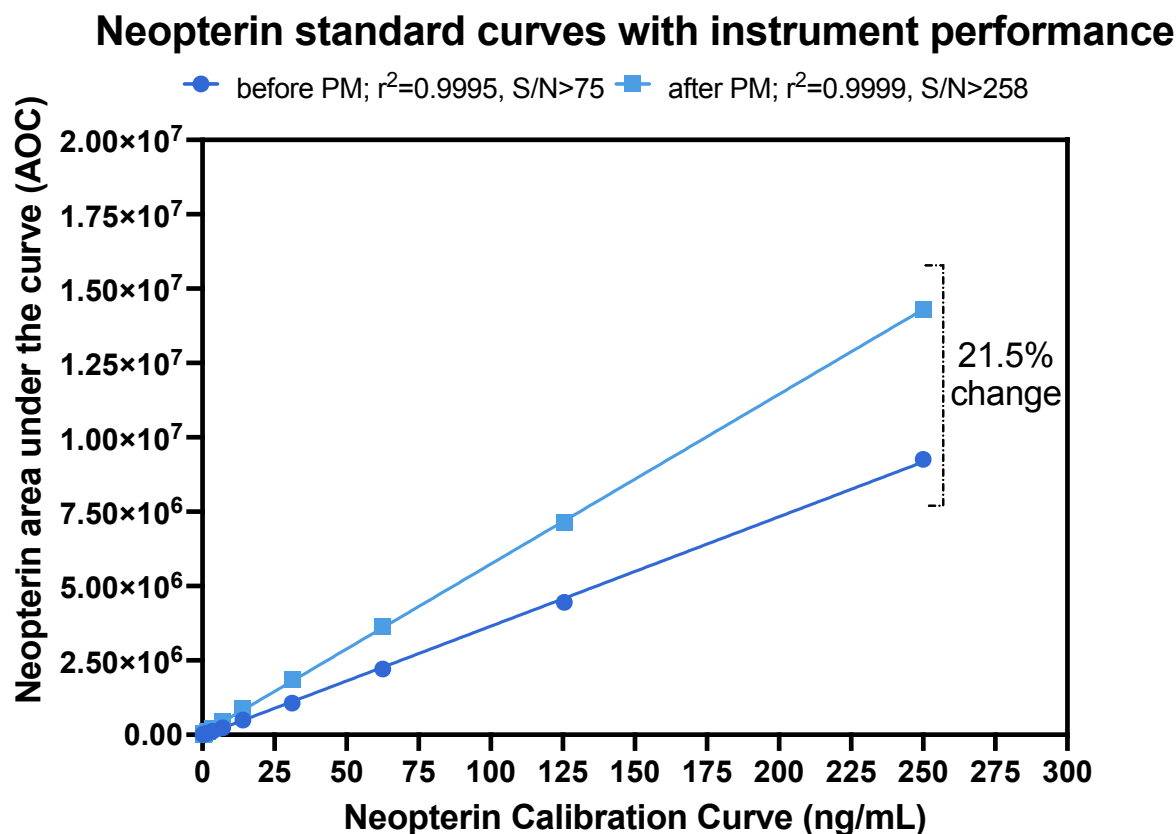


Figure 3.13: *D*-neopterin standard calibration curve changes with instrument performance

The performance of the Altis Triple Quadrupole mass spectrometer reported day-to-day variability. The major source of this variation was by contamination of the ion optics caused by repeated sampling. The instrument was routinely serviced by planned maintenance (PM) to eliminate contaminants and improve sensitivity of quantification. *D*-neopterin standard calibration curves were reported before and after PM, reporting a 21.5% increase in signal sensitivity post-maintenance, with signal-to-noise ratios changing from 75 before PM to 258 after PM. Despite changes in instrument signal sensitivity, both pre- and post- PM standard calibration curves remained highly linear ($r^2=0.999$), indicating that all quantification of *D*-neopterin in human urine remained valid.

3.3.8 Final method performance for neopterin detection in human urine

Performance characteristics of the adapted LCMS method are summarised in **Table 3.7**. Analytical sensitivity, assessed in terms of method detection limits (MDLs) and lower limits of quantification (LLoQ), were comparable to (Burton et al., 2016) for neopterin quantification with an MDL of 220 pg/mL. Pteridine signal response exhibited excellent linearity ($r^2 > 0.999$) over a wide linear range that spanned over three orders of magnitude, over multiple standard curves ($n=10$), with a limit of detection from 0.22 ng/mL to 225 µg/mL at a retention time of ~4.30 minutes. The method was found to be highly sensitive with a LLoQ of 330 pg/mL, being the lower limit to which the analytical response was reproducible ($n=3$) for this study. The lowest calibration standard was 40% of the equivalent LLoQ, such that MDL values are unlikely to change due to instrument performance. The abundance of *D*-neopterin in human urine necessitated an operating range well within the 12-point calibration curve, in the absence of ion suppression up to a concentration of 1 µg/mL in H₂O/MeOH (**Figure 3.9D**, $r^2=0.997$) that was well outside the detection limit of neopterin in human urine. Method accuracy was evaluated with spiked recoveries in a range of urine fold-dilutions which ranged from 73.3% in 100-fold and to 65% in 80-fold diluted urine in a linear fashion (**Figure 3.11A**, $r^2=0.612$). Hence, 100-fold dilution of human urine specimens were used throughout this study design. The method processed excellent inter-assay precision that was reported at 10.9% ($n=31$, **Table 3.5**) over extended testing of biological replicates, and an on average 10.9% for intra-daily precision, that was consistent across high ($n=6$), medium ($n=31$), and low ($n=6$) concentrations of endogenous neopterin in human urine from people with MND.

Table 3.7: Parameters of method performance

Linear range, IDLs, MDLs, LLoQ, retention times, encountered ranges in real urine, using the adapted LCMS and MRM method.

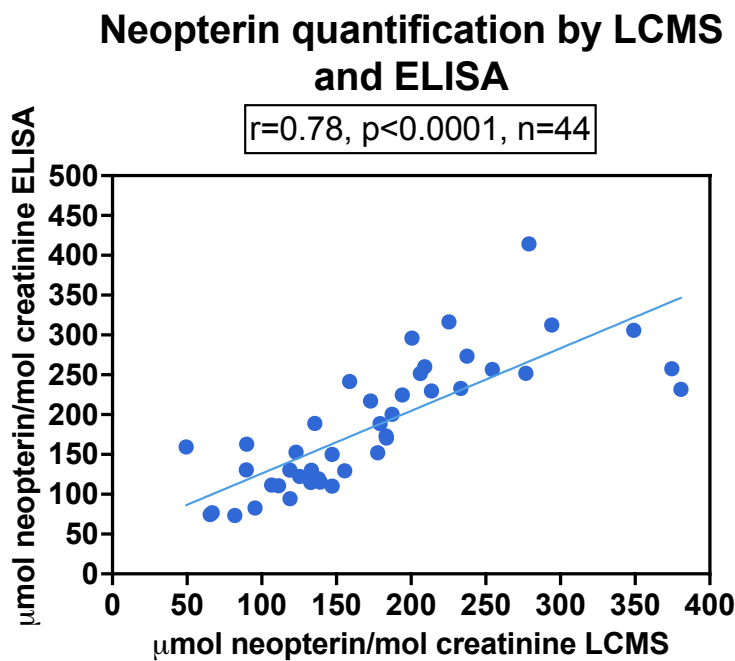
Compound	Linear range (ng/mL)	Linearity r^2	MDL in H ₂ O/MeOH (ng/mL)	MDL in 100-fold urine (ng/mL)	LLoQ (pg/mL)	RT (min)	Encountered range (ng/mL)
<i>D</i> -neopterin	0.22-1000	>0.999	0.025-225	0.025-225	330	4.34	0.33 to 6.9
MDL=method detection limit (ng/mL). LLoQ=lower limit of quantitation (ng/mL). RT=retention time (min). Linear range=standard calibration curve (ng/mL). Encountered range=neopterin quantified in human urine (ng/mL).							

3.3.9 Neopterin quantification in human urine by LCMS and ELISA

The performance of the final LCMS method was characterised by the quantification of urinary neopterin in the same cohort of people with MND (MND, $n=44$) and healthy individuals (HI, $n=19$) from previously published results by IBL ELISA in (Shepherd et al., 2022) described in **Table 3.1**. The accuracy of the LCMS and MRM method was assessed in **Figure 3.14A**. The concentration of urinary neopterin normalised to creatinine, in μmol neopterin/mol creatinine, was compared from detection by IBL competition ELISA to the adapted LCMS method by Pearson's correlation in Prism 10. There was a significant correlation between urinary neopterin detection by ELISA and LCMS in people with MND at baseline (**Figure 3.14A**, $r=0.78$, $p<0.0001$, $n=44$), providing confidence in neopterin detection by LCMS herein.

The concentration of urinary neopterin in μmol neopterin/mol creatinine in people with MND (MND, $n=44$) was compared to healthy individuals (HI, $n=19$) from detection by LCMS in **Figure 3.14B** and compared to published ELISA results in **Figure 3.2A** (MND, $n=44$, $p=0.0004$, 2-way T-test). Baseline urinary neopterin quantified by LCMS was significantly increased in 44 people with MND (174.4 ± 79.2 μmol neopterin/mol creatinine, $p=0.0002$ Welch's T-test) compared to 19 healthy individuals (116.3 ± 38.7 μmol neopterin/mol creatinine). Further, baseline urinary neopterin quantified by IBL competition ELISA was significantly increased in 44 people with MND (184.5 ± 79.1 μmol neopterin/mol creatinine, $p=0.0004$, Welch's T-test) compared to 19 healthy individuals (116.4 ± 58.8 μmol neopterin/mol creatinine). This indicates that both methods independently confirm that urinary neopterin is significantly increased in people with MND at baseline. The consistency between these methods supports the validity and reliability of both assays, performing cross-platform validation to generate reproducible data.

A



B

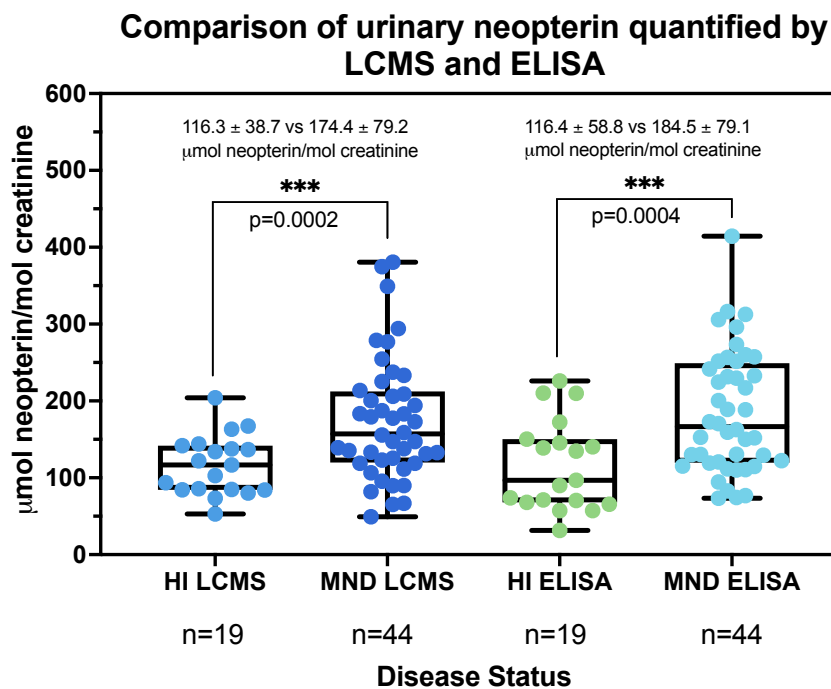


Figure 3.14: Comparison of the urinary neopterin quantified by LCMS and IBL competition ELISA

(A) The quantification of urinary neopterin normalised to creatinine ($\mu\text{mol neopterin/mol creatinine}$) by LCMS was significantly correlated to published ELISA results ($r=0.78, p<0.0001$) in 44 people with MND by Pearson's correlation. In (B) the results from published ELISA results were compared to urinary neopterin quantified by LCMS in 44 people with MND and 19 healthy individuals (HI) at baseline. Neopterin quantified by LCMS ($p=0.0002$) and by inhibition ELISA ($p=0.0004$) was significantly increased in people with MND at baseline.

3.4 Discussion

Through this investigation, a rapid, sensitive, and reproducible liquid chromatography mass spectrometry (LCMS) and multiple reaction monitoring (MRM) method was adapted from (Burton et al., 2016) and optimised for the simultaneous detection of *D*-neopterin and *D*-neopterin- $^{13}\text{C}_5$ in the urine of people with MND. The design of a high-throughput LCMS assay for urinary pteridine detection has not previously been reported to detect and quantify neopterin in the urine of people with MND. The aim of this investigation was to adapt, design, and integrate an LCMS assay for the detection and quantification of urinary neopterin in MND urine to refine and validate the quantification of urinary neopterin as a candidate predictive biomarker of pro-inflammation in people with MND compared to the IBL competition ELISA (Shepherd et al., 2022).

Measurement of neopterin, as we described in (Shepherd et al., 2022), demonstrated that neopterin was poorly prognostic for people with MND in a small cohort study, but did change over disease progression at a measurable rate of 6.8 μmol neopterin/mol creatinine per month from diagnosis. Using a multivariate survival model, urinary neopterin could not add prognostic value beyond what could be determined by the ΔFRS in the clinic. However, the results suggest utility in predicting a pro-inflammatory state in people with MND. Neopterin is a surrogate for interferon-gamma (Murr et al., 2002), and therefore this marker could be predictive for pro-inflammation in people with MND (Berdowska and Zwirska-Korczala, 2001, Molero-Luis et al., 2013). Receptor bound interferon-gamma is rapidly internalised (Anderson et al., 1983), making neopterin as a downstream product of the interferon response a viable candidate for detection. Neopterin levels are related to disease as shown by a significant correlation to the ALSFRS-R at baseline. Thus, urinary neopterin levels could predict people with MND that enter clinical trials in a pro-inflammatory state and stratify those most likely to respond to anti-inflammatory treatments in future clinical trials.

Adding to the already described progression biomarker urinary p75^{ECD} , urinary neopterin also changes over a measurable rate over MND progression. Both urinary p75^{ECD} and urinary neopterin are worthy of being investigated as progression biomarkers in larger cohorts of samples. Urinary neopterin is thus a candidate predictive and progression biomarker that may be used in a panel with urinary p75^{ECD} as a progression biomarker (Shepherd et al., 2017) and serum NfL (Benatar et al., 2024b) as a pharmacodynamic biomarker in MND clinical trials. However, one limitation of this study was the small number of samples measured. Future studies aim to measure urinary neopterin in large cohorts at

baseline and over disease progression using the optimised mass spectrometry and MRM assay to determine the usefulness of neopterin in clinical trials.

We identified that urinary neopterin was associated with older age in people with MND, but when restricted to an age-range below 72 years this association was negligent. Correlation between urinary neopterin and age have been previously reported in individuals under the age of 18, and over the age of 75 (Werner et al., 1987). In this study, restricting the age to individuals under 72 years was sufficient to remove this association. Urinary neopterin was analysed using a Thermo Altis Triple Quadrupole mass spectrometer and an adapted MRM technique from (Burton et al., 2016), capable of the simultaneous detection of *D*-neopterin, *D*-neopterin- $^{13}\text{C}_5$ in a single 9-minute gradient. The Phenomenex Luna Phenyl-Hexyl analytical column was previously found to separate pteridines from other compounds with superior resolution under a flow rate of 0.15-0.40 mL/min, with a mobile phase consisting of 0-0.2% formic acid in water, acetonitrile, and methanol, compared to a LUNA amino column by (Allegri et al., 2012). Stable isotope-labelled internal standard, *D*-neopterin- $^{13}\text{C}_5$, was introduced into the methodology to co-ionise and co-elute with endogenous *D*-neopterin to account for instrument performance losses, sample preparation losses, and matrix ionisation effects during high-throughput sampling of urinary neopterin.

D-neopterin and *D*-neopterin- $^{13}\text{C}_5$ detection and quantification by MRM mass spectrometry were unique to this method using four optimised product ions for each compound. Product ion 206.11 m/z was used for *D*-neopterin confirmation, and product ion 210.13 m/z was used for *D*-Neopterin- $^{13}\text{C}_5$ confirmation for their highest reported signal. This method was validated through the evaluation of spiked-recovery experiments, recovering $73\% \pm 7.1\%$ of spiked-in *D*-neopterin in 100-fold diluted urine, while achieving high reproducibility across multiple assays. The method demonstrated an average intra-assay variability coefficient of 3.1%, and inter-assay (over 6 urine samples) variability coefficient of 10.3% by LCMS. This was comparable to the IBL competition ELISA, reporting an average intra-assay variability coefficient of 7.2%, and inter-assay variability coefficient of 11.2% in the same individuals, indicating that the cross-platform quantification of neopterin was robust and repeatable. The intra-assay precision was excellent (<5%) using LCMS, consistent with published findings for other targeted metabolites quantified in human urine (Arioli et al., 2022, Cao et al., 2015). This suggests consistent results within runs that deterred variability from sample preparation (i.e., pipetting, serial dilutions), instrument performance, or urine heterogeneity. An inter-assay precision of <15% on average is widely accepted in bioanalytical method validation (Food and Drug, 2001). For neopterin measured in all 44 people with MND, the average

inter-assay variability coefficient was $9.0\% \pm 5.8\%$ over 3-6 assays by LCMS, compared to $12.2\% \pm 5.6\%$ by IBL competition ELISA. This indicated reproducible and stable quantification of neopterin in MND urine by LCMS, with marginal improvement, over time and across conditions.

The desired *D*-neopterin standard calibration range was determined by previous studies exploring urinary neopterin by HPLC-MS techniques (Burton et al., 2013), HPLC and fluorescent detection (Lunetta et al., 2020), and by neopterin previously detected by IBL competition ELISA (Shepherd et al., 2022). *D*-neopterin was reported with a linear range of 0.025-1000 $\mu\text{g/L}$, and with an encountered sample range of 115-3,816 $\mu\text{g/L}$ by (Burton et al., 2013) in 100-fold diluted human urine. In comparison, the IBL competition ELISA was limited to a detection limit between 0.034 and 28 $\mu\text{g/L}$ (Shepherd et al., 2022) using a logarithmic response curve that risked misinterpretation at both ends of the curve due to non-linearity. This was a caveat of the IBL competition ELISA having a narrow dynamic range using a logarithmic response curve that was open to the risk of large changes in response (nmol/L neopterin) to small changes in absorbance (450 nm). The 12-point *D*-neopterin calibration curve by LCMS was designed to reach lower detectable limits than the ELISA at 225 ng/mL, taking advantage of a linear calibration curve to reduce baseline noise by mass spectrometry with a signal-to-noise ratio.

Stable isotope internal standard *D*-neopterin- $^{13}\text{C}_5$ was spiked in to each human urine sample and standard calibrant at a desired concentration of 14 ng/mL with an approximate signal of 1.3E^5 (**Figure 10B**), reflecting the average intensity of endogenous neopterin quantified in MND urine (7.27E^4 , **Figure 8B**) and to avoid contributing towards ion suppression with a compound that co-ionises with *D*-neopterin in standards and human urine samples. Both compounds were highly linear, with linear regression coefficients above $r^2 > 0.99$ within the selected analytical range for *D*-neopterin and *D*-neopterin- $^{13}\text{C}_5$. The encountered range of *D*-neopterin quantification in MND urine specimens from this study was 0.033 to 0.69 $\mu\text{g/L}$, comparable to previously reported HPLC-MS, fluorescent detection, or ELISA techniques for urinary neopterin detection (Burton et al., 2013, Burton et al., 2016, Shepherd et al., 2022, Lunetta et al., 2020).

Urinary creatinine was optimised as a compound to be simultaneously detected with neopterin pteridines but was not quantified by LCMS in this investigation. This was because creatinine measurement performed at the same time as neopterin using LCMS was unreliable, due to being 1000-fold higher than that of *D*-neopterin in 100-fold diluted urine,

reported by (Burton et al., 2013). Hence, creatinine quantification was performed independently as per *section 2.5.1*. Further caveats included that the stability and possible inter-conversion of reduced neopterin derivatives in human urine was not explored in this investigation. It was previously reported by (Burton et al., 2016) that although the stability of semi-reduced pteridines is poor, no oxidative pre-treatment of urine specimens was necessary for HPLC-MS/MS detection of *D*-neopterin in the absence of fluorescent detection. However, neopterin is subject to adduct formation in human urine, and the efficiency of fragmentation can be distributed across multiple sodium (Na^+) or ammonium (NH_4^+) ions, for example $[\text{D-neopterin} + \text{Na}]^+$. Ammonium oxalate is commonly used for its sodium scavenging properties by reducing the solubility of ammonium salts (Buttke et al., 2016), which is an approach that may be investigated in future studies to confirm the absence of *D*-neopterin adducts in MND urine.

The detection and quantification of *D*-neopterin in this study may have been complicated by the presence of its structural isomer, *D*-monapterin, which shares the same chemical formula and precursor ion mass to differ in stereochemistry at the 2' position (Feirer et al., 2015). It has been previously reported that due to their isobaric nature and similar biochemical properties, the incomplete chromatographic separation of these two pteridine compounds can be a significant analytical challenge (Burton et al., 2016). Another caveat of this study was the absence of a *D*-monapterin chemical standard to optimise the triple quadrupole mass spectrometer and characterise any chromatographic peaks that may have represented *D*-monapterin. To combat this, the method was optimised in several ways to provide confidence in adequate *D*-neopterin separation and quantitation from the Phenyl-Hexyl analytical column. The separation of *D*-neopterin/*D*-monapterin isomer pairs can be proportionally affected by solvent composition, suggested by (Burton et al., 2016) that high formic acid concentrations above 0.1% v/v promoted separation by more efficient analyte ionisation. Further, *D*-neopterin- $^{13}\text{C}_5$ was optimised to co-elute and co-ionise with *D*-neopterin, providing confidence in interpolating the chromatographic peak produced from *D*-neopterin at 4.31 minutes. This result was confirmed by the linearity of *D*-neopterin from each product ion, confirming that *D*-neopterin was sufficiently targeted and quantitated from this workflow.

This adapted liquid chromatography mass spectrometry method was applied to the urine of 44 people with MND and 19 healthy individuals using the IBL competition ELISA. The results were normalised to urinary creatinine as a renal dilution correction factor to normalise for hydration status in spot urine specimens. Neopterin quantified by the MRM technique

provided a strong and significant correlation to quantification by the IBL competition ELISA, providing robust evidence that urinary neopterin is changing in people with MND by two independent techniques. The advantages of the IBL competition ELISA were in its high-throughput 96-well plate format with great ease of use and low up-front cost, however, the LCMS and MRM assay was advantageous in time efficiency and high-throughput design, using a linear standard curve to achieve a wide dynamic range for sensitive and specific neopterin detection using the *D*-neopterin- $^{13}\text{C}_5$ internal calibrant for neopterin quantification. The aim of this study was to adapt, design, and integrate an LCMS assay for neopterin quantification in MND urine and should be interpreted within the context of the current sample size. Despite the strong concordance between LCMS and ELISA-based quantification of urinary neopterin, cross-platform validation studies with larger cohorts are required and on-going to confirm the robustness, repeatability, sensitivity, and accuracy of LCMS-based neopterin quantification, to provide insight into its clinical utility as a candidate biomarker of pro-inflammation for MND.

CHAPTER 4. URINARY NEOPTERIN QUANTIFICATION AS A BIOMARKER OF MOTOR NEURON DISEASE

4.1 Introduction

Urinary neopterin has been characterised in the urine of people with MND as a candidate predictive biomarker of pro-inflammation (Shepherd et al., 2022). Urinary neopterin was also shown to be a promising candidate biomarker of disease progression in people with MND using an IBL competition ELISA, that requires a more rigorous assay. A novel multiple reaction monitoring (MRM) and mass spectrometry technique has been developed to selectively target and measure neopterin in MND urine. The application of MRM-MS to MND biomarker verification is still an emerging approach. We now aim to use this technique to further validate neopterin as a biomarker for MND in larger sample sizes.

This chapter reports on a larger cohort of people tested to refine urinary neopterin quantification as a biomarker in people with MND that can predict a pro-inflammatory state. The first criteria of a biomarker candidate is to determine its discriminatory value between a disease state and healthy individuals (FDA-NIH, 2021). To achieve this, urinary neopterin was quantified in a larger cohort of people with MND at baseline (i.e., first visit to the neurologist) and in healthy individuals using the mass spectrometry and MRM technique described in *Chapter 3*. This study also completed the measurement of neopterin in a pilot cohort of people with other disease and condition states to get a snapshot of the utility of neopterin as a biomarker candidate of pro-inflammatory processes.

To determine if urinary neopterin levels are prognostic for survival and predictive of pro-inflammation in people with MND, neopterin concentrations need to be measured in larger numbers of samples at baseline and compared to currently available measures of disease prognosis. Indicators of disease prognosis include the ALSFRS-R, Δ FRS, site of disease onset (i.e., bulbar onset MND), gender, and urinary p75^{ECD}, and recently serum NfL (Benatar et al., 2024b). Prognostic biomarkers are baseline characteristics that allow people with MND to be stratified based on their expected survival (Group, 2001), categorising people with MND into groups of short and long survival expectancy in clinical trials. Further, predicting people with high neopterin may indicate a pro-inflammatory state that may be useful as a predictive biomarker of anti-inflammatory therapeutics in phase 2 clinical trials.

This chapter focuses on characterising the utility of urinary neopterin as a biomarker for MND by using mass spectrometry and MRM in larger sample sizes. The comparison of urinary neopterin levels to clinical and neurological characteristics will determine the utility of neopterin as a candidate prognostic and predictive biomarker of pro-inflammation in people with MND.

4.2 Neopterin measurement by mass spectrometry

4.2.1 Clinical characteristics of people with MND and healthy individuals

Initially, ninety people with MND were recruited as per (Shepherd et al., 2017) and described in *Chapter 2*, with individuals diagnosed as either 'clinically definite' or 'clinically probable' MND in accordance with the El Escorial Criteria (Brooks et al., 2000) of MND diagnosis. From this initial sample size, assortment of inclusion criteria selected 76 people with MND between the ages of 40 to 85 years of age from baseline, and with a controlled hydration status of 0.3 – 3.0 mg/mL urinary creatinine to standardise urinary dilutions from sport urine samples. Further, sixty-nine healthy individuals were recruited for this study locally and internationally, which was reduced to thirty-nine age-matched healthy individuals to meet the inclusion criteria in *section 2.1.1* for this study.

Urine collection and neurological assessment was completed at baseline, and disease characteristics of 76 people with MND was reported in **Table 4.1**. 46 men (61%) and 30 women (39%) presented with MND, and the site of disease onset varied in the baseline cohort having 29 people described with bulbar onset, and the remaining 47 described with limb onset disease. 97% of the study participants had sporadic disease, with a median baseline age of 67.5 years. The time between symptom onset and MND diagnosis ranged up to 41.0 months, with an average diagnostic delay of 10.1 ± 8.1 months. Within this cohort, 74 individuals died, and 1 individual was dependent on permanent assisted ventilation (PAV), within an average time of 19.3 ± 14.3 months. The estimated decrease in ALSFRS-R (Δ FRS) between symptoms and baseline was between 0.31 and 0.88 points per month. Most MND was sporadic ($n=74$, 97%), with no family history. Genetic mutations were identified in two patients, one with a C9orf72 repeat expansion, and one other with an E101G (E100G) mutation in the SOD1 gene.

18 healthy individuals were recruited locally from the 4th generation MND clinic (Adelaide, South Australia), with an additional 12 individuals from Macquarie University (Sydney, Australia), 9 individuals recruited from overseas cohorts from the University of Miami (Miami, USA) and PreModi (pre-symptomatic MND) study healthy individuals (Germany, courtesy of Paul Lingor). The total cohort of 39 healthy individuals consisted of 17 (44%) women and 22 (56%) men with a median age of 64.5 years described in **Table 4.1**. Individuals with impaired kidney function and/or diabetes were excluded from this study.

Table 4.1: Baseline cohort of study participants for neopterin detection by LCMS

All disease characteristics and clinical information for people with MND and healthy individuals at baseline were reported. Abbreviations: ALSFRS-R: revised amyotrophic lateral sclerosis functional rating scale. Δ FRS: estimated rate of progression (change in ALSFRS-R per month from symptom onset to baseline). Range: 25th–75th percentile.

Disease Characteristic		Healthy Individuals (n=39)	People with MND (n=76)
Gender (Male, Female)	Male, (%)	22, (56%)	46, (61%)
Site of onset (Bulbar, Limb)	Bulbar, (%)	N/A	29, (38%)
Age at symptoms (years)	Mean $\pm$ SD (Range)*	N/A	65.0 $\pm$ 10.6 (57.3 – 74.5)
Age at diagnosis (years)	Mean $\pm$ SD (Range)*	N/A	65.9 $\pm$ 10.6 (58.4 – 74.5)
Age at sample (years)	Mean $\pm$ SD (Range)*	62.3 $\pm$ 10.7 (55.0 – 70.0) NS	66.3 $\pm$ 10.7 (58.8 – 75.1) ^{NS}
Baseline ALSFRS-R	Mean $\pm$ SD (Range)*	48	40.0 $\pm$ 4.5 (37.0 – 44.0)
Δ FRS	Mean $\pm$ SD (Range)*	N/A	0.66 $\pm$ 0.48 (0.31 – 0.88)
Diagnostic delay (months)	Mean $\pm$ SD (Range)*	N/A	10.1 $\pm$ 8.1 (5.1 – 11.7)
Time to death from sample	Mean $\pm$ SD (Range)*	N/A	19.3 $\pm$ 14.3 (8.7 – 25.6)
Deceased	Deceased, (%)	N/A	75, (99%)
Disease phenotype (Familial, Sporadic)	Familial, (%)	N/A	2, (3%)
Riluzole treatment	Riluzole, (%)	N/A	56, (74%)
*Range is 25 th to 75 th percentiles. Values presented as Mean $\pm$ SD (range). NS: $p=0.061$, unpaired T-test with Welch's correction between MND and HI. Also, Δ FRS=48 – (ALSFRS-R at sample collection)/(time from symptom onset) (Kimura et al., 2006).			

4.2.2 Urinary neopterin in people with MND and healthy individuals at baseline

The adapted pterinomic workflow from *Chapter 4* was applied to clinical urine specimens from 76 people with MND and 39 healthy individuals, and urinary neopterin as a prognostic biomarker of MND was investigated at baseline. Using a 100-fold dilution of urine specimens, the encountered concentration range for neopterin at baseline ranged from 380 pg/mL (1.5 nM) to 3.20 ng/mL (12.6 nM) in 76 people with MND, and from 130 pg/mL (0.51 nM) to 6.9 ng/mL (27.3 nM) in 39 healthy individuals (HI). Urinary neopterin levels were significantly higher in people with MND at baseline compared to healthy individuals after standardisation of urinary dilutions to urinary creatinine (nmol/L). People with MND reported an average ($\pm$ SD) of 170.7 ± 73.2 μ mol neopterin/mol creatinine, and healthy individuals reported a quantity of 135.2 ± 44.7 μ mol urinary neopterin/mol creatinine. An independent unpaired T-test reported a significant difference between people with MND and healthy individuals (**Figure 4.1A**, $p=0.0017$).

The sensitivity and specificity of neopterin in people with MND was examined by receiver operating characteristic (ROC) analysis. Using the AUC, the cut-off levels for sensitivity and specificity of the ROC curve were determined using the Youden index to plot the true positive rate (sensitivity) against the false positive rate (1-specificity) (Ruopp et al., 2008). ROC analysis using the AUC indicated that urinary neopterin modestly distinguished 76 people with MND from 39 healthy individuals 63% of the time ($p=0.0252$, 95% CI: 0.53-0.73), with 52% sensitivity (**Figure 4.1B**). To further investigate any differences in urinary neopterin variability within the cohort of people with MND, study participants were categorised into subgroups and normalised neopterin concentration was compared between bulbar and limb onset MND, above and below the median age at baseline (± 67.5 years), and between males and females. Since disease outcomes and prognosis have previously shown to change between the site of disease onset and gender (Chiò et al., 2009), and neopterin has increased with age when detected by ELISA in MND (Shepherd et al., 2022), these clinical characteristics were re-evaluated in a larger cohort to determine if disease onset, age at baseline, and gender were confounding factors driving MND prognosis.

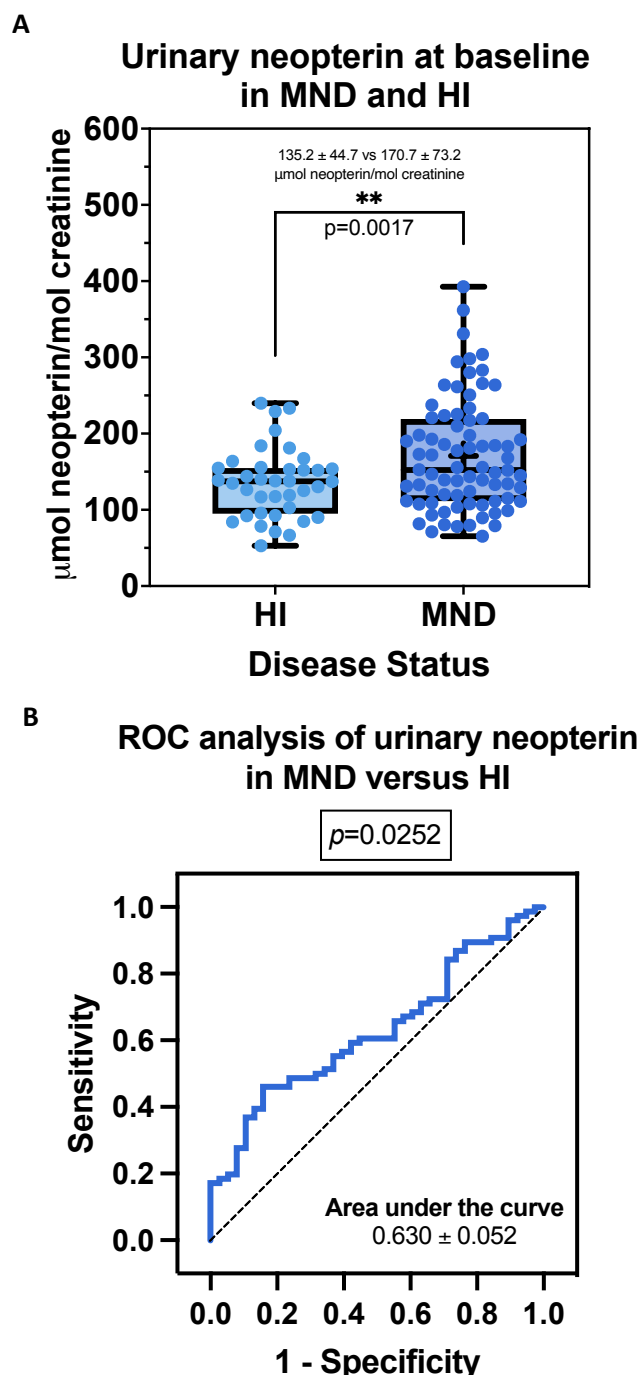


Figure 4.1: Urinary neopterin levels in people with MND and healthy individuals quantified by LCMS

(**A**) Urinary neopterin levels were quantified in people with MND (MND, ●, $n=76$) and healthy individuals (HI, ●, $n=39$) at baseline using the optimised LCMS workflow from *Chapter 4* (2 technical replicates per assay, minimum 3 assays per sample). Urinary neopterin was normalised to creatinine, and a significant difference in neopterin measurement between healthy individuals ($135.2 \pm 44.7 \mu\text{mol neopterin/mol creatinine}$) and people with MND ($170.7 \pm 73.2 \mu\text{mol neopterin/mol creatinine}$) was reported ($p=0.0017$) by independent T-test with unequal SD (Welch's T-test). (**B**) ROC analysis of urinary neopterin by LCMS reported a modest ability to discriminate between people with MND and healthy individuals using LCMS, with an area under the curve of 0.630 ± 0.052 , indicating a specificity of 63%, with 52% sensitivity ($p=0.0252$, Wilson–Brown method). All statistical analyses performed in Prism 10.

4.2.3 Relationship of neopterin to the ALSFRS-R

Figure 4.2A demonstrates a significant correlation between the measurement of urinary neopterin and ALSFRS-R scores for 76 people with MND at baseline ($r=-0.251$, $p=0.029$, Pearson's correlation), reporting increasing levels of urinary neopterin as the ALSFRS-R score decreases and symptoms of disease progressively worsen. Given that urinary neopterin was measured in spot urine samples and required standardisation to hydration status, urinary creatinine and osmolality as renal dilution correction factors were compared to ALSFRS-R scores at baseline. Urinary osmolality was compared to urinary creatinine within its detectable range of 0.3 and 3.0 mg/mL creatinine in **Figure 4.2B**. Urinary creatinine and osmolality reported a significant correlation in 76 people with MND ($r=0.70$, $p<0.0001$, Pearson's correlation). The average concentration of urinary creatinine was 1.0 ± 0.42 mg/mL, and osmolality was 608 ± 225 mOsm/kg (**Table 4.1**), and urinary creatinine levels in 76 people with MND shared no significant correlation with ALSFRS-R score ($r=-0.055$, $p=0.64$) in **Figure 4.2C**, and urinary osmolality shared no significant correlation to ALSFRS-R scores ($r=-0.012$, $p=0.92$) in **Figure 4.2D** by Pearson's correlation. This reinforced that the significance of urinary neopterin as a candidate biomarker of MND prognosis, and its relationship to clinical characteristics, was not being driven by renal dilution correction factors in a muscle wastage disease.

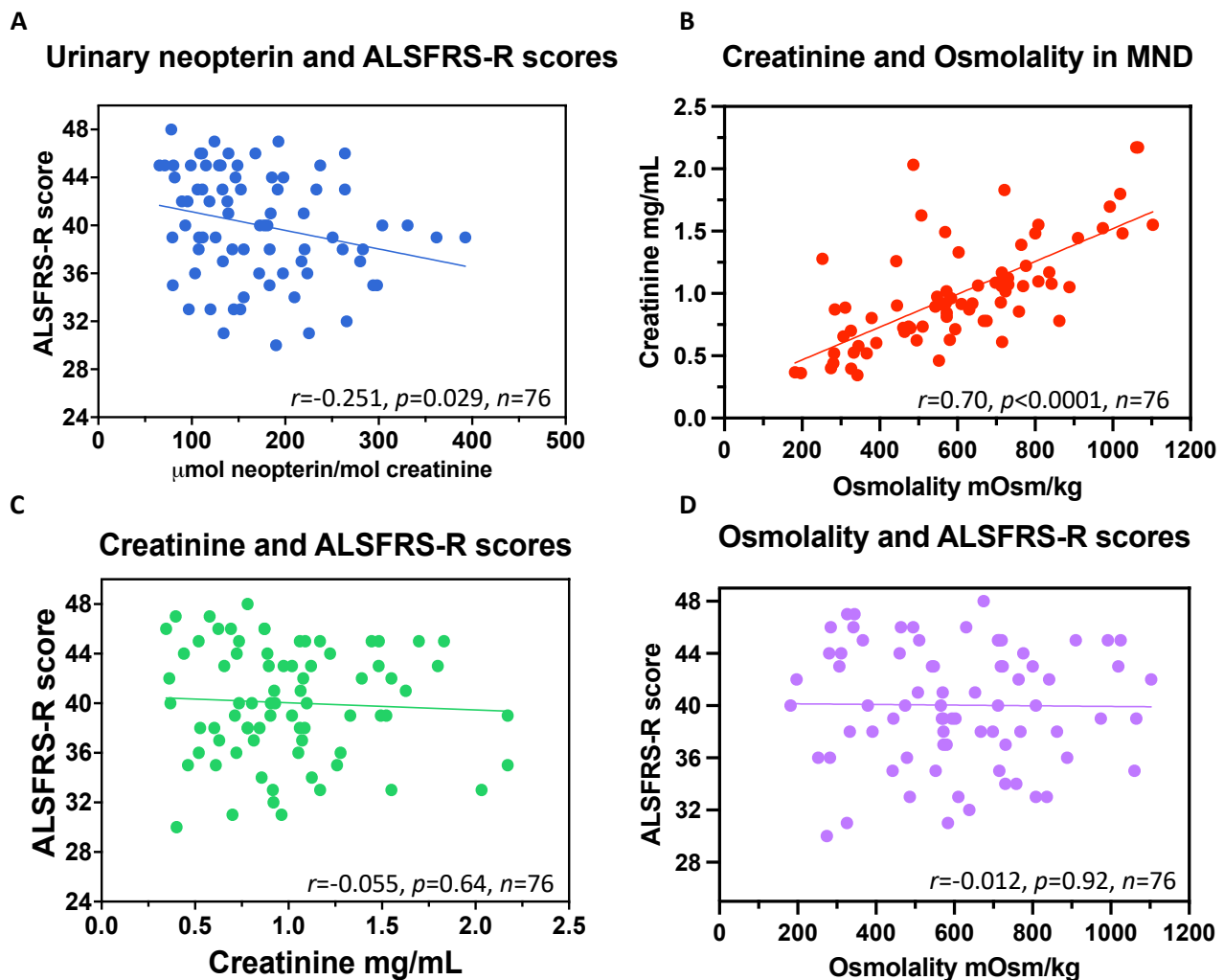


Figure 4.2: Urinary neopterin compared to ALSFRS-R scores, creatinine, and osmolality

(A) Urinary neopterin levels were quantified at baseline in people with MND ($\bullet$, $n=76$). There was a significant negative correlation between urinary neopterin levels and ALSFRS-R scores ($r=-0.251$, $p=0.029$). (B) There was a significant correlation between urinary creatinine and osmolality in people with MND ($\bullet$, $n=76$) as a renal dilution correction factor ($r=0.70$, $p<0.0001$). (C) There was no significant correlation between urinary creatinine and ALSFRS-R scores ($\bullet$, $r=-0.055$, $p=0.64$), or in (D) between urinary osmolality and ALSFRS-R scores in people with MND ($\bullet$, $r=-0.012$, $p=0.92$). Statistical significance was determined using Pearson's correlation, Prism 10.

4.2.4 Clinical characteristics between site of disease onset and gender

The site of disease onset and gender of people with MND were explored in this cohort (**Table 4.1**). There was no statistical difference in urinary neopterin measurement between 47 people with limb onset MND and 29 people with bulbar onset MND ($p=0.259$, Welch's T-test), reported in **Figure 4.3A**. Further, urinary neopterin was not significantly changed between 46 males and 30 females with MND ($p=0.826$, Welch's T-test) in **Figure 4.3B**. Correlation to the ALSFRS-R was also explored in people with limb and bulbar onset MND, and between males and females with MND. Limb onset MND was not significantly correlated to ALSFRS-R scores ($r=-0.20$, $p=0.175$) in **Figure 4.4A**, or in bulbar onset MND in **Figure 4.4B** ($r=-0.34$, $p=0.075$, Pearson's correlation). Neopterin in males with MND was not significantly correlated to ALSFRS-R scores in **Figure 4.4C** ($r=-0.25$, $p=0.094$), nor did females in **Figure 4.4D** ($r=-0.26$, $p=0.165$, Pearson's correlation) when compared to ALSFRS-R scores at baseline.

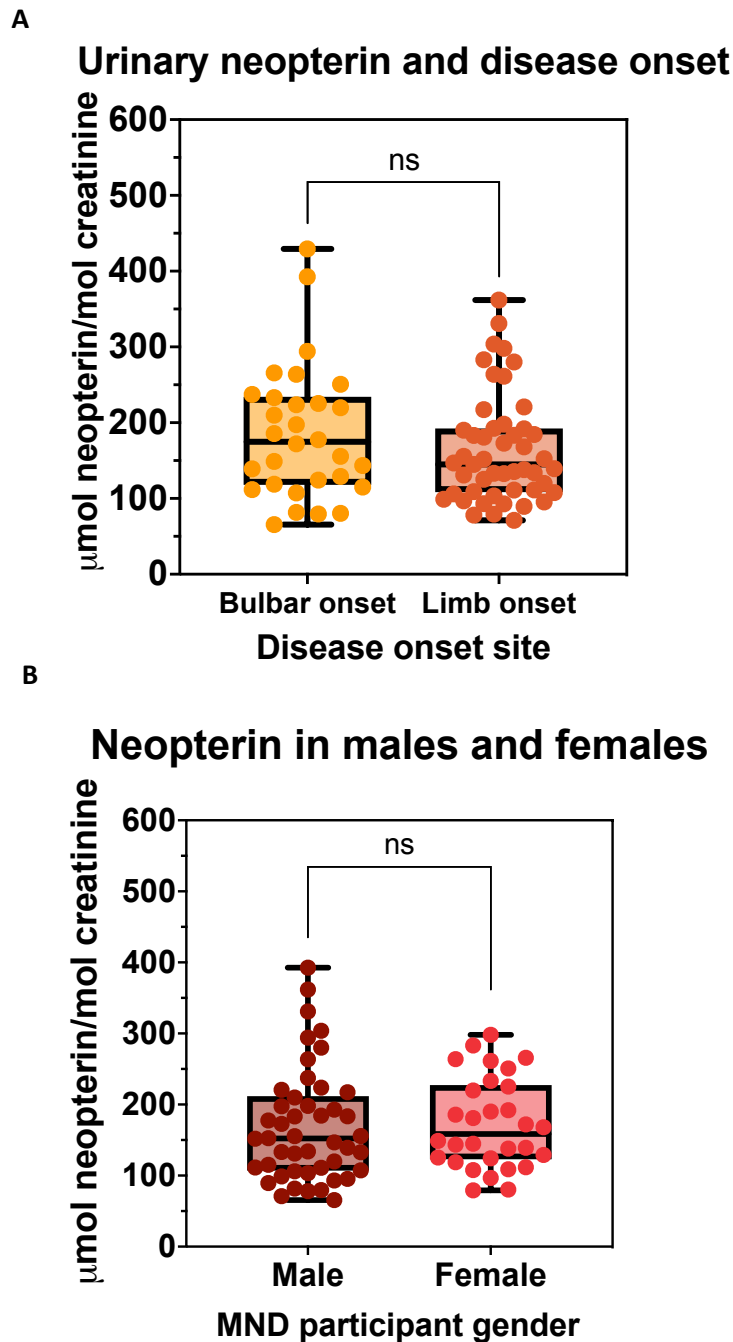


Figure 4.3: Comparing site of disease onset and gender to urinary neopterin at baseline in people with MND

(**A**) Urinary neopterin levels were quantified in people with bulbar onset (●, $n=29$) compared to limb onset MND (●, $n=47$), indicating no significant difference (ns) by Independent T-test with equal SD ($p=0.259$). (**B**) Urinary neopterin levels were quantified in males (●, $n=46$) and compared to females (●, $n=30$) with MND, indicating no significant difference (ns) by Independent T-test with equal SD $p=0.826$). Statistical significance was determined using Prism 10.

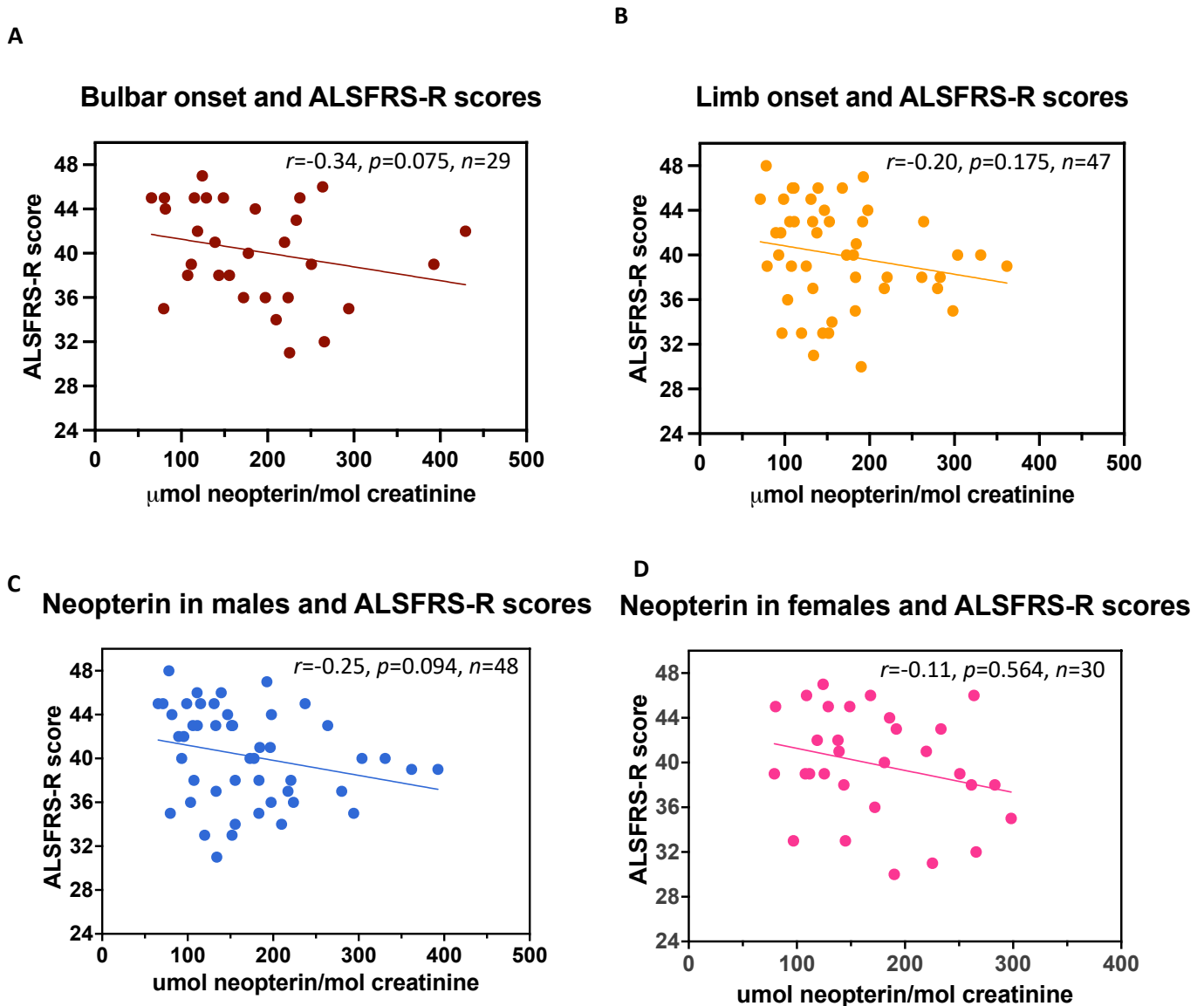


Figure 4.4: Urinary neopterin in type of onset (bulbar and limb) and sex (males and females) compared to ALSFRS-R

Urinary neopterin levels were observed in people with limb ($n=47$) and bulbar onset ($n=29$) MND, and between males ($n=48$) and females ($n=30$). **(A)** In 29 people with bulbar onset MND, neopterin measurement was not significantly correlated to ALSFRS-R scores ($\bullet$, $r=-0.34$, $p=0.075$) and in **(B)** no significant correlation was observed between urinary neopterin measurement and ALSFRS-R scores in 47 people with limb onset MND ($\bullet$, $r=-0.20$, $p=0.175$). **(C)** No significant correlation was reported between urinary neopterin and ALSFRS-R scores in 48 males with MND ($\bullet$, $r=-0.25$, $p=0.094$), and in **(D)** no significant correlation was reported between urinary neopterin and ALSFRS-R scores in 30 females with MND ($\bullet$, $r=-0.26$, $p=0.165$). Statistical significance was determined using Pearson's correlation in Prism 10

4.2.5 Clinical characteristics between median age at sample collection

Urinary neopterin was compared to age in 76 people with MND at baseline to determine if age was a clinical covariate. The relationship between urinary neopterin and age was investigated in **Figure 4.5A**, reporting a significant correlation ($p=0.0006$, $r=0.39$). This validated previous findings from (Shepheard et al., 2022), indicating that age is correlated to neopterin levels in people with MND. The measurement of urinary neopterin was compared in 76 people with MND above and below the median age of 67.5 years in **Figure 4.5B**, reporting an average ($\pm$ SD) concentration of neopterin below the median age of 147.7 ± 61.3 μmol neopterin/mol creatinine, compared to 193.7 ± 77.6 μmol neopterin/mol creatinine above the median. Urinary neopterin levels were significantly different between 38 people with MND that were below, and 38 people with MND that were above the median age of 67.5 years ($p=0.0055$, Welch's T-test). Interestingly in 39 healthy individuals, no significant correlation was reported between age and neopterin at sample collection in **Figure 4.5C** ($p=0.080$, $r=0.28$), and neither when testing 26 healthy individuals above, and 14 below the median age of 67.5 years ($p=0.48$, Welch's T-test) in **Figure 4.5D**.

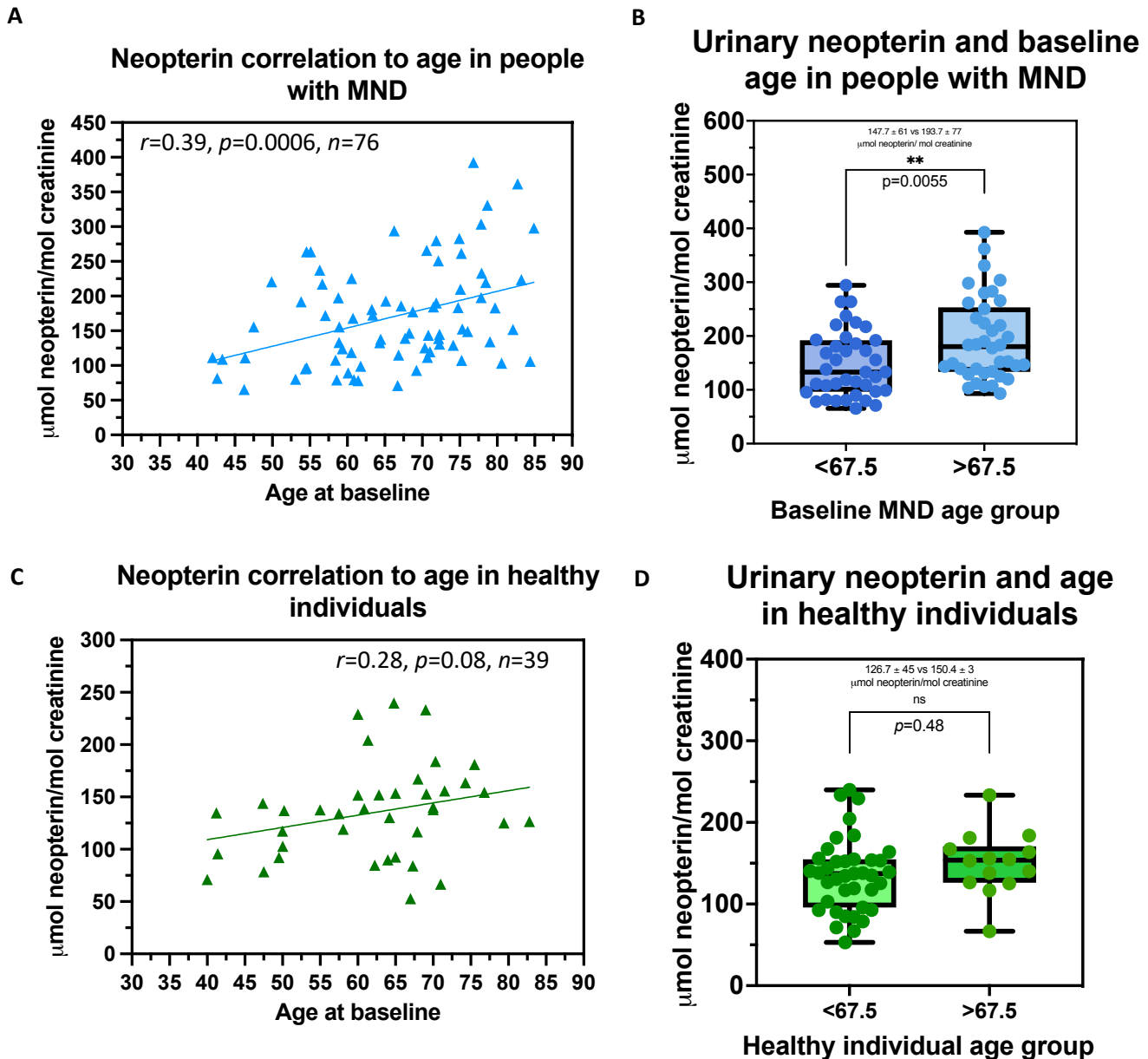


Figure 4.5: Comparing age at collection to urinary neopterin at baseline in people with MND

(A) Urinary neopterin levels were significantly correlated to the age of 76 people with MND at baseline by Pearson's correlation ($r=0.39, p=0.0006$). In (B), urinary neopterin quantified in people with MND at baseline was separated above (●, $n=39$) and below (●, $n=39$) the median age of 67.5 years. A significant difference was reported in neopterin measurement between the age of 76 people with MND above and below the median ($p=0.0055$) by independent T-test with unequal SD (Welch's T-test). This was repeated in 39 HI (C), showing no significant correlation to age at baseline by Pearson's correlation ($r=0.28, p=0.08$), and no change (D) above and below the median age of 67.5 years ($p=0.48$). Statistical significance was determined using Prism 10.

4.2.6 Prognostic factors at baseline

Table 4.2 outlines that urinary neopterin measurements were significantly correlated with ALSFRS-R scores. However, in people with MND, significant correlations were found between neopterin concentrations and the age of people with MND at baseline. Importantly, urinary neopterin concentrations were not correlated to the age or gender of healthy individuals that participated in this study. With the aim of refining neopterin quantification by LCMS as a candidate biomarker of predicting pro-inflammation and prognosis (Shepherd et al., 2022), Cox proportional hazards models were performed in SPSS to compare neopterin to known prognostic factors in people with MND, as done in previous studies (Shepherd et al., 2017).

Univariate Cox-Regression survival analyses were performed, comparing known prognostic factors of MND in 76 people (across all ages) with MND. In this study, 75 people with MND had reached end-stage disease or PAV. Prognostic factors were individually tested in **Table 4.3**, and the analyses included the prognostic variables; site of disease onset (bulbar compared to limb onset), change in ALSFRS-R per month from symptom onset (Δ FRS), gender (male and females), age at sample collection, baseline urinary p75^{ECD}, and baseline urinary neopterin. **Table 4.3** demonstrated that age at baseline (HR: 1.03, 95% CI: 1.01-1.06, $p=0.018$), Δ FRS (HR: 2.97, 95% CI: 1.91-4.62, $p<0.001$), urinary neopterin (HR: 1.004, 95% CI: 1.001-1.008, $p=0.018$), and urinary p75^{ECD} (HR: 1.15, 95% CI: 1.06-1.25, $p<0.001$) were all prognostic indicators of disease survival by univariate analysis.

To make an interpretation of the impact these variables had on predicting prognosis together, a multivariate Cox-Regression analysis was performed where more than one predictor was included in the model. In the same cohort of 75 people with MND, **Table 4.3** demonstrated that only the Δ FRS (HR: 3.69, 95% CI: 2.17-6.27, $p<0.001$), urinary p75^{ECD} (HR: 1.12, 95% CI: 1.02-1.24, $p=0.024$), and bulbar onset (HR: 1.91, 95% CI: 1.12-3.26, $p=0.017$) were significant predictors of prognosis in a multivariate model. Disease onset was aligned with the literature that prognosis can differ based on limb or bulbar onset MND (Chiò et al., 2009). This indicated that gender, baseline age, or neopterin measurements by LCMS did not provide any greater prognostic value compared to the significance of Δ FRS scores in the clinic, site of disease onset, or urinary p75^{ECD} concentrations as predictors of survival in people with MND.

Table 4.2: Correlation of urinary neopterin to clinical characteristics at baseline

Urinary neopterin measurements were compared by Pearson's correlation and Welch's T-tests to characteristics of disease. These characteristics included of gender, site of disease onset, ALSFRS-R and Δ FRS scores, age at various time points of disease, diagnostic delay, genetic involvement, and the treatment by Riluzole in 76 people with MND and 39 healthy individuals.

Disease Characteristic	Significance (<i>p</i> value)
Gender: MND	^B 0.826
Gender: HI	^B 0.118
Age: MND	^A 0.0006*
Age: HI	^A 0.084
Site of onset	^B 0.259
Symptom onset: age	^A 0.0004*
Diagnosis: age	^A 0.0006*
Baseline ALSFRS-R	^A 0.0287*
Δ FRS	^A 0.0349*
Diagnostic delay (months)	^A 0.256
Genetic involvement (familial vs sporadic)	^B 0.615
Riluzole treatment administered	^B 0.466
A=Pearson's correlation. B=Welch's T-test. *Age in MND from baseline, symptom onset, diagnosis, ALSFRS-R scores, and Δ FRS scores all significant to neopterin at $p<0.05$ threshold	

Table 4.3: Baseline cohort and survival by Cox proportional hazards models for urinary neopterin

By univariate Cox-Regression analysis, urinary p75^{ECD} (HR: 1.15, 95% CI: 1.06-1.25, $p < 0.001$), neopterin (HR: 1.00, 95% CI: 1.00-1.01, $p = 0.016$), Δ FRS (HR: 3.01, 95% CI: 1.95-4.66, $p < 0.001$), and age at sample collection (HR: 1.03, 95% CI: 1.00-1.05, $p = 0.036$) were prognostic for survival in 75 people with MND at baseline. By multivariate Cox-Regression analysis, disease onset (HR: 1.78, 95% CI: 1.09-2.91, $p = 0.022$), Δ FRS (HR: 3.70, 95% CI: 2.2-6.1, $p < 0.001$), and p75^{ECD} (HR: 1.12, 95% CI: 1.02-1.23, $p = 0.022$) were prognostic for survival.

	Univariate Cox-Regression				Multivariate Cox-Regression			
			95% Confidence Interval				95% Confidence Interval	
Parameter	<i>p</i> value	Hazard ratio	Upper	Lower	<i>p</i> value	Hazard ratio	Upper	Lower
Gender	0.580	1.145	0.709	1.847	0.740	1.098	0.631	1.911
Disease onset	0.131	1.445	0.896	2.330	0.022	1.778	1.086	2.912
Baseline age	0.036	1.028	1.002	1.054	0.117	1.023	0.994	1.051
Δ FRS	<0.001	3.012	1.947	4.659	<0.001	3.693	2.221	6.141
Neopterin	0.016	1.004	1.001	1.008	0.609	1.001	0.997	1.005
p75 ^{ECD}	<0.001	1.148	1.059	1.245	0.022	1.119	1.017	1.232

4.2.7 Age at baseline as a prognostic factor in MND

Age was correlated to urinary neopterin levels in people with MND (**Figure 4.5A-B**), but not in healthy individuals (**Figure 4.5C-D**). This is an observation that has previously been reported in neopterin detection in people with MND (Shepherd et al., 2022) that needed to be addressed in this cohort. Univariate Cox proportional hazards analysis also indicated that age at sample collection was a significant predictor of survival at baseline (**Table 4.4**). Therefore, age was filtered for a maximum of 78 years at baseline for the remaining survival analyses similar to (Shepherd et al., 2022). This reduced the number of study participants to 65 people with MND within a range of 42 to 78 years of age at baseline. The disease characteristics of 65 people with MND and 39 healthy individuals were reported in **Table 4.4**. The cohort was divided between 37 men (57%) and 28 women (53%), with 26 people described as having bulbar onset, and 39 people with limb onset disease. The average age of the cohort was 63.8 years, which was age-matched to the 39 healthy individuals previously described with an average age of 62.3 years ($p=0.468$, Welch's T-test). 98% of participants had sporadic disease, with only one individual presenting with a C9orf72 repeat expansion gene mutation. The decrease in ALSFRS-R (Δ FRS) between symptom onset and baseline was between 0.30 – 0.83 points per month, and within this cohort, 64 people reached end-stage disease by PAV dependency or death, with an average disease duration of 20.7 ± 14.8 months. Baseline age was no longer a significant prognostic factor driving survival by univariate (HR: 1.02, 95% CI: 0.99-1.05, $p=0.30$) or multivariate (HR: 1.01, 95% CI: 0.98-1.04, $p=0.59$) Cox proportional hazards models in **Table 4.5**. Herein, the cohort of 65 people with MND within this age range described in **Table 4.4** was used to assess neopterin as a candidate prognostic marker amongst other clinical and neurological characteristics, without age contributing as a confounding of disease survival.

Table 4.4: Baseline cohort of study participants between 42-78 years of age

All disease characteristics and clinical information for 65 people with MND and 39 healthy individuals at baseline between 42-78 years of age were reported. Abbreviations: ALSFRS-R, revised amyotrophic lateral sclerosis functional rating scale. Δ FRS, estimated rate of progression (change in ALSFRS-R per month from symptom onset to baseline). Results reported as described, range is 25th–75th percentile.

Disease Characteristic		Healthy Individuals (n=39)	People with MND (n=65)
Gender (Male, Female)	Male, (%)	22, (56%)	37 Male, 28 Female
Site of onset (Bulbar, Limb)	Bulbar, (%)	N/A	26 Bulbar, 39 Limb
Age at symptoms (years)	Mean $\pm$ SD (Range)*	N/A	62.5 $\pm$ 9.3 (56.3 – 70.4)
Age at diagnosis (years)	Mean $\pm$ SD (Range)*	N/A	63.4 $\pm$ 9.4 (57.3 – 71.3)
Age at sample (years)	Mean $\pm$ SD (Range)*	62.3 $\pm$ 10.7 (55.0 – 70.0) ^{NS}	63.8 $\pm$ 9.5 (57.8 – 71.9) ^{NS}
Baseline ALSFRS-R	Mean $\pm$ SD (Range)*	48	40.5 $\pm$ 4.5 (38.0 – 45.0)
Δ FRS	Mean $\pm$ SD (Range)*	N/A	0.63 $\pm$ 0.49 (0.30 – 0.83)
Diagnostic delay (months)	Mean $\pm$ SD (Range)*	N/A	10.2 $\pm$ 8.4 (5.2 – 11.6)
Time to death from sample	Mean $\pm$ SD (Range)*	N/A	20.7 $\pm$ 14.8 (9.6 – 28.0)
Deceased	Deceased, (%)	N/A	57 Deceased, 8 Alive
Disease phenotype (Familial, Sporadic)	Familial, (%)	N/A	64 Sporadic, 1 Familial
Riluzole treatment	Riluzole, (%)	N/A	47 Riluzole, 18 None
*Range is 25 th to 75 th percentiles. Values presented as Mean $\pm$ SD (range). NS: $p=0.468$, unpaired T-test with Welch's correction between MND and HI. Also, Δ FRS=48 – (ALSFRS-R at sample collection)/(time from symptom onset) (Kimura et al., 2006).			

4.2.8 Prognostic factors of disease in age-restricted cohort

Table 4.5 reported by univariate Cox proportional hazards analysis that the Δ FRS (HR: 3.03, 95% CI: 1.91-4.82, $p < 0.001$), urinary neopterin (HR: 1.005, 95% CI: 1.001-1.010, $p = 0.019$), and urinary p75^{ECD} (HR: 1.14, 95% CI: 1.05-1.25, $p = 0.003$) were prognostic factors for disease survival in 65 people with MND at baseline. The multivariate Cox proportional hazards model examined all clinical covariates together, reporting that disease onset (HR: 1.80, 95% CI: 1.05-3.10, $p = 0.033$), Δ FRS (HR: 3.91, 95% CI: 2.23-6.84, $p < 0.001$), and urinary p75^{ECD} (HR: 1.13, 95% CI: 1.02-1.25, $p = 0.020$) predicted survival, indicating that in this cohort only disease onset and urinary p75^{ECD} added prognostic value beyond what can be determined based on the Δ FRS in 65 people with MND at baseline. Clinical covariates including gender, age at sample collection, and urinary neopterin measurements by LCMS did not provide greater prognostic value compared to the significance of disease onset, Δ FRS, and urinary p75^{ECD} recorded by the clinician at baseline as predictors of survival in people with MND.

Table 4.5: Baseline cohort and survival by Cox proportional hazards models for neopterin with age range between 42-78 years of age

In 65 people with MND at baseline univariate Cox proportional hazard analysis reported clinical factors predictive of survival. Δ FRS (HR: 3.03, 95% CI: 1.91-4.82, $p < 0.001$), urinary neopterin (HR: 1.005, 95% CI: 1.001-1.010, $p = 0.019$), and urinary p75^{ECD} (HR: 1.14, 95% CI: 1.05-1.25, $p = 0.003$) were prognostic factors for disease survival. By multivariate Cox proportional hazards model, disease onset (HR: 1.80, 95% CI: 1.05-3.10, $p = 0.033$), Δ FRS (HR: 3.91, 95% CI: 2.23-6.84, $p < 0.001$), and urinary p75^{ECD} (HR: 1.13, 95% CI: 1.02-1.25, $p = 0.020$) predicted survival in people with MND at baseline.

	Univariate Cox-Regression				Multivariate Cox-Regression			
			95% Confidence Interval				95% Confidence Interval	
Parameter	<i>p</i> value	Hazard ratio	Upper	Lower	<i>p</i> value	Hazard ratio	Upper	Lower
Gender	0.359	1.271	0.761	2.124	0.881	1.043	0.600	1.184
Disease onset	0.064	1.622	0.972	2.707	0.033	1.803	1.050	3.095
Baseline age	0.329	1.015	0.986	1.044	0.600	1.009	0.977	1.041
Δ FRS	<0.001	3.030	1.906	4.816	<0.001	3.910	2.234	6.844
Neopterin	0.019	1.005	1.001	1.010	0.083	1.004	0.977	1.041
p75 ^{ECD}	0.003	1.141	1.045	1.246	0.020	1.130	1.020	1.251

4.3 Baseline urinary neopterin as a predictor of survival

The relationship between urinary neopterin levels and survival probability by univariate analysis was also explored through a Kaplan-Meier survival curve in **Figure 4.6** on 64 people with MND that had passed away or reached PAV in retrospect to this study. People with MND were separated into two groups by their median urinary neopterin values, above and below 146.7 μmol neopterin/mol creatinine ($n=32$). **Figure 4.6** demonstrated a significant difference in projected survival times ($p=0.0202$; 12.0 vs 22.5 months) by Gehan-Breslow-Wilcoxon test. Individuals with lower than the median urinary neopterin level, on average, survived 22.5 months from baseline to time of death, which was significantly different to individuals with higher median urinary neopterin values surviving, on average, 12.0 months from baseline to time of death, indicating that neopterin as a candidate prognostic biomarker of MND prognosis may have some utility in this cohort in predicting survival outcomes from baseline.

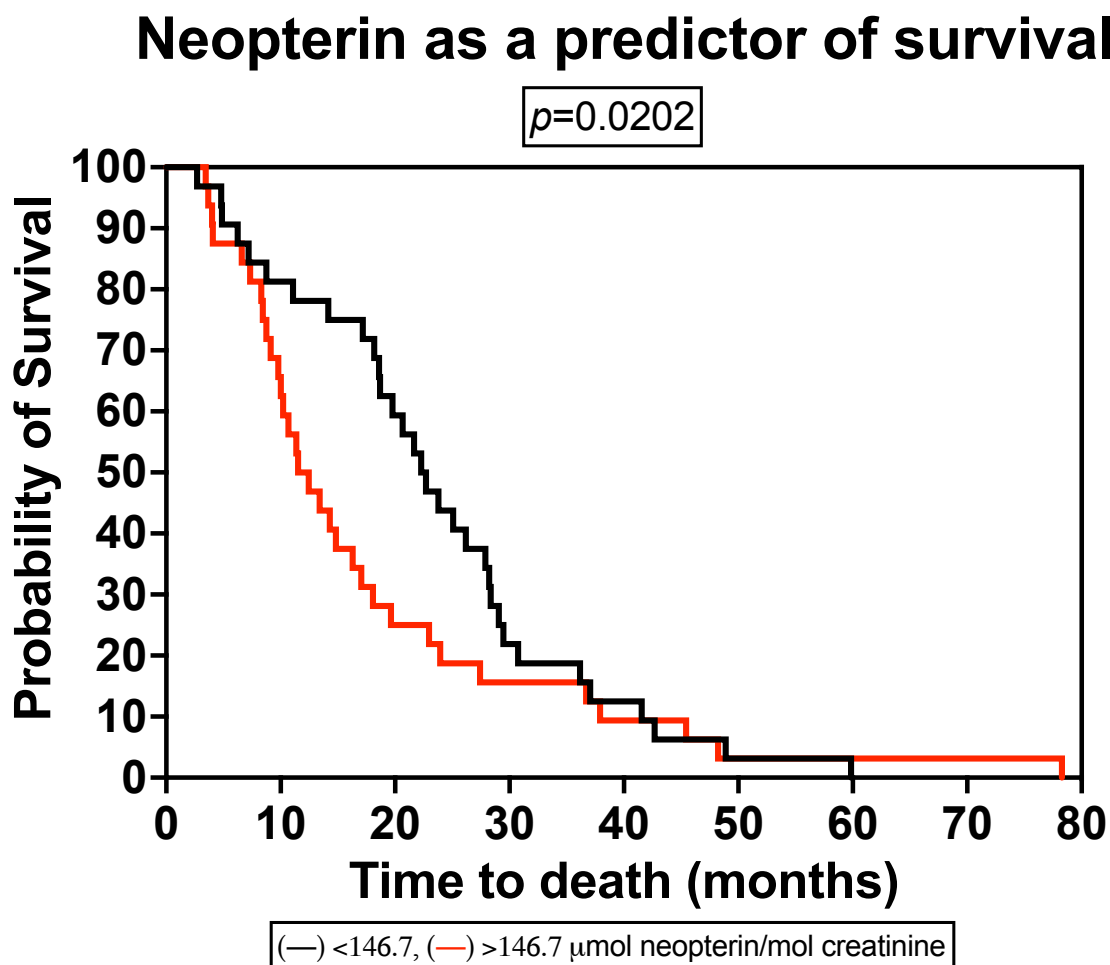


Figure 4.6: Urinary neopterin levels and estimation of survival in people with MND

Kaplan-Meier survival analysis was used to comparing 32 people with MND who had baseline urinary neopterin below (—) and 32 people above (—) the median (146.7 μmol neopterin/mol creatinine; $n=64$), showing a statistically significant change in time to death from baseline (months) with higher urinary neopterin levels ($p=0.0202$). The median survival in people with MND that were above the median was 12.0 months, which was significantly different to 22.5 months in people lower than the median (Wilcoxon test, $p=0.0202$). Statistical significance was determined using Gehan-Breslow-Wilcoxon tests, Prism 10.

4.4 Baseline p75^{ECD} quantification

4.4.1 Urinary p75^{ECD} in people with MND compared to healthy individuals

To reduce MND heterogeneity, multiple biomarkers are needed to reflect disease pathology. To complement urinary neopterin, urinary p75^{ECD}, a candidate biomarker of motor neuron death previously described by (Shepherd et al., 2017) was investigated. Urinary p75^{ECD} was quantified using the sandwich ELISA (see *section 2.5*) as described in (Shepherd et al., 2022) in the same 65 people with MND between the age range of 42-78 years, however, only 27 healthy individuals were available from the cohort in **Table 4.1**. Although the sample size in healthy individuals was limited, this did not affect the analysis between exploring urinary neopterin and p75^{ECD} as candidate biomarkers of MND prognosis. Urinary p75^{ECD} levels were significantly higher in 65 people with MND at baseline compared to 27 healthy individuals after standardisation by urinary creatinine. People with MND had an average ($\pm$ SD) urinary p75^{ECD} measurement of 5.5 ± 2.5 ng p75^{ECD}/mg creatinine compared to healthy individuals, with an average concentration of 3.1 ± 1.1 ng p75^{ECD}/mg creatinine in **Figure 4.7A** ($p < 0.0001$, Welch's T-test)

Urinary p75^{ECD} was compared to ALSFRS-R scores in 65 people with MND in **Figure 4.7B**, reporting a significant correlation ($r = -0.268$, $p = 0.0178$) with functional disease decline as symptoms of disease progressively worsen (Shepherd et al., 2017, Shepherd et al., 2022). There was no statistical difference in urinary p75^{ECD} measurement between 47 people with limb onset MND and 29 people with bulbar onset MND ($p = 0.259$, Welch's T-test), reported in **Figure 4.7C**, and ROC analysis using area under the curve indicated that urinary p75^{ECD} distinguished between MND and healthy individuals 82.5% of the time ($p < 0.0001$) with 48% sensitivity in **Figure 4.7D**. There was no reported difference between urinary p75^{ECD} above and below the median age of 64.5 years within this cohort ($p = 0.113$, Welch's T-test).

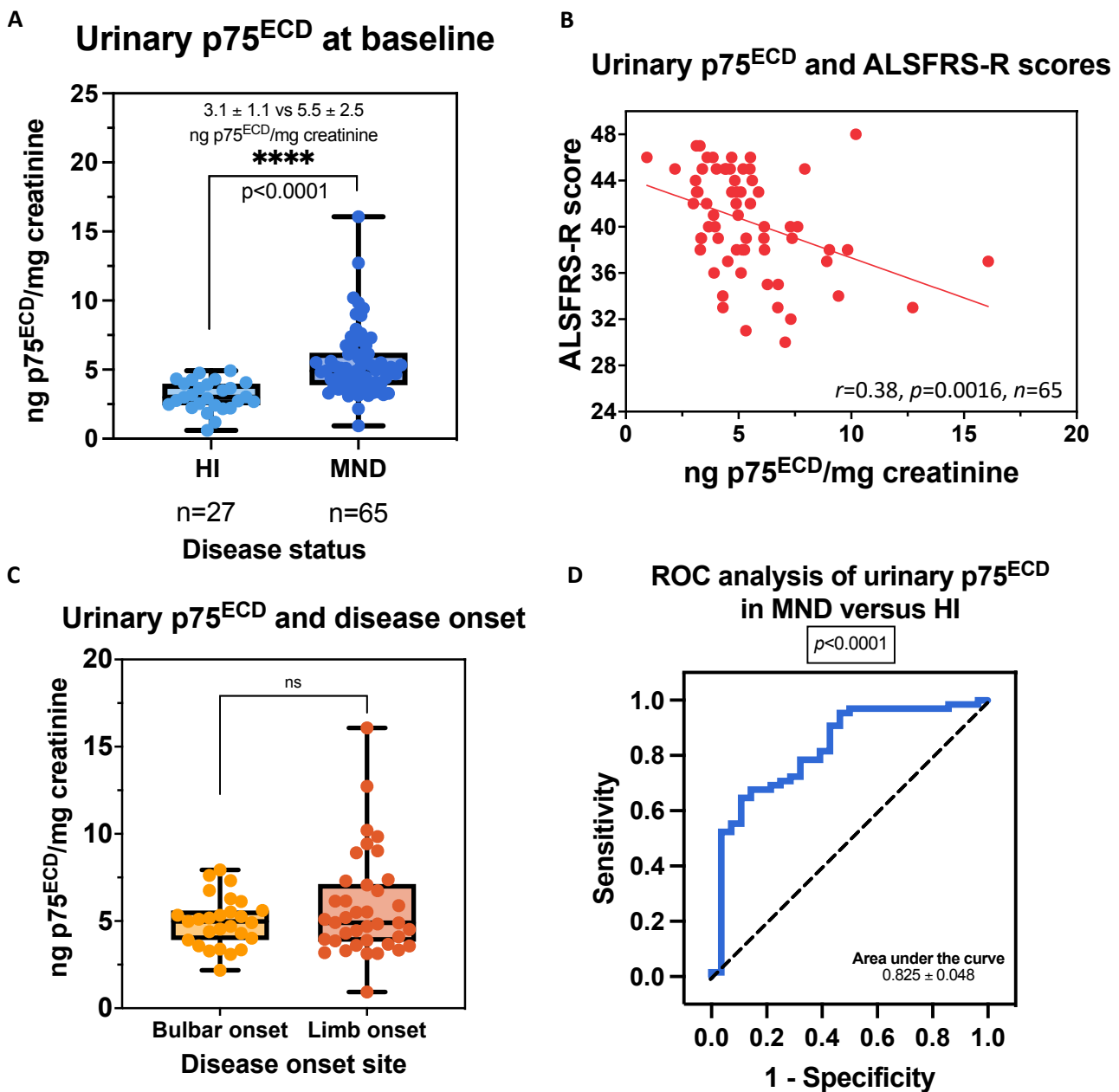


Figure 4.7: Urinary p75^{ECD} at baseline compared to ALSFRS-R and disease onset in people with MND

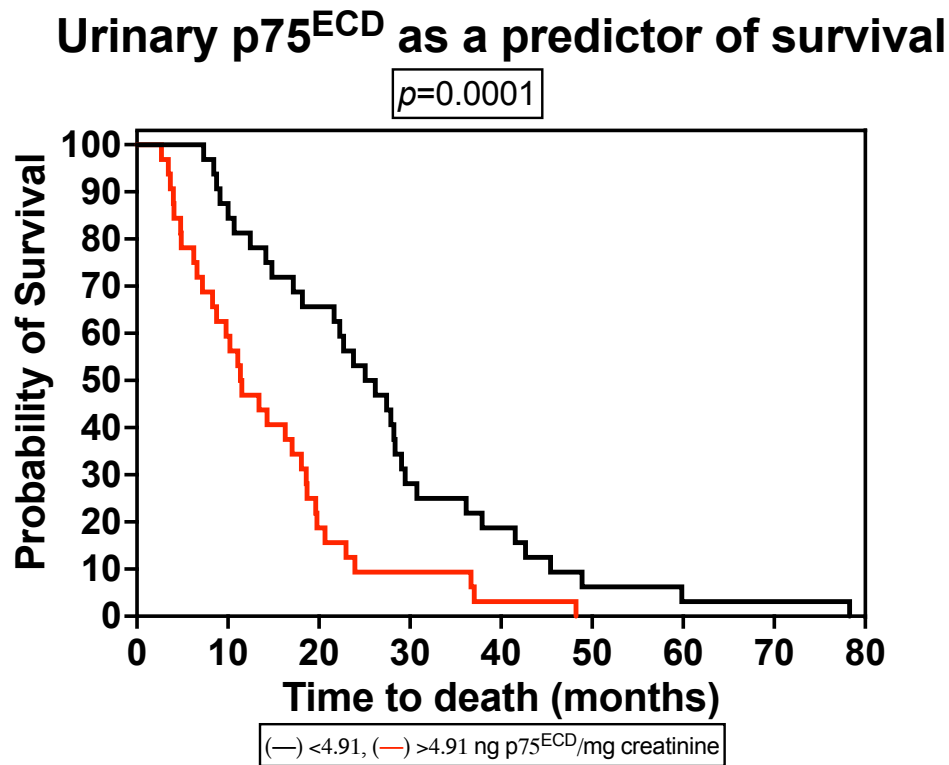
(A) Urinary p75^{ECD} levels were quantified in people with MND (●, $n=65$) and healthy individuals (●, $n=27$) at baseline using the p75^{ECD} sandwich ELISA ($n=3$ replicates per assay, 3-8 assays per sample). Urinary p75^{ECD} was normalised to urinary creatinine, and there was a significant difference in p75^{ECD} between MND and healthy individuals ($p < 0.0001$) by Independent T-test with unequal SD (Welch's T-test, Prism 10). (B) Urinary p75^{ECD} displayed a significant correlation between ALSFRS-R scores at baseline (●, $r = -0.38$, $p = 0.0016$), however, (C) Urinary p75^{ECD} levels were quantified in people with bulbar onset (●, $n=27$) compared to limb onset MND (●, $n=38$), indicating no significant difference (ns) by Independent T-test with equal SD ($p = 0.135$). (D) ROC analysis of urinary p75^{ECD} by determined the discriminatory power of urinary p75^{ECD} between MND and healthy individuals as 82.5% with 48% sensitivity, with an area under the curve of 0.825 ($p < 0.0001$) (Wilson-Brown method, Prism 10).

4.4.2 Baseline p75^{ECD} as a predictor of survival

Urinary p75^{ECD} has previously been described as a candidate biomarker of disease prognosis by (Shepherd et al., 2017), shown to estimate the life expectancy of people with MND from baseline. In univariate and multivariate Cox proportional hazards models, urinary p75^{ECD} at baseline was a significant prognosticator for survival. To demonstrate the prognostic capability of urinary p75^{ECD}, a Kaplan Meier survival analysis was performed on 64 people with MND who passed away or reached PAV in retrospect to this study. The survival analysis was performed by separating individuals by median urinary p75^{ECD} values, above and below 4.91 ng p75^{ECD}/mg creatinine, with 32 people with MND in each group. The Kaplan-Meier survival curve in **Figure 4.8A** showed a significant difference in projected survival times ($p=0.0001$, 11.5 vs 25.6 months) by Gehan-Breslow-Wilcoxon test. Individuals with lower than the median urinary p75^{ECD} level, on average, survived 25.6 months from baseline to time of death, which was significantly different to individuals with higher median urinary p75^{ECD} values surviving, on average, 11.5 months from baseline to time of death.

4.4.3 Baseline urinary neopterin and p75^{ECD}

To aid in the development of a panel of candidate prognostic biomarkers with the aim of reducing heterogeneity MND clinical trials, urinary neopterin and p75^{ECD} were explored for their utility together. The contribution of both biomarkers could help in identifying disease sub-groups to better stratify individuals with MND based on the level of urinary p75^{ECD} (i.e., neurodegeneration), compared to neopterin (i.e., pro-inflammation). The levels of urinary neopterin in μmol neopterin/mol creatinine and urinary p75^{ECD} in ng p75^{ECD}/mg creatinine were plotted on **Figure 4.8B** and reported a significant correlation ($n=65$, $r=0.27$, $p=0.030$, Pearson's correlation)



B

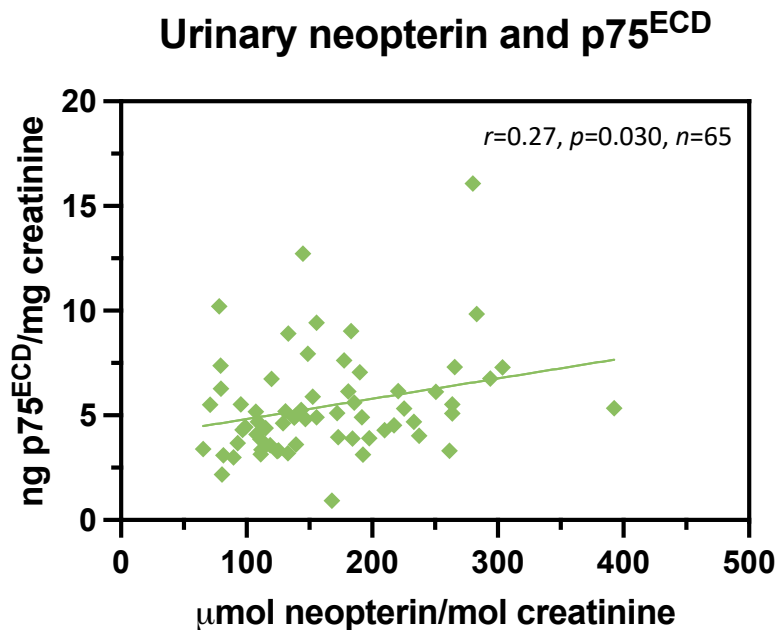


Figure 4.8: Estimating survival by urinary p75^{ECD} and comparing urinary neopterin at baseline

(A) Kaplan-Meier survival analysis was used to compare 32 people with MND who had baseline urinary neopterin below (—) and 32 people above (—) the median (4.91 ng p75^{ECD}/mg creatinine; $n=64$). Those with higher p75^{ECD} had a significantly shorter median survival time compared to people with lower p75^{ECD} (25.6 vs 11.5 months; Wilcoxon test, $p=0.0001$). Levels of urinary neopterin and p75^{ECD} were quantified in 65 people with MND at baseline. (B) There was a significant correlation between neopterin and p75^{ECD} (●, $r=0.27$, $p=0.030$). Statistical significance was determined using Gehan-Breslow-Wilcoxon test and Pearson's correlation, Prism 10.

4.5 Candidate biomarkers of MND in other conditions

4.5.1 Clinical characteristics of people with other conditions

To determine if urinary neopterin also increases in people with other conditions, urine samples were obtained from a cohort of 18 individuals outlined in **Table 4.6**. These people were recruited from the Technical University of Munich (Germany) at the time of urine collection. The cohort included 7 females (39%) and 11 males (61%) with an average age ($\pm$ SD) of 58.5 ± 17.6 years at sample collection (range: 26-83). These individuals presented with a variety of conditions, which ranged in severity. Five people were categorised into stroke events, including middle cerebral artery infarction, superior cerebellar artery infarction, retinal artery occlusion, vertebrobasilar stroke, and temporary hemihypaesthesia. Four people represented the cohort with headaches from unknown causes. Two people with immune conditions were characterised with myasthenia gravis and dysimmune axonal sensorimotor neuropathy. Two people presented with hydrocephalus by meningiomatosis with intracranial hypertension and normal pressure hydrocephalus. The remaining other conditions were represented by one individual only. These conditions included meningitis (streptococcus), diplopia, scanning speech of unknown origin, abducens nerve palsy, and amnesia (see **Table 4.6**). Individuals with impaired kidney function and/or diabetes were excluded from this study. Hydration status was controlled to a measurement of 0.3 – 3 mg/mL of urinary creatinine to standardise urinary dilutions from sport-urine samples.

The average ($\pm$ SD) neopterin measurement in other conditions was 213.5 ± 150.1 μ mol neopterin/mol creatinine, which was significantly higher ($p=0.040$, Welch's T-test) than 39 healthy individuals who had an average measurement of 135.2 ± 44.7 μ mol neopterin/mol creatinine (see **Figure 4.1A**). However, there was a large range of neopterin measurement reported across various conditions, mostly because concentrations were high in people who had strokes, infectious meningitis, immune conditions, and nerve palsy, but were reportedly low in people with headaches, diplopia, hydrocephalus, amnesia, and scanning speech of unknown origin (see **Table 4.5**). The low number of participants in each category was a limitation of this investigation, but this pilot study on other conditions gave indication that pro-inflammatory conditions and immune conditions increase urinary neopterin concentrations compared to healthy individuals.

Table 4.6: Cohort description of study participants from people other conditions

All disease characteristics and clinical information for 18 people with other conditions, including urinary neopterin and p75^{ECD} measurement for each condition.

Disease Characteristics			Neopterin by LCMS (μmol neopterin/mol creatinine) $\pm$ SD	p75 ^{ECD} (ng p75 ^{ECD} /mg creatinine) $\pm$ SD
Total number of participants		18	213.5 $\pm$ 150.1	4.5 $\pm$ 1.0
Gender	Male, (%)	11, (73%)	N/A	N/A
Age at collection	Mean $\pm$ SD (Range; IQR)	58.5 $\pm$ 17.6 (26-84; 45.0-72.7)	N/A	N/A
Stroke	N; mean age $\pm$ SD	5; 55.7 $\pm$ 22.6	369.7 $\pm$ 169	5.2 $\pm$ 2.1
Headache	N; mean age $\pm$ SD	4; 52.2 $\pm$ 18.7	127.3 $\pm$ 53.6	4.0 $\pm$ 0.6
Immune conditions	N; mean age $\pm$ SD	2; 62.5 $\pm$ 14.4	173.2 $\pm$ 3.3	3.2 $\pm$ 4.7
Hydrocephalus	N; mean age $\pm$ SD	2; 69.3 $\pm$ 17.5	128.4 $\pm$ 62.8	2.7 $\pm$ 0.4
Infection (streptococcus)	N; mean age	1; 39.1	369.4	8.1
Diplopia	N; mean age	1; 69.7	98.6	4.5
Scanning speech of unknown origin	N; mean age	1; 50.9	113.4	2.6
Abducens nerve palsy	N; mean age	1; 67.8	178.9	6.0
Amnesia	N; mean age	1; 73.0	130.9	3.8

4.5.2 Urinary p75^{ECD} in people with MND and healthy individuals compared to other conditions.

The levels of urinary p75^{ECD} in 18 people with other conditions (OC) reported a significant difference to 27 healthy individuals (OC: 4.5 ± 1.8 ng/mg creatinine vs HI: 3.25 ± 1.0 ng/mg creatinine, $p=0.010$; **Table 4.6**). Urinary p75^{ECD} ranged between highest values reported in one individual with meningitis (8.1 ng p75^{ECD}/mg creatinine) and with abducens nerve palsy (6.0 ng p75^{ECD}/mg creatinine) compared to other conditions, however, only one sample was tested for those conditions. The lowest p75^{ECD} measurements were reported in scanning speech of unknown origin, hydrocephalus, immune disorders, and people with headaches.

4.5.3 Urinary neopterin concentrations detected by LCMS and ELISA compared in people with MND, healthy individuals, and other conditions.

Urinary neopterin was measured by inhibition ELISA and LCMS to quantify neopterin in human urine ($\mu\text{mol/L}$), normalised to urinary creatinine to standardise for spot urine dilution factors ($\mu\text{mol neopterin/mol creatinine}$). To compare the cross-platform methods of neopterin quantification, neopterin was quantified in all baseline samples from this study, including 78 people with MND, 39 healthy individuals, and 18 people with other conditions (total, $n=133$) was compared by LCMS and ELISA techniques. Neopterin quantified by LCMS and ELISA was tested in duplicate on each assay, enduring 3-5 repeat biological measurements. In **Figure 4.9A**, neat measurement ($\mu\text{mol/L}$) of urinary neopterin quantified by ELISA and LCMS reported a significant correlation in 133 tested study participants ($r=0.61$, $p<0.0001$, $n=133$). In **Figure 4.9B**, the measurement of urinary neopterin by ELISA and LCMS normalised to creatinine ($\mu\text{mol neopterin/mol creatinine}$) reported a significant correlation in 133 people ($r=0.78$, $p<0.0001$, $n=133$). This contributed towards the validation of urinary neopterin detection across both analytical methods of detection.

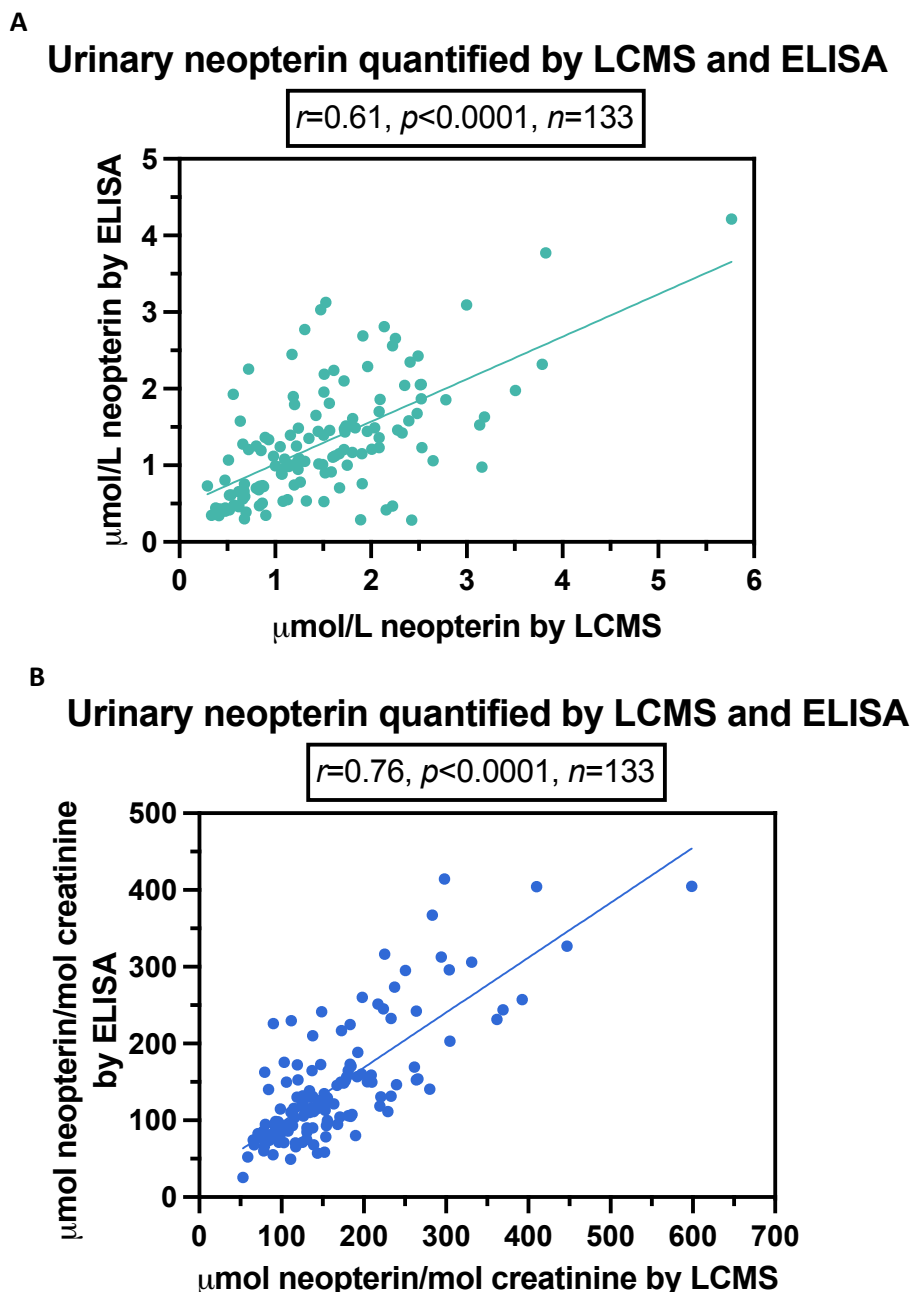


Figure 4.9: Urinary neopterin quantified by LCMS assay compared to IBL competition ELISA

To compare cross-platform methods of neopterin quantification, the measurement urinary neopterin quantified in all baseline samples from 78 people with MND, 39 HI, and 18 OC (total $n=133$) using LCMS ($n=2$ replicates per test, 3-5 tests per sample) and ELISA ($n=2$ replicates per ELISA, 3-5 ELISAs per sample). **(A)** There was a significant correlation between urinary neopterin ($\mu\text{mol/L}$) quantified by LCMS and ELISA in 133 individuals ($r=0.61, p<0.0001$), and **(B)** a significant correlation was reported between urinary neopterin standardised to creatinine to account for spot urine dilution ($\mu\text{mol neopterin/mol creatinine}$) quantified by LCMS and ELISA in 133 individuals ($r=0.76, p<0.0001$). Statistical significance was determined using Pearson's correlation in Prism 10.

4.5.4 Cox proportional hazards model of urinary neopterin and other clinical variates in people with MND

Urinary neopterin quantified by ELISA and LCMS, and other clinical covariates were compared in a Cox proportional hazards model for their ability to predict survival in 65 people with MND. The potential utility of urinary neopterin quantified by two techniques was investigated for the first time in a univariate Cox regression analysis. In **Table 4.7**, clinical factors that were independently predictive of survival included Δ FRS (HR: 3.03, 95% CI: 1.91-4.82, $p < 0.001$), neopterin by ELISA (HR: 1.004, 95% CI: 1.000-1.008, $p = 0.033$), neopterin by LCMS (HR: 1.005, 95% CI: 1.001-1.010, $p = 0.019$), and urinary p75^{ECD} (HR: 1.14, 95% CI: 1.05-1.25, $p = 0.003$). Univariate analysis suggested that whether urinary neopterin was quantified by ELISA or LCMS techniques, neopterin had modest prognostic value in a cohort of 65 people with MND between the ages of 42 and 78 years of age.

To perform a multivariate Cox proportional hazards model for these clinical covariates in 65 people with MND, statistical guidelines were followed from (Eliason, 1993). The rule suggested that for every 10 samples, one clinical covariate should be included in the multivariate regression model to prevent overfitting and ensure the model remains statistically valid. With a sample size of 65 people with MND, no more than 7 covariates were included in the model at one time, including gender, site of disease onset, age at sample collection, Δ FRS, urinary neopterin detected by ELISA and LCMS, and urinary p75^{ECD}. Multivariate Cox proportional hazards model revealed that only disease onset (HR: 1.80, 95% CI: 1.05-3.10, $p = 0.033$), Δ FRS (HR: 3.91, 95% CI: 2.23-6.86, $p < 0.001$), and urinary p75^{ECD} (HR: 1.12, 95% CI: 1.01-1.25, $p = 0.040$) predicted survival in 65 people with MND at baseline, and that neither neopterin quantified by ELISA or LCMS techniques could add prognostic value beyond what could be discerned from the site of disease onset, Δ FRS, and urinary p75^{ECD}. This highlights the need to further validate neopterin as a candidate prognostic biomarker for people with MND.

Table 4.7: Baseline cohort and survival by Cox proportional hazards models for neopterin detected by LCMS and IBL competition ELISA

In 65 people with MND at baseline univariate Cox proportional hazard analysis reported clinical factors predictive of survival, including urinary neopterin detected by LCMS and ELISA. Δ FRS (HR: 3.03, 95% CI: 1.91-4.82, $p < 0.001$), neopterin by ELISA (HR: 1.004, 95% CI: 1.000-1.008, $p = 0.033$), neopterin by LCMS (HR: 1.005, 95% CI: 1.001-1.010, $p = 0.019$), and urinary $p75^{\text{ECD}}$ (HR: 1.14, 95% CI: 1.05-1.25, $p = 0.003$) were prognostic factors for disease survival. By multivariate Cox proportional hazards model, disease onset (HR: 1.80, 95% CI: 1.05-3.10, $p = 0.033$), Δ FRS (HR: 3.91, 95% CI: 2.23-6.86, $p < 0.001$), and urinary $p75^{\text{ECD}}$ (HR: 1.12, 95% CI: 1.01-1.25, $p = 0.040$) predicted survival in people with MND at baseline.

	Univariate Cox-Regression				Multivariate Cox-Regression			
			95% Confidence Interval				95% Confidence Interval	
Parameter	<i>p</i> value	Hazard ratio	Upper	Lower	<i>p</i> value	Hazard ratio	Upper	Lower
Gender	0.359	1.271	0.761	2.124	0.867	1.049	0.603	1.824
Disease onset	0.064	1.622	0.972	2.707	0.033	1.802	1.050	3.093
Baseline age	0.329	1.015	0.986	1.044	0.610	1.008	0.977	1.041
Δ FRS	<0.001	3.030	1.906	4.816	<0.001	3.913	2.231	6.862
Neopterin by ELISA	0.033	1.004	1.000	1.008	0.276	1.003	0.977	1.009
Neopterin by LCMS	0.019	1.005	1.001	1.010	0.748	1.001	0.995	1.007
$p75^{\text{ECD}}$	0.003	1.141	1.045	1.246	0.040	1.122	1.005	1.252

4.6 Discussion

The aim of this investigation was to outline the application of a liquid chromatography mass spectrometry (LCMS) technique to quantify neopterin in the urine of people with MND at baseline as a candidate biomarker of disease prognosis. The proposed LCMS method from *Chapter 3* was applied in a large cohort of people with MND to separate and identify neopterin in human urine, using a rapid and robust sample preparation technique for the high-throughput quantification of neopterin in MND urine at baseline. To our knowledge, this is the first time a targeted analysis using LCMS has been applied to the urine of people with MND to characterise urinary neopterin. The result of this investigation was comparable to neopterin quantified in the same cohort of people by the commercial IBL competition ELISA, such that with the addition of future validation studies, urinary neopterin may eventually be a prognostic and/or predictive biomarker.

The proposed LCMS workflow was applied to 76 clinical urine specimens from people with MND at baseline, 39 healthy individuals, and 18 people with other conditions. The participants for this study were selected based on the inclusion criteria outlined in *section 2.1.1*, and their involvement in prior research from (Shepherd et al., 2022). The aim was to evaluate urinary neopterin as a candidate biomarker of disease prognosis by sampling urine from people with MND at baseline using a targeted LCMS technique and compare this result to quantification by commercial IBL competition ELISA. This strategy of enrolment was used to compare the cross-platform quantification of urinary neopterin to cross-validate methods of neopterin detection in people with MND and the robustness of neopterin as a candidate biomarker of prognosis. Initially, urinary neopterin was sampled in 76 people with MND at baseline, or first visit to the neurologist, to compare neopterin measurement to other clinical and neurological characteristics defined in **Table 4.1** that may be prognostic for survival. These characteristics included gender, site of disease onset, age at sample collection, ALSFRS-R scores, and the estimated decrease in ALSFRS-R (Δ FRS) between symptoms and baseline. Urinary creatinine was a reliable measure of urinary dilution based on a strong correlation with urinary osmolality and people with MND were age-matched to healthy individuals (**Table 4.1**, $p=0.061$). Confirming previous findings (Lunetta et al., 2020, Shepherd et al., 2022), urinary neopterin was higher in people with MND (170.7 ± 73.2 μ mol neopterin/mol creatinine) compared to healthy individuals (135.2 ± 44.7 μ mol neopterin/mol creatinine, $p=0.0017$), and was significantly correlated with the revised ALS functional rating scale (ALSFRS-R) at first evaluation. This was consistent with a study that quantified neopterin by high performance liquid chromatography (HPLC) with fluorescent

detection (FD) at 355 nm. In 81 people with MND and 68 healthy individuals, the average concentration of urinary neopterin was 263.9 μmol neopterin/mol creatinine (Lunetta et al., 2020) compared to 169.6 μmol neopterin/mol creatinine in this study. Higher levels of neopterin can be accredited to the average time from symptom onset to sample collection, reported at 26.4 months by (Lunetta et al., 2020) compared to 15.2 months in this study. People with MND later in their disease course may account for higher documented neopterin levels as reported by (Shepherd et al., 2022), and seen in advanced stages of cancer (Reibnegger et al., 1991).

Neopterin production increases in various diseases and infections associated with increased oxidative stress (Widner et al., 2002a), or when T-cells, macrophages, or microglia are activated in a neuroinflammatory response (Melichar et al., 2006). High neopterin concentrations have been documented in various conditions such as cancer or characteristics of infectious, autoimmune, neurological, or cardiovascular diseases involved with a common immune cell activation indicated by increased neopterin production (Murr et al., 2002). Reference values for neopterin in human urine have been reported between 119 ± 39 and 133 ± 38 μmol neopterin/mol creatinine in healthy individuals between 55 and >65 years of age (Murr et al., 2002), corresponding well with our measurement of 135.2 ± 44.7 μmol neopterin/mol creatinine in 39 healthy individuals with an average age of 62.3 years.

To validate whether disease survival was influenced by age-related differences from (Shepherd et al., 2022), the analysis was restricted to a narrower age range from 42 to 78 years of age to minimize potential confounding by advanced age. Within this restricted range, age at sample collection was no longer significantly associated with disease survival outcomes in Cox proportional hazards models ($p=0.33$) and the restricted cohort was subsequently used to control for age-related inflammation where age is a dominant risk factor. To investigate the potential utility of urinary neopterin and $p75^{\text{ECD}}$ at baseline as predictors of prognosis, survival analyses were performed on 65 people with MND, 64 of whom passed away during follow-up to this study. Univariate survival models reported that the ΔFRS , neopterin, and $p75^{\text{ECD}}$ predicted shorter survival. Urinary neopterin and $p75^{\text{ECD}}$ were dichotomized above or below their median baseline values, and their Kaplan Meier survival plots longer median survival from baseline below the median value for neopterin ($p=0.0202$) and $p75^{\text{ECD}}$ ($p=0.0001$). However, by multivariate Cox proportional hazards model, neopterin could not add prognostic value beyond what could be determined based on the ΔFRS and urinary $p75^{\text{ECD}}$ measurement at baseline. These findings are consistent with the results of a meta-regression analysis published by (Hu et al., 2017), revealing that

candidate biomarkers of inflammation (i.e., TNF- α , MCP-1, and various interleukins) are only prognostic when powered sufficiently with large sample sizes from 102-456 people with MND. Meta-regression analyses on age reported significant associations with serum TNF- α and age as did neopterin in this study. This suggests that the utility of inflammatory biomarker candidates is in predicting the pro-inflammatory state and stratifying individuals into those most likely to respond to anti-inflammatory treatments in clinical trials, rather than predicting survival outcomes from baseline. We found no relationship between gender, although site of onset was associated with prognosis in this cohort which was consistent with previous reports (Benatar et al., 2020, Chiò et al., 2009).

Despite multiple ongoing efforts in the space of MND biomarker research and clinical trial design, only serum neurofilament light (NfL) has emerged as a validated biomarker that is prognostic for people with MND at baseline (Benatar et al., 2024b). Serum NfL was recently validated as a powerful prognostic and responsive tool that can predict survival and rate of ALSFRS-R decline in people with MND (Miller et al., 2022, Benatar et al., 2023, Huang et al., 2020, Thompson et al., 2022, Benatar et al., 2024b) that can add to the design and analysis for all forth-coming MND clinical trials and to the European Network to Cure ALS (ENCALS) prediction model (Benatar et al., 2020, Benatar et al., 2024b). Although the prognostic power of urinary p75^{ECD} is modest at baseline compared to serum NfL (Benatar et al., 2024b), in this cohort, urinary p75^{ECD} was prognostic in a multivariate analysis. To push past the urgent challenge of failed phase 2 clinical trial design, it is proposed that multiple biomarkers reflective of different disease mechanisms are needed to predict survival outcomes, characterize disease pathology, and reduce disease heterogeneity. In this investigation, urinary p75^{ECD} was significantly elevated in 65 people with MND compared to 27 healthy individuals, supporting previous results (Shepherd et al., 2017, Shepherd et al., 2022). Urinary p75^{ECD} is a candidate biomarker of neurodegeneration that has been implemented in multiple phase 2 clinical trials (Gold et al., 2019, Vucic et al., 2020b, Henderson et al., 2021, Koch et al., 2024, Paganoni et al., 2025), with future prospects of phase 3 clinical trials investigating experimental therapeutics in people with MND. To understand where urinary neopterin might fit into this model as a candidate biomarker of pro-inflammation, we identified that neopterin was significantly correlated to p75^{ECD} ($r=0.27$, $p=0.030$). This implied that disease-specific inflammation was observed with an association with neurodegeneration at baseline, and both biomarkers could be useful for disease stratification in future clinical trials.

The application of these biomarkers in people with MND and healthy individuals was compared to people with various other conditions described in *section 4.6.1*. The observed differences and correlations of urinary neopterin and p75^{ECD} in the urine of people with MND and healthy individuals at baseline supports their candidate utility as prognostic biomarkers, suggesting these markers may reflect underlying disease pathophysiology of neuroinflammation and neurodegeneration in MND. However, neither urinary neopterin or p75^{ECD} were able to differentiate MND from other conditions. This is an important observation, as although both biomarkers may reflect disease-specific changes in neurological pathology in people with MND, they cannot distinguish between different disease aetiologies. Although there are limited effective treatments approved by the Therapeutic Goods Administration (TGA) in Australia for people with sporadic MND (Khamaysa and Pradat, 2022), the need for additional disease-specific diagnostic biomarker candidates alongside clinical characteristics, neuroimaging, and the revised El Escorial diagnostic criteria is important in the age of therapies such as Tofersen (Ludolph et al., 2015). With over 179 experimental therapeutics tested in phase 2 clinical trials for sporadic MND over the last 30 years, only 26 made it to phase 3 clinical trials (Pupillo et al., 2024) and only Riluzole has been approved as a disease modifier in all countries, extending lifespan by 2-3 months (Bensimon et al., 1994). Edaravone is the only other treatment approved by the TGA for use in Australia, which has inconclusive and poor clinical evidence (O'Neill et al., 2024b). Tofersen is a treatment available only to people who carry mutations in the SOD1 gene (Miller et al., 2022), but is not yet approved by the TGA. The importance of aiding the development of experimental therapeutics and reducing heterogeneity in phase 2 and 3 clinical trials by the discovery, characterisation, and validation of prognostic, pharmacodynamic, progression, and predictive biomarkers is of utmost importance for future studies (Goyal et al., 2020).

With an overarching aim of contributing towards clinically sensitive, accurate, and robust biomarker detection, the robustness and comparability of urinary neopterin quantification across platforms was evaluated. From 78 people with MND, 39 healthy individuals, and 18 people with other conditions, neopterin concentrations measured by ELISA and LCMS were assessed in a total cohort of 133 samples. Strong and statistically significant correlations were observed between both methods when measuring neat neopterin ($r=0.61$, $p<0.0001$), indicating good agreement between both techniques. This correlation improved upon normalisation to urinary creatinine ($r=0.76$, $p<0.0001$), highlighting the importance of accounting for spot-urine hydration variability. Urinary neopterin quantified by IBL

competition ELISA and LCMS were equally compared in a final Cox proportional hazards model that ultimately demonstrated neopterin was not a strong predictor of survival in people with MND at baseline compared to the likes of bulbar onset MND, Δ FRS, and urinary p75^{ECD}, consistent with previous findings (Shepherd et al., 2022).

The largest caveat of this investigation was the lack of longitudinal sampling using the LCMS technique as done with the IBL competition ELISA (Shepherd et al., 2022). Neopterin has previously demonstrated greater ability as a candidate progression and predictive biomarker that changes longitudinally and can predict pro-inflammation in people with MND further in their disease course. To contribute towards the clinical implementation of urinary neopterin as a candidate predictive biomarker of pro-inflammation in phase 2 clinical trials and beyond, future studies incorporating longitudinal sampling and therapeutic monitoring of anti-inflammatory experimental therapeutics will be critical to establishing the functional utility of neopterin in the clinic, beyond its ability to stratify people with MND by predicting survival from baseline. Future studies should examine people at risk of developing MND symptoms in a pre-symptomatic familial state (pre-fMND) to help determine the duration of pre-symptomatic disease, and if neopterin can characterize changes in the inflammatory state leading up to disease.

MND is a highly heterogenous disease and multiple biomarkers are needed to reflect disease pathology. MND can be characterised by serum NfL which has emerged as the leading prognostic and response biomarker in clinical trials that can reflect axonal degradation in people with MND (Benatar et al., 2020, Huang et al., 2020, Miller et al., 2022, Thompson et al., 2022, Benatar et al., 2023). Candidate biomarkers including urinary p75^{ECD} (Shepherd et al., 2017) reflective of motor neuron degeneration, microRNA-181 (Magen et al., 2021) reflecting changes in gene expression, and urinary neopterin that may be predictive of pro-inflammation in people with MND require future clinical validation to establish them as useful contributors to clinical trial design, analysis, and result interpretation. Other candidate biomarkers have found themselves involved with past, present, and future phase 2 clinical trials, involving experimental therapeutics targeting the inflammatory response by increasing anti-inflammatory T-regulatory cells using Tecfidera (Vucic et al., 2020b), using monoclonal antibodies against monocyte CD14 (IC14) receptors (Henderson et al., 2021), and targeting endogenous retrovirus and HERV-K involvement associated with TDP-43 and inflammation using Triumeq (Gold et al., 2019) by mechanisms that remain unclear (Dubowsky et al., 2023). These are all examples of phase 2 clinical trials that, with further refinement and validation of neopterin quantification as a candidate

biomarker of pro-inflammation in people with MND, may provide significant clinical benefit alongside serum NfL, urinary p75^{ECD}, the ALSFRS-R, and others in a panel to better prioritize drug candidates for phase 3 clinical trials and translate to successful clinical outcomes for people living with MND.

CHAPTER 5.
DETECTION AND DISCOVERY OF
URINARY PROTEINS AS
BIOMARKERS OF MOTOR NEURON
DISEASE BY MASS
SPECTROMETRY

5.1 Introduction

Urine is a relatively unexplored biofluid for biomarker discovery in MND. Thus far, *Chapters 3 and 4* has demonstrated the ability to perform targeted measurement of biomarker candidates in MND urine, including neopterin. Previous research in our laboratory has found the extracellular domain of the neurotrophin receptor p75 (p75^{ECD}) as a targeted candidate protein-based biomarker (Shepherd et al., 2014, Shepherd et al., 2017, Shepherd et al., 2022). Urinary p75^{ECD} has been shown to be a candidate biomarker of prognosis and progression (Shepherd et al., 2017, Shepherd et al., 2022, Benatar et al., 2024b), and neopterin as a candidate biomarker of disease progression and predictive of pro-inflammation in people with MND (Shepherd et al., 2022). To complement these biomarkers, the aim of this study was to use un-targeted techniques to isolate the urinary proteome and identify urinary proteins in people with MND that were differentially changed at baseline compared to healthy individuals, and are related to disease processes, which could be further validated as candidate biomarkers of MND prognosis or predictive of immune dysfunction. To achieve this, urinary proteins were isolated from the urine of healthy individuals and people with MND sampled at baseline (i.e., first visit to the neurologist), where neurological and clinical features were recorded. Liquid chromatography-mass spectrometry (LCMS) and gene ontology techniques were then used to identify urinary protein biomarker candidates in people with MND.

Urine contains a less complex proteome compared to serum and cerebrospinal fluid (CSF) (Rogers et al., 2023). Urine is composed of water (95%), urea (2%), creatinine (0.1%), uric acid (0.03%), and trace amounts of other ions, cells, electrolytes, extracellular micro-vesicles, metabolites, and proteins. Approximately 20-30% of systemic serum proteins are discoverable in urine, while metabolites are common between both biofluids (Eaton and Pooler, 2009, Rogers et al., 2023). The renal system reabsorbs most proteins resulting in spot-urine protein concentrations of ~0.2 mg protein/mL (Eaton and Pooler, 2009, Ogobuiro and Tuma, 2024), compared to the 60-70 mg protein/mL seen in serum and plasma (Simon et al., 2022). Serum is dominated by proteins like albumin (55%) and immunoglobulins (IgGs) that are unrelated to MND pathology (Tirumalai et al., 2003). Proteins with a molecular weight between 20-50 kDa are mostly freely filtered into the urine, and higher molecular weight proteins above 60 kDa are largely reabsorbed (Rogers et al., 2023). However, some larger cationic proteins are more freely filtered compared to anionic proteins in the glomerulus (Kanwar et al., 1980). This charge-based filtration means that anionic proteins of the same molecular weight can be filtered by up to 50% less (Julian et al., 2009).

Although urine contains kidney and urinary tract proteins, like uromodulin derived exclusively from the ascending loop of Henle (Bachmann et al., 1990), approximately 20-30% of filtered plasma proteins originating from distal organs, including the brain (Jia et al., 2009), have been detected in urine. The advantage of sampling urine is that it is a non-invasive and readily accessible source of proteins that can be sampled repeatedly and collected in large volumes (Scott and Quaggin, 2015, Rogers et al., 2023).

The human proteome project globally has identified approximately 4,500 potential protein groups in human urine (UniProt search) (Rogers et al., 2023). On average, 2,000 of these protein groups can be detected in urine using LCMS techniques (Muntel et al., 2015, Swensen et al., 2021). When this is compared with other biofluids, only ~300-600 proteins in serum, and ~1,500 proteins in CSF are detectable by LCMS from the approximate ~4,500 detectable protein groups (Shao et al., 2021) (proteomeatlas.org/humanproteome, (Rogers et al., 2023). The higher number of identified protein groups in urine is attributed to its dynamic range (Dayon et al., 2022) and lesser amounts of albumin and immunoglobulins (IgGs) in urine compared to serum (Anderson and Anderson, 2002). This is an advantage in that low abundance proteins are more easily detectable in urine when compared to serum and are relatively under explored in the field of MND biomarker research (Rogers et al., 2023, Dayon et al., 2022).

However, unlike serum and CSF, the volume and protein concentration of spot-urine is not under homeostatic control and varies with hydration status (Thomas et al., 2016). Thus, using renal dilution correction factors such as urinary creatinine (mg/mL) or osmolality (mOsm/kg) are needed to account for the hydration status of each participant. In addition, the WHO guidelines indicate that urinary creatinine concentrations below 0.3 ± 0.03 mg/mL is reflective of urine that is too dilute and may distort the detection of low levels of analyte measurement, and concentrations above 3.0 ± 0.3 mg/mL indicate severe dehydration or renal filtration issues (Mikheev and Lowry, 1996). Therefore, study participants were limited to concentrations between 0.3 ± 0.03 and 3.0 ± 0.3 mg/mL of urinary creatinine. In this chapter, the urinary proteome was explored as a biomarker source for MND, and the relationship to clinical prognosis, as well as pathological processes by gene ontology.

5.2 Methods

Urinary proteins were isolated from samples by diafiltration using 3 kDa spin-columns, such that urinary proteins >3 kDa in size remained in PBS solution and were concentrated up to 30 times (see *section 2.6.1*). Protein concentration was estimated by EZQ protein assay described in *section 2.6.5*. Accurate protein concentration is important to ensure equal injection of peptides, and to prevent experimental artifacts such as changes in chromatography and peak shape between samples. The concentration of protein was confirmed by SDS-PAGE and ImageLab in *section 2.6.6*. Completing SDS-PAGE of urinary proteins on stain-free gels provides an additional level of normalisation to provide a more consistent total ion current (TIC) and base peak intensity of peptides in the mass spectrometer. To confirm protein concentration by EZQ assay, the saturation of each SDS-PAGE protein lane was normalised to a 10 µg positive-control. The EZQ result was adjusted to ensure protein equivalence across all samples.

Urinary proteins were digested into peptides using a Trypsin Protease (Burkhart et al., 2012, Gundry et al., 2010) (see *section 2.6.7*) following reduction by DTT and alkylation by IAA to prevent the formation of disulphide bonds. Liquid chromatography was performed on a 25 cm custom pulled tip column, manufactured in-house using fused silica (360 µm outer diameter, 75 µm inner diameter) incorporating a spray tip. The tip was pulled using a Sutter 2000 tip puller, polished using a Sutter BV-10N tip polisher. The 3 µm C18 beads (Dr. Maisch) were packed into the column using a HPLC pump, pushing 50% acetonitrile into a pressure vessel containing the beads and subsequently into the capillary uHPLC column. The column was placed in a column heater (Sonation) immediately in-front of the mass spectrometer inlet and heater to 50°C. The flow rate of 300 nanoliters per minute (nL/min) was provided by a Dionex Ultimate 3000 uHPLC system with an autosampler. Samples were injected using a trap and elute method which uses one pump to load the samples onto a small C18 column (trap) at 10 µL/min which binds the peptides. The trap is brought into line with the main pump so that an increasing acetonitrile gradient will elute the peptides from the trap to the column, and subsequently into the mass spectrometer. With a gradient of increasing acetonitrile and formic acid, peptides were eluted from the C18 column based on their hydrophobicity and affinity, optimizing the separation of a wide range of peptide sizes and sequences (Mant et al., 2007).

Exploris 480 and Fusion Lumos mass spectrometers were operated in positive ion mode to detect positively charged peptides protonated on their primary amine groups at the amino

terminus, and on lysine or arginine residues located at the C-terminus (Steckel and Schlosser, 2019) where they were ionized by the high voltage electrospray ionization source, generally limited to sequencing peptides of less than 40 amino acids in length (Coon et al., 2005). Proteome Discoverer v2.4 (Orsburn, 2021) and Spectronaut v19.0 (Bruderer et al., 2015) were used to analyse the resulting mass spectra, identifying peptides based on their mass and charge by comparing them to a database of known protein sequences. The peptide sequence coverage of each protein is determined by product (y^+) ions in the MS2 scan, identifying protein groups compared to a spectral library. The intensity of the MS1 scan determines how much of those proteins were present, generating a relative quantification between people with MND and healthy individuals (Orsburn, 2021), and protein quantities were corrected for renal dilution by normalisation to urinary creatinine (mg/mL) and analysed in MatLab. Bioinformatics tool PANTHER (Thomas et al., 2022) was used to describe protein functions by gene ontology (GO) over-representation analysis, while SPSS and Prism 10 were used to perform statistical and survival analyses to determine the relationship between candidate proteins with neurological and clinical outcomes.

5.3 Differentially expressed proteins in people with MND

5.3.1 Clinical characteristics of people with MND and healthy individuals

Thirty-nine people with MND were recruited who were diagnosed as either ‘clinically definite’ or ‘clinically probable’ MND in accordance with the El Escorial Criteria (Brooks et al., 2000) of MND diagnosis. Twenty-six healthy individuals were recruited that met the inclusion criteria for this study. Healthy individuals were assessed as having no neurological conditions or illness and had healthy kidney function. Spot-urine from people with MND and healthy individuals were collected at baseline from the South Australian 4th Generation MND clinic, as per human ethics 229.11. The maximum age of participation was 78 years of age to prevent age being a confounding factor for immunological biomarkers. All participants, including people with MND and healthy individuals, were selected between 40 and 78 years of age, and with a controlled hydration status of 0.3 – 3.0 mg/mL urinary creatinine to standardise urinary dilutions from spot-urine samples as per WHO guidelines (Mikheev and Lowry, 1996). Assessment by MultiStix analyses (see *section 2.1.1*) was performed to ensure participants did not have diabetes or urinary infection, and neurological and clinical data was available including ALSFRS-R scores, latencies from symptom onset, date of baseline visit and diagnosis, as well as age, gender, and site of disease onset (bulbar or limb).

5.3.2 Cross sectional analysis

Urine collection and neurological assessment was completed at first visit to the neurologist (baseline), and disease characteristics that were able to predict the survival of the MND population are reported in **Table 5.1** including the ALSFRS-R, latencies from symptom onset and diagnosis to sample collection, monthly change in ALSFRS-R (Δ FRS), and time to death were compared to urinary p75^{ECD}. The cohort consisted of 10 females (26%) and 29 males (74%), with 15 people with bulbar onset, and 24 people with limb onset that was consistent with previous studies (Shepherd et al., 2022) and (Benatar et al., 2024b). The average baseline age was 64.0 ± 10.5 years in people with MND, with an average diagnostic delay of 17.4 ± 11.6 months. The average time from sample collection to death was 19.3 (range; 8.3-26.1) months, where within this cohort, 38 individuals reached dependency on permanent assisted ventilation (PAV) or death. The average baseline ALSFRS-R score was 38.8 (range; 35.0-47.0), and the Δ FRS was 0.71 (range; 0.31-1.03) points per month. 85% of people with MND were treated with Riluzole, and one person had familial MND with a C9orf72 repeat expansion compared to the 38 remaining participants (97%) having sporadic MND.

The cohort of 26 healthy individuals consisted of 15 (58%) women and 11 (42%) men with an average age of 62.2 ± 11.9 years, that was age-matched to people with MND. Urinary p75^{ECD} measured by in-house ELISA was reported as significantly different between people with MND and healthy individuals. People with MND had an average of 5.51 ± 2.20 ng p75^{ECD}/mg creatinine compared to healthy individuals, with an average presentation of 2.94 ± 1.0 ng p75^{ECD}/mg creatinine ($p < 0.0001$, Welch's T-test, **Table 5.1**). To reinforce that the differential abundance of urinary proteins from this study was not being driven by urinary characteristics of people with MND, urinary creatinine, osmolality, and protein concentrations were analysed to rule out any potential confounding factors. No significant differences were seen between the urinary creatinine, osmolality, and protein levels in people with MND compared to healthy individuals (**Table 5.1**). Creatinine had a significant correlation with urinary osmolality in people with MND ($r = 0.85$, $p < 0.0001$, **Figure 5.1A**). Further, urinary creatinine (mg/mL) was not correlated to ALSFRS-R scores at baseline ($r = -0.183$, $p = 0.265$, **Figure 5.1B**), nor was urinary osmolality (mOsm/kg) in **Figure 1C** ($r = -0.147$, $p = 0.372$), or urinary protein in **Figure 5.1D** ($r = -0.026$, $p = 0.873$). This highlights that disease was not correlated to creatinine, osmolality, or change in protein concentration. Thus, creatinine as previously described in (Shepherd et al., 2022, Shepherd et al., 2017) is valid for correcting for urinary dilution, and there is no association of protein in urine with disease inferring healthy kidney function in people with MND.

Table 5.1: Baseline cohort of study participants for untargeted protein discovery by mass spectrometry

All disease characteristics and clinical information for people with MND and healthy individuals at baseline were reported. The ALSFRS-R is the Revised ALS Functional Rating Scale at baseline, and Δ FRS is the rate of change in ALSFRS-R per month from symptom onset to sample collection. Results were reported as mean $\pm$ standard deviation, or percentages where applicable.

Disease Characteristic		Healthy Individuals (n=26)	People with MND (n=39)
Gender (male, female)	Male, (%)	11, (42.3%)	29, (74.3%)
Site of onset (Bulbar, Limb)	Bulbar, (%)	N/A	15, (38.5%)
Age at Sample (years)	Mean $\pm$ SD (range)*	62.2 $\pm$ 11.9 (56.0-79.4)	64.0 $\pm$ 10.5 (58.6-77.9)
Baseline ALSFRS-R	Mean $\pm$ SD (range)*	N/A	38.8 $\pm$ 4.9 (35.0-47.0)
Δ FRS	Mean $\pm$ SD (range)*	N/A	0.73 $\pm$ 0.55 (0.31-1.03)
Diagnostic delay (months)	Mean $\pm$ SD (range)*	N/A	17.4 $\pm$ 11.6 (5.9-48.6)
Time from sample to death (months)	Mean $\pm$ SD (range)*	N/A	19.3 $\pm$ 17.0 (8.3-26.1)
Disease phenotype (familial, sporadic)	Familial, (%)	N/A	1, (2.5%)
Death	Deceased, (%)	N/A	38, (97%)
Riluzole treatment	Riluzole, (%)	N/A	28, (85%)
Urinary p75 ^{ECD} ELISA (ng p75 ^{ECD} /mg creatinine)	Mean $\pm$ SD (range)*	2.94 $\pm$ 1.0 (2.2 – 4.9) ^x	5.51 $\pm$ 2.2 (3.8– 6.65) ****
Creatinine, (mg/mL)	Mean $\pm$ SD (range)*	1.44 $\pm$ 0.84 (0.8 – 2)	1.07 $\pm$ 0.39 (0.78 – 1.92) ^{NS}
Osmolality (mOsm/mg)	Mean $\pm$ SD (range)*	0.69 $\pm$ 0.27 (0.48 – 0.87)	0.69 $\pm$ 0.22 (0.55 – 0.86) ^{NS}
Protein, (mg/mL)	Mean $\pm$ SD (range)*	0.070 $\pm$ 0.045 (0.038- 0.09)	0.087 $\pm$ 0.050 (0.05 – 0.12) ^{NS}
*Range is 25th, and 75th percentiles. NS=not significant, ****=<0.0001. Welch's T-test between MND and HI at baseline. Also, Δ FRS=(48–ALSFRS–R at sample collection))/(Time from symptom onset) (Kimura et al., 2006). Urinary creatinine, osmolality, and protein were measured as per methods. ^x =Only have p75 ^{ECD} values for 15 healthy individuals.			

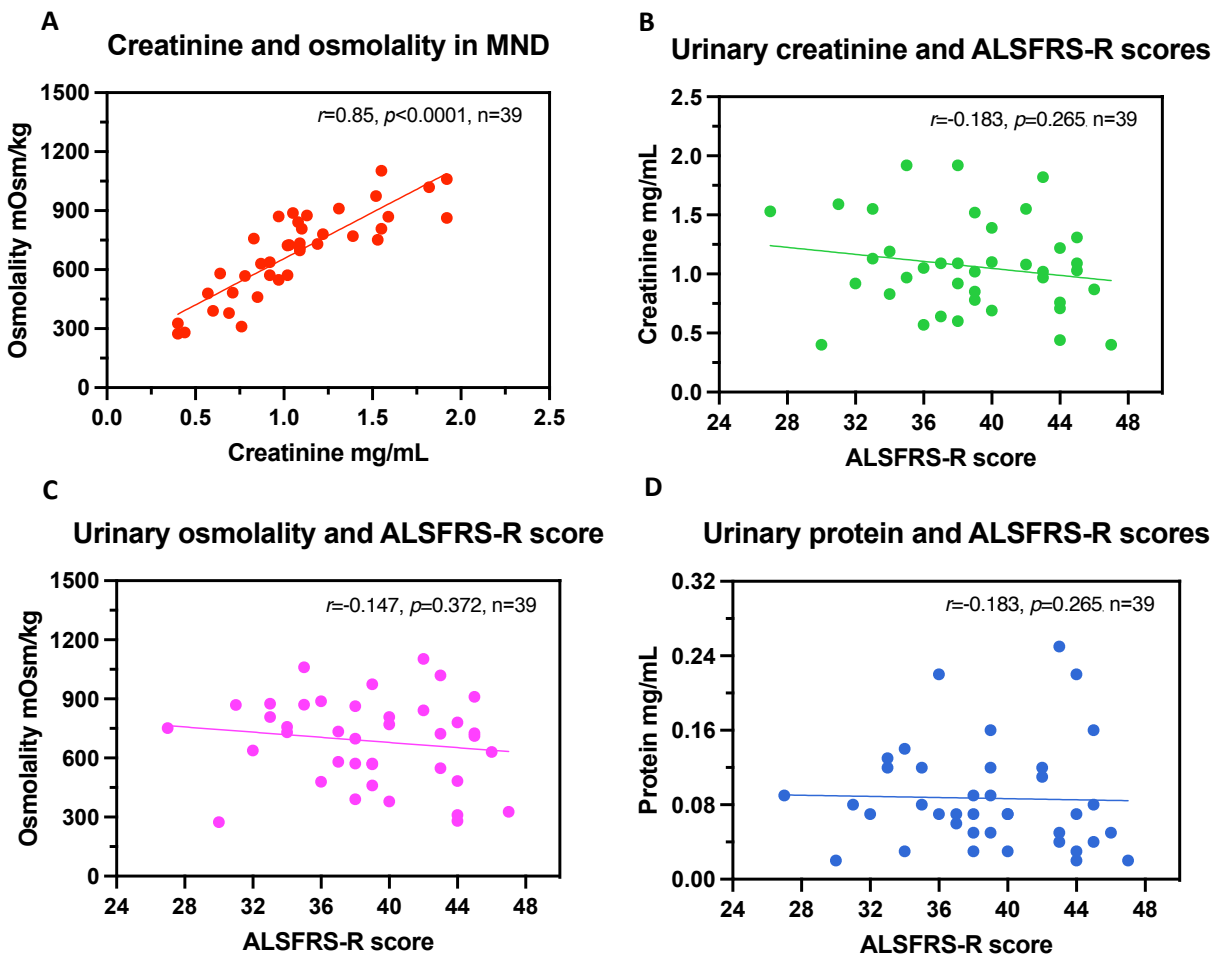


Figure 5.1: Urinary creatinine, protein, osmolality, and ALSFRS-R for people with MND protein biomarker discovery

(A) There was a significant correlation between urinary creatinine and osmolality in people with MND (●, $n=39$) as renal dilution normalisation factors ($r=0.85$, $p<0.0001$). (B) There was no significant correlation between urinary creatinine and ALSFRS-R scores (●, $r=-0.183$, $p=0.265$), or in (C) between urinary osmolality and ALSFRS-R scores in people with MND (●, $r=-0.147$, $p=0.372$). Lastly, (D) reported no significant correlation between urinary protein concentration and ALSFRS-R scores (●, $r=-0.183$, $p=0.265$). Statistical significance was determined using Pearson's correlation, Prism 10.

5.3.3 Prognostic characteristics of people with MND

Interestingly, urinary p75^{ECD} measured by in-house ELISA was correlated to ALSFRS-R scores at baseline in **Figure 5.2** ($r=-0.33$, $p=0.041$), indicating that individuals with poorer functional status exhibited higher urinary p75^{ECD} levels at baseline.

The association between prognostic characteristics at baseline and survival was evaluated by a Cox-Regression analysis in SPSS to determine the factors that influenced survival. Firstly, univariate Cox-Regression survival analyses were performed on 39 people with MND. In this cohort, 38 people with MND had reached end-stage disease or PAV, with one person censored at last sample collection (8.5 months from sample collection). By univariate Cox-Regression analysis model, the covariates: site of disease onset (bulbar compared to limb onset), change in ALSFRS-R per month from symptom onset (Δ FRS), gender (male or female), age at sample collection, and baseline urinary p75^{ECD} were independently assessed as predictors of survival from time to death from sample collection (months). **Table 5.2** demonstrates that urinary p75^{ECD} (HR: 1.27, 95% CI: 1.08-1.50, $p=0.004$), and Δ FRS (HR: 2.60, 95% CI: 1.49-4.55, $p<0.001$) were both independent prognostic factors for survival. Multivariate Cox-Regression analysis was then performed to determine if urinary p75^{ECD} was able to add value to the Δ FRS was and site of onset as prognosticators. **Table 5.2** demonstrated that the Δ FRS (HR: 2.81, 95% CI: 1.40-5.63, $p=0.004$) and urinary p75^{ECD} (HR: 1.22, 95% CI: 1.03-1.44, $p=0.023$) were significant predictors of survival by multivariate cox proportional hazards model, showing that urinary p75^{ECD} was able to add prognostic value to clinical assessment by the Δ FRS, consistent with previous analysis of urinary p75^{ECD} (Shepherd et al., 2017, Shepherd et al., 2022, Benatar et al., 2024b).

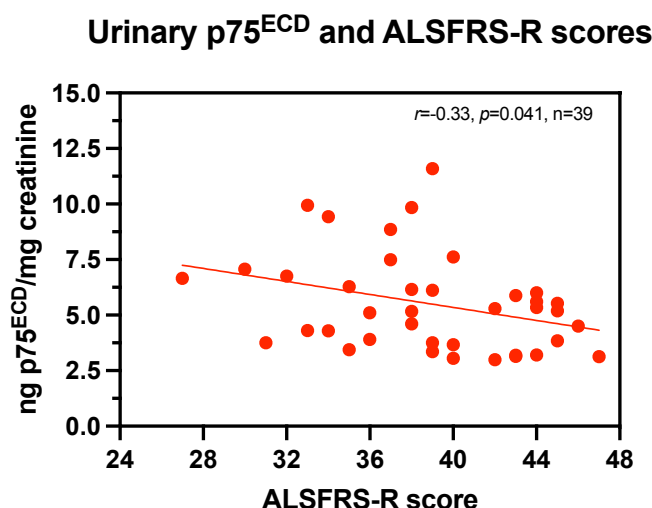


Figure 5.2: Urinary p75^{ECD} by ELISA compared to ALSFRS-R scores

The relationship between urinary p75^{ECD} and functional disease decline by ALSFRS-R score at baseline was explored. Urinary p75^{ECD} detected by in-house ELISA and ALSFRS-R scores recorded at baseline were significantly correlated in 39 people with MND (●, $r=-0.33$, $p=0.041$). Statistical significance was determined using Pearson's correlation, Prism 10.

	Univariate Cox-Regression				Multivariate Cox-Regression			
			95% Confidence Interval				95% Confidence Interval	
Parameter	<i>p</i> value	Hazard ratio	Upper	Lower	<i>p</i> value	Hazard ratio	Upper	Lower
Gender	0.400	1.396	0.642	3.035	0.19	1.82	0.74	4.49
Site of onset	0.135	1.683	0.850	3.331	0.086	1.90	0.91	3.97
Baseline age	0.748	0.990	0.962	1.03	0.894	0.998	0.960	1.036
ΔFRS	<0.001	2.601	1.488	4.548	0.004	2.806	1.399	5.627
p75 ^{ECD} ELISA	0.004	1.269	1.081	1.490	0.023	1.218	1.028	1.443

Table 5.2: Baseline cohort for protein discovery and survival by Cox proportional hazards models

By univariate Cox-Regression survival analysis, urinary p75^{ECD} (HR: 1.27, 95% CI: 1.08-1.50, $p=0.004$) and ΔFRS (HR: 2.60, 95% CI: 1.49-4.55, $p<0.001$) were both prognostic for survival in people with MND at baseline ($n=39$). By multivariate Cox-Regression analysis, urinary p75^{ECD} (HR: 1.22, 95% CI: 1.03-1.44, $p=0.023$) and the ΔFRS (HR: 2.81, 95% CI: 1.40-5.63, $p=0.004$) remained significant as the only predictors of survival at baseline.

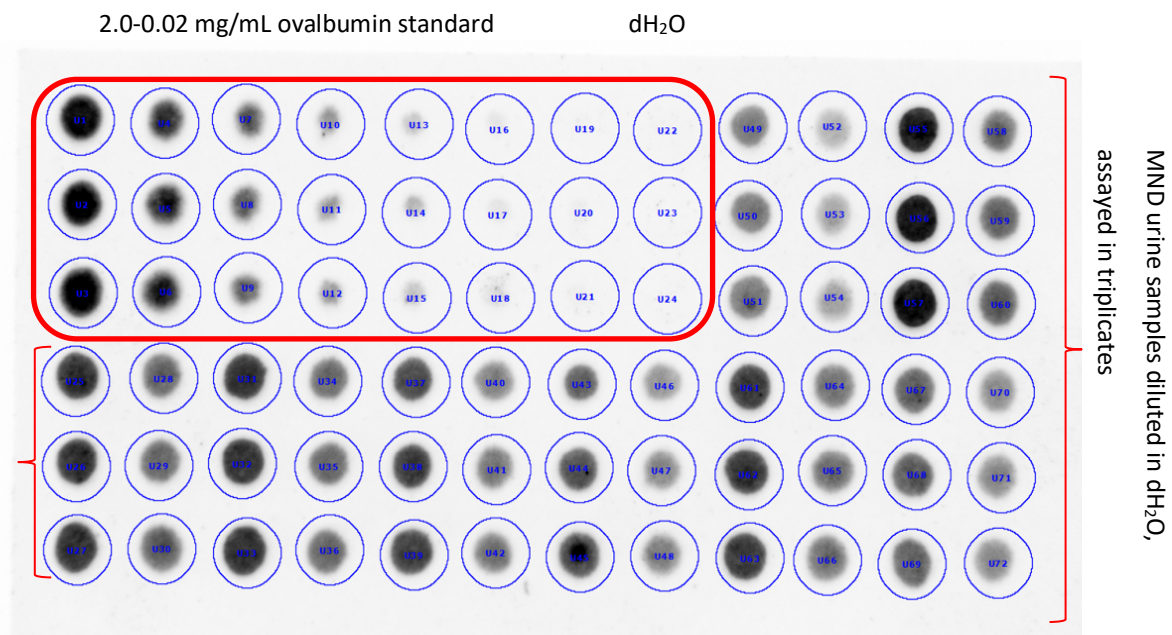
5.3.4 Isolating the urinary proteome

To isolate and concentrate the proteome from human urine in a way that removed unwanted salts and debris, while achieving the desired concentration factor for optimal discovery, the diafiltration technique (described in *section 2.6.1*) was applied to separate, retain, and concentrate proteins above 3 kDa from the remaining urinary matrix. The protein concentration of all 65 study participants was estimated using the EZQ assay described in *section 2.6.5* and shown in **Figure 5.3A**. By using an ovalbumin standard curve in the EZQ assay between 0.02 and 2 mg/mL, the average final protein concentration for 65 people was 1.46 ± 0.98 mg/mL (**Table 5.1**). This concentrated urinary proteins to a volume that was optimal for protein estimation, normalisation, tryptic digestion, and mass spectrometer loading.

5.3.5 Estimating and normalising urinary protein concentrations

To ensure the protein concentration of all samples were reliable for equal loading onto the mass spectrometer, urinary proteins were then normalised by SDS-PAGE (described in *section 2.6.6*). This was achieved by loading 10 ug of proteins from each sample onto 4-20% stain-free gels, and ImageLab was used to compare the fluorescent area of each protein lane in **Figure 5.3B** to normalise for differences in protein loading. In total, 10 stain-free gels were used to normalise 65 samples to the reference sample (Lane 1, **Figure 5.3B**). Protein concentrations were corrected from factors ranging from 0.4 times, up to 2.8 times the concentration of the reference sample to ensure that equal protein across all samples was loaded onto the mass spectrometer.

A



B

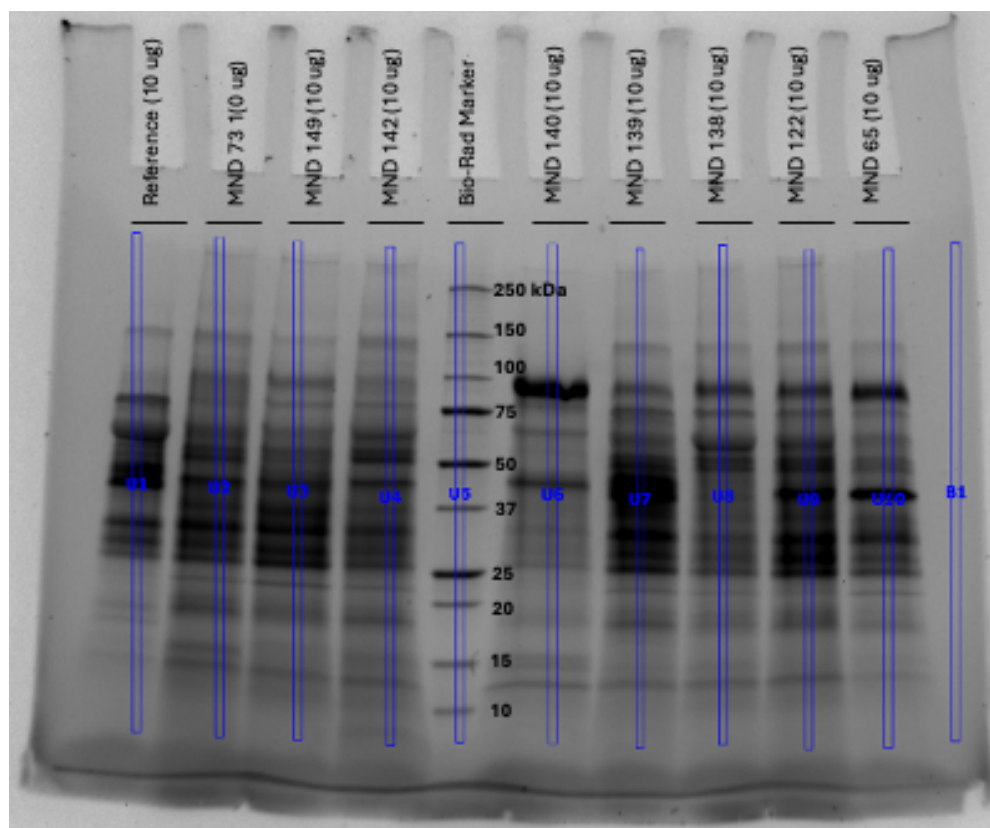


Figure 5.3: Urinary protein concentration, estimation, and normalisation

(A) Urinary protein concentrations were estimated by EZQ Protein Estimation Kit by testing in triplicates and interpolation against ovalbumin standard curve from 2.0 to 0.02 mg/mL protein. (B) Protein concentrations from EZQ estimation were normalised to 10 µg reference (Lane 1) using SDS-PAGE. Bio-Rad Criterion TGX Stain-Free Precast Gels were used (10-well comb, 50 µL, 1.0mm, Any kDa) with 5 µL of a Bio-Rad 250–10 kDa molecular weight ladder (Lane 5). All fluorescent screening was performed by ImageLab, images were set to auto-expose upon activation of each stain-free gel.

5.3.6 Tryptic digest and mass spectrometry detection of urinary proteins

Each protein sample was digested into peptides using a Trypsin Protease, after being reduced by DTT and alkylated by IAA to unravel the tertiary structure of the proteins allowing Trypsin to cleave protein sequences at the lysine and arginine amino acid sites. Trypsin was used in a 1:50 ratio to total protein, and at an absolute ratio of 200 ng per 10 μ g protein. This resulted in a Trypsin concentration of 100 ng/ μ L, in a final digest volume of 200 μ L, at 37°C overnight. The temperature, concentration, and trypsin/protein ratio ensured the optimal working efficacy of Trypsin, with minimized missed-cleavages and non-specific cleavages of urinary proteins.

5.3.7 Library generation of urinary proteins

Following the tryptic digest of urinary proteins in 39 people with MND and 26 healthy individuals ($n=65$), 1 μ g of tryptic peptides were pooled together for a total of 65 μ g to generate a combined spectral library from 8 reversed phase high pH library fractions (described in *section 2.7.3*). 1 μ g of fractionated peptides were loaded onto the Thermo Exploris 480 mass spectrometer that was run in data dependent acquisition (DDA) mode. All spectra were searched against the UniProt human database (*UniProt human reviewed September 2023*), which generated a library of approximately 1,970 protein groups with a 1% false discovery rate (FDR). This allowed for a maximum of 2 missed cleavages and a maximum mass error of 0.02 Da (20 ppm). Using the MGF script (see *section 2.8.3*), this data generated 45 variable m/z windows for data independent acquisition (DIA) mode between the mass ranges of 350.5 m/z to 1,199 m/z for the optimal quantification of protein groups based on their abundance and peptide specific matches (PSMs) in DDA mode (**Appendix 8.3**).

5.3.8 Differentially changed urinary proteins in people with MND at baseline

Following library generation, the urinary proteome prepared from 39 people with MND, and 26 age-matched healthy individuals were then individually analysed in DIA mode on the Thermo Exploris 480 mass spectrometer. 1 μ g of urinary peptides were loaded onto a C18 trap-column and eluted onto a 25 cm C18 analytical column. 45 variable m/z windows were analysed over a gradient of 150-minutes, and Spectronaut was used to analyse the MS1 and MS2 spectra against the generated spectral library to identify an average of 1,270 protein groups per sample ($n=65$). Spectronaut applied a normalisation factor based on the total ion current (TIC) of each sample to account for variances in injection load such that

equal amounts of peptides were analysed, providing a box-plot of the sensitivity responses from all samples before (**Figure 5.4A**) and after (**Figure 5.4B**) normalisation.

Initially, 1,760 differentially changed protein groups were identified in all 65 participants. Using peptide precursor filtering, Spectronaut was programmed to exclude protein groups in less than 50% of total number of samples ($n=65$), reporting a final number of 1,350 protein groups for down-stream analysis. The remaining 410 protein groups were deemed low-confidence and may have been discovered in low abundance (i.e., NaN PG.Quantity output), identified as single hits (i.e., discovered in MND only), or false positives below the 50% discovery threshold. Notably, this filter did not discount the differential abundance of said protein groups, where single hits found in MND, or healthy individuals only, were found particularly interesting and explored later in this analysis. However, for the purpose of this study, prognostic biomarker candidates must have been present in at least 50% of the pilot population to be confident in their differential abundance and that changes in target protein abundance were resulting from disease. The principal component analysis (PCA) plot in **Figure 5.5** displayed the clustering of samples from people with MND (red, ♦) compared to healthy individuals (green, ◆) based on their protein profiles, with the largest component of variance in the data being up to 9.71% (PC1), explaining the expected overlap in protein abundance between all individuals while highlighting some variation between people with MND and healthy individuals. This data analysis performed in Spectronaut.

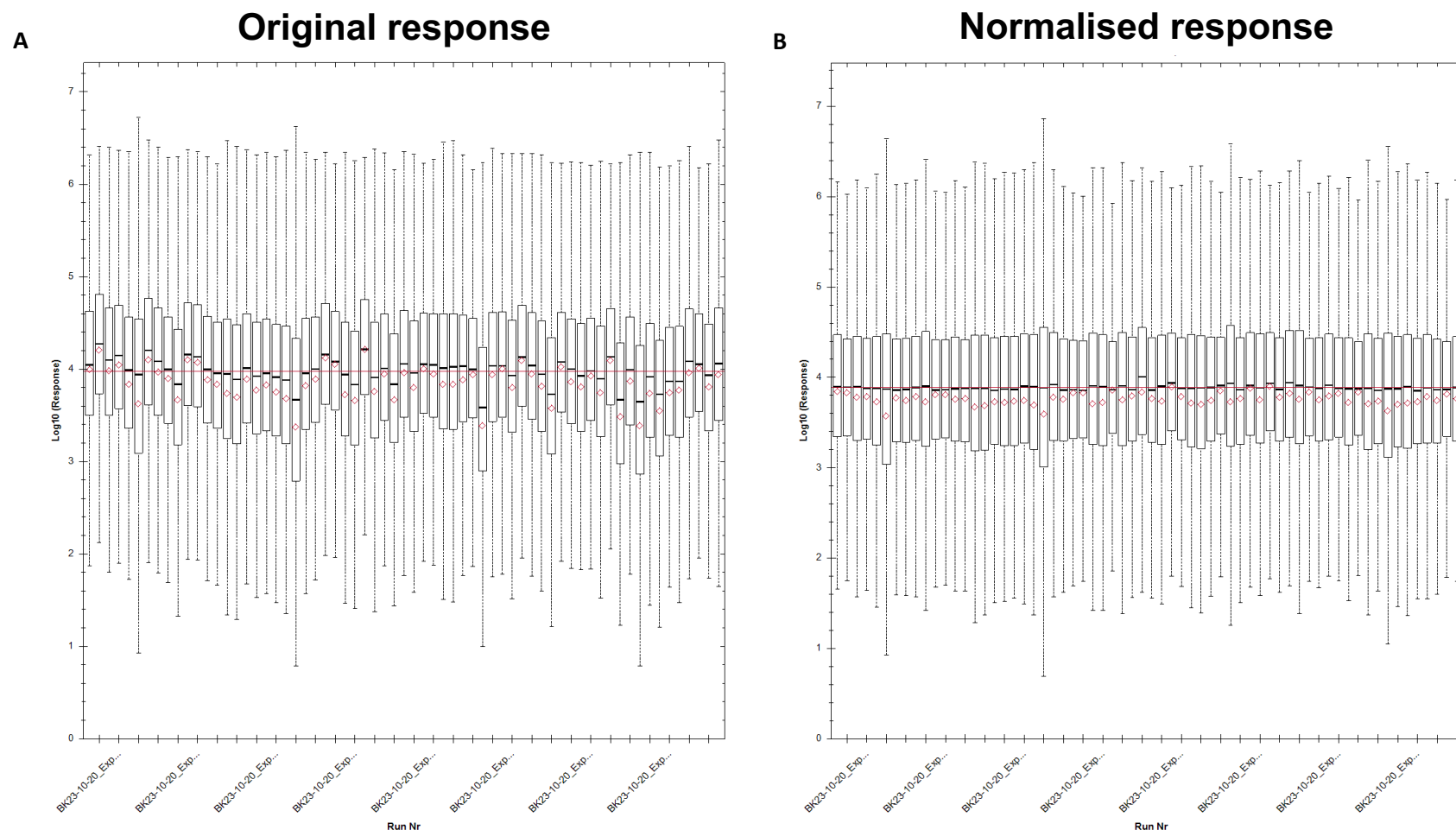


Figure 5.4: Mass spectrometer loading and Spectronaut normalisation

Original response (**A**) and normalised response (**B**) plots were calculated by Spectronaut after an 'equal' 1 μ g of urinary peptides were loaded onto the 25 cm C18 analytical column after estimation and normalisation. Before Spectronaut analysed the spectral data, Spectronaut normalised the intensity of the total ion current (TIC) to match the median Log₁₀ (response, y-axis) across all individual samples loaded (x-axis). The red line represents the median Log₁₀ precursor response, or TIC, that each sample was normalised to. Statistics were performed by Spectronaut, represented as box and whisker plots.

Principal component analysis

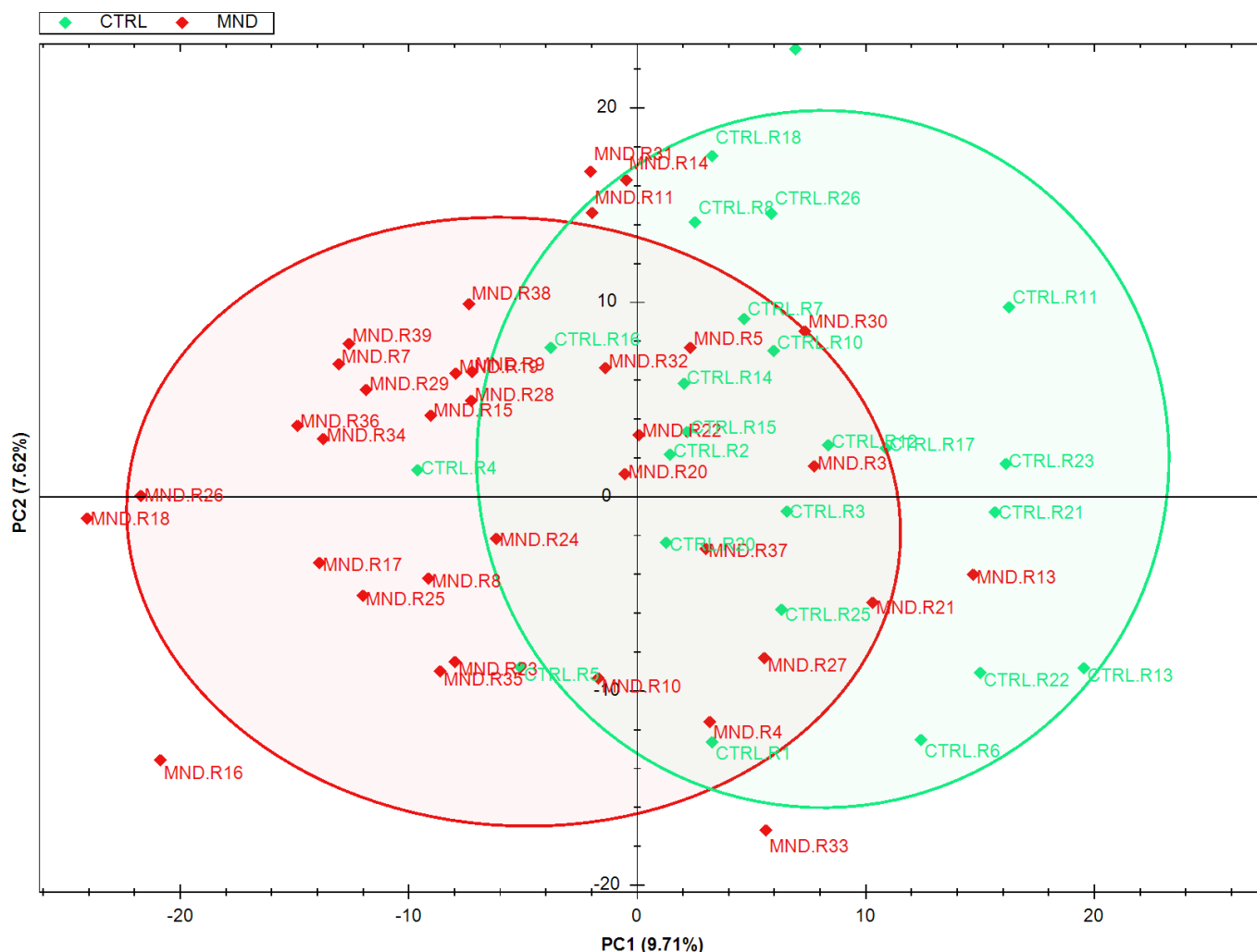


Figure 5.5: Principal component analysis (PCA) from Spectronaut

This scatter plot shows a principal component analysis (PCA) made on the protein abundance profiles of 65 samples from DIA performed on a 25cm C18 analytical column. The x- and y-axes represent the first two principal components, PC1 and PC2 respectively, which together explain 17.33% of the total variance. Samples coming from people with MND are shown in red (♦) compared to healthy individuals shown in green (♦). The variance of the first principal component, PC1, was 9.71%, accounting for the most amount of variance reported between the two groups, explaining some variation in protein abundance in people with MND. The variance on the second principal component, PC2, was 7.62%. Statistics were performed by Spectronaut.

5.3.9 Differentially changed protein groups normalised to creatinine

The protein quantities for 1,350 protein groups discovered by DIA analysis and Spectronaut were adjusted for renal dilution by urinary creatinine to account for inter-individual variances in hydration status, time of day, and protein concentrations, ensuring changes in protein biomarkers reflect true biological changes of disease rather than variances in urine dilution. Urinary creatinine was quantified in neat urine prior to diafiltration, and the concentration did not exceed 3.0 ± 0.3 mg/mL. For correct normalisation, the absolute quantity of peptides injected onto the mass spectrometer was calculated. This produced a unique 'hypothetical' injection volume that was loaded onto the mass spectrometer to achieve 1 μ g of protein load for each sample, had it not been concentrated. This calculation was described in **Appendix 8.6**, using the diafiltration concentration factor and instrument injection volume to infer the quantity of neat peptides loaded onto the mass spectrometer. The 'PG.Quantity' of each protein group was divided by the 'hypothetical' injection volume, and by renal dilution correction factor creatinine (mg/mL), generating a 'PG.Quantity/mg creatinine.' This produced a normalised protein group quantity to creatinine that reflected the neat concentration of urinary proteins, prior to diafiltration, to avoid circumstances of a statistically incorrect 'double normalisation.'

Using MatLab, a script was generated to group, normalise, and analyse all 1,350 differentially expressed protein groups. Protein accession numbers, unique to each protein group, were sorted into 'MND' ($n=39 \times 1,350$ proteins) or 'CTRL' ($n=26 \times 1,350$ proteins) groups based on their normalised quantity. This created two condition groups, to perform unique two-tailed unpaired T-tests for each unique protein identified. The two-tailed T-test between 1,350 protein groups common between people with MND and healthy individuals identified 751 significantly changed proteins ($p<0.05$). To control for type 1 error, the data from multiple T-tests was corrected for multiple comparisons by false discovery rate (FDR). The p -values were ranked in ascending order, applying the Benjamini-Hochberg (BH) technique to control for FDR. This was used to apply a stricter $q<0.01$, and as a result, the two-tailed T-tests of unique proteins between people with MND and healthy individuals identified 257 significantly changed proteins normalised to creatinine ($q<0.01$, **Figure 5.6**) using MatLab. Of the 1,350 total proteins, proteins in red (●) represented 257 significantly changed proteins with most up-regulated, and 6 down-regulated protein groups. Proteins of interest were highlighted in green (●). **Table 5.3** listed each of the significantly changed protein candidates at baseline (●, $q<0.01$, $-\text{Log}_{10} q$ value ≥ 2.00 , **Figure 5.6**) that were identified by DIA-mass spectrometry for downstream analyses.

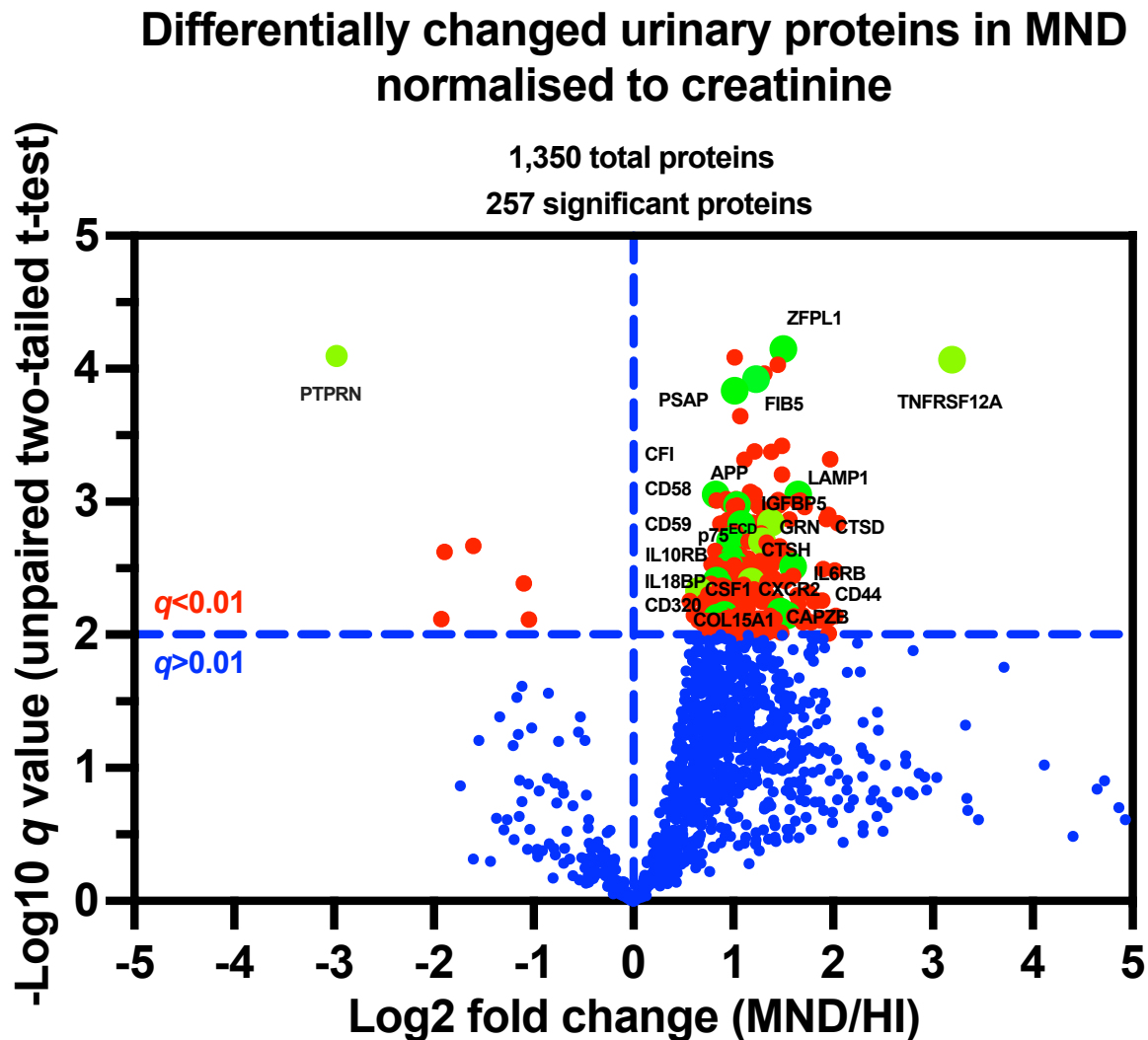


Figure 5.6: Differentially changed protein groups in people with MND compared to healthy individuals normalised to creatinine

Volcano plot of urinary proteins from 39 people with MND and 26 healthy individuals (HI) by DIA mode and normalised to creatinine using MatLab (2-way T-test). 1,350 total protein groups were identified. 1,093 proteins shown in blue (●) were not significantly changed, and 257 protein groups were significantly changed in people with MND (2-way T-test, multiple comparisons corrected by FDR and BH, ●, $q < 0.01$), when considering protein groups are in at least 50% of all study participants. Protein groups were organised by their $-\text{Log}_{10} q$ value (threshold, $q < 0.01$, $-\text{Log}_{10} q$ value ≥ 2.00), compared to their Log_2 fold change in people with MND and HI. 257 protein groups were significantly changed with most up-regulated, and 6 down-regulated protein groups. 24 proteins of interest are named by gene and highlighted in green (●) for further discussion and validation.

Legend; ZFPL1: zinc finger protein-like 1 (O95159), PTPRN: receptor-type tyrosine-protein phosphatase-like N (Q16849), TNFRSF12A: tumour necrosis factor receptor superfamily member 12A (Q9NP84), FIB5: fibulin-5 (Q9UBX5), PSAP: prosaposin (P07602), CTSH: pro-cathepsin H (P09668), CD58: lymphocyte function-associated antigen 3 (P19256), CD59: CD59 glycoprotein (P13987), CTSD: cathepsin D (P07339), APP: amyloid-beta precursor protein (P05067), IGFBP5: insulin-like growth factor-binding protein 5 (P24593), LAMP1: lysosome-associated membrane glycoprotein 1 (P11279), p75ECD: tumour necrosis factor receptor superfamily member 16 (P08138), CSF1: macrophage colony-stimulating factor 1 (P09603), COL15A1: collagen alpha01(XV) chain (P39059), CXCR2: C-X-C chemokine receptor type 2 (P25025), CD320: CD320 antigen (Q9NPF0), IL6RB: interleukin-6 receptor subunit beta (P40189), PAP: progranulin (P28799), IL10RB: interleukin-10 receptor subunit beta (Q08334), CD44: CD44 antigen (P16070), CFI: complement factor I (P05156), CAPZB: F-actin-capping protein subunit beta (P47756), IL18BP: interleukin-18 binding protein (O95998).

Table 5.3: Significantly differentially changed urinary proteins in people with MND compared to healthy individuals

The 257 unique protein groups that were identified as significantly changed in 39 people with MND compared to 26 healthy individuals from *section 5.3.7* on a 25 cm analytical column were reported after normalisation to creatinine using MatLab. The order of significance was generated by correction for multiple comparisons (FDR), post 2-way T-test in MatLab. Each candidate was summarized by their unique protein accession number (UniProt ID), brief protein description, *q*-value from two-tailed T-test (MatLab) corrected for FDR by BH, and their log2-fold change (+, upregulated, -, downregulated). Unique protein groups highlighted in green (*n*=24) from **Figure 5.6** were further explored in *section 5.4.2*.

Protein Accession	Protein Description	<i>q</i> -value (post 2-way T-test)	Log2 fold change
O95159	Zinc finger protein-like 1	7.08E-05	1.50E+00
Q16849	Receptor-type tyrosine-protein phosphatase-like N	8.00E-05	-2.97E+00
P01833	Polymeric immunoglobulin receptor	8.21E-05	1.01E+00
Q9NP84	Tumour necrosis factor receptor superfamily member 12A	8.54E-05	3.19E+00
Q6GTX8	Leukocyte-associated immunoglobulin-like receptor 1	9.34E-05	1.44E+00
O00144	Frizzled-9	1.09E-04	1.31E+00
Q9UBX5	Fibulin-5	1.20E-04	1.23E+00
P07602	Prosaposin	1.46E-04	1.01E+00
Q8TDQ0	Hepatitis A virus cellular receptor 2	2.27E-04	1.07E+00
P01591	Immunoglobulin J chain	3.81E-04	1.49E+00
Q04721	Neurogenic locus notch homolog protein 2	4.18E-04	1.22E+00
Q9NS15	Latent-transforming growth factor beta-binding protein 3	4.21E-04	1.38E+00
P49767	Vascular endothelial growth factor C	4.79E-04	1.97E+00
Q9NWX8	BRIS and BRCA1-A complex member 1	4.81E-04	1.11E+00
P07996	Thrombospondin-1	6.22E-04	1.49E+00
Q9GZM5	Protein YIPF3	8.44E-04	1.18E+00
P02655	Apolipoprotein C-II	8.48E-04	1.17E+00
P09668	Pro-cathepsin H	8.78E-04	1.65E+00
P19256	Lymphocyte function-associated antigen 3	8.80E-04	8.24E-01
Q8TBP5	Membrane protein FAM174A	8.81E-04	1.21E+00
P04275	von Willebrand factor	9.55E-04	9.30E-01
Q13308	Inactive tyrosine-protein kinase 7	9.56E-04	1.03E+00
P07711	Procathepsin L	9.66E-04	1.45E+00

Q15149	Plectin	9.75E-04	1.67E+00
Q8N2S1	Latent-transforming growth factor beta-binding protein 4	9.80E-04	8.34E-01
Q9UMX5	Neudesin	9.83E-04	1.12E+00
P06865	Beta-hexosaminidase subunit alpha	9.91E-04	1.20E+00
Q9ULI3	Protein HEG homolog 1	9.92E-04	9.80E-01
Q6NUS6	Tectonic-3	1.00E-03	1.64E+00
P15144	Aminopeptidase N	1.01E-03	1.49E+00
P35442	Thrombospondin-2	1.02E-03	1.10E+00
Q14767	Latent-transforming growth factor beta-binding protein 2	1.02E-03	1.08E+00
Q8NDC0	MAPK-interacting and spindle-stabilizing protein-like	1.04E-03	9.65E-01
P35613	Basigin	1.04E-03	9.50E-01
P08519	Apolipoprotein(a)	1.05E-03	1.41E+00
P13987	CD59 glycoprotein	1.06E-03	1.04E+00
Q92956	Tumor necrosis factor receptor superfamily member 14	1.07E-03	1.04E+00
Q13561	Dynactin subunit 2	1.07E-03	1.46E+00
Q5VSL9	Striatin-interacting protein 1	1.08E-03	1.25E+00
P11215	Integrin alpha-M	1.08E-03	1.00E+00
Q96DR8	Mucin-like protein 1	1.10E-03	1.72E+00
Q9H013	Disintegrin and metalloproteinase domain-containing protein 19	1.25E-03	1.95E+00
Q8IZA0	Dyslexia-associated protein KIAA0319-like protein	1.27E-03	1.39E+00
P09237	Matrilysin	1.34E-03	1.93E+00
Q07954	Prolow-density lipoprotein receptor-related protein 1	1.34E-03	1.30E+00
O76076	CCN family member 5	1.35E-03	1.01E+00
Q96ID5	Immunoglobulin superfamily member 21	1.36E-03	1.56E+00
Q8IXJ6	NAD-dependent protein deacetylase sirtuin-2	1.37E-03	9.50E-01
P21709	Ephrin type-A receptor 1	1.42E-03	9.23E-01
O95967	EGF-containing fibulin-like extracellular matrix protein 2	1.43E-03	1.20E+00
P07339	Cathepsin D	1.43E-03	1.38E+00
Q13753	Laminin subunit gamma-2	1.44E-03	9.82E-01
O94910	Adhesion G protein-coupled receptor L1	1.45E-03	1.04E+00
Q99102	Mucin-4	1.45E-03	2.05E+00
P35555	Fibrillin-1	1.46E-03	8.71E-01

P05067	Amyloid-beta precursor protein	1.47E-03	1.08E+00
Q13477	Mucosal addressin cell adhesion molecule 1	1.68E-03	1.01E+00
Q6UXD5	Seizure 6-like protein 2	1.69E-03	1.03E+00
Q9Y6H1	Coiled-coil-helix-coiled-coil-helix domain-containing protein 2	1.72E-03	1.28E+00
P24593	Insulin-like growth factor-binding protein 5	1.97E-03	9.67E-01
Q9HCN6	Platelet glycoprotein VI	1.98E-03	1.15E+00
P11279	Lysosome-associated membrane glycoprotein 1	1.98E-03	1.29E+00
Q9UKU6	Thyrotropin-releasing hormone-degrading ectoenzyme	2.03E-03	1.33E+00
Q96AP7	Endothelial cell-selective adhesion molecule	2.14E-03	-1.60E+00
Q96RF0	Sorting nexin-18	2.15E-03	1.46E+00
A6NI73	Leukocyte immunoglobulin-like receptor subfamily A member 5	2.33E-03	8.18E-01
Q9NZ53	Podocalyxin-like protein 2	2.39E-03	-1.89E+00
P05060	Secretogranin-1	2.42E-03	8.28E-01
P15814	Immunoglobulin lambda-like polypeptide 1	2.42E-03	1.40E+00
Q6UY14	ADAMTS-like protein 4	2.49E-03	1.07E+00
P15311	Ezrin	2.51E-03	1.55E+00
P08138	Tumor necrosis factor receptor superfamily member 16	2.51E-03	9.85E-01
Q9HAV5	Tumor necrosis factor receptor superfamily member 27	2.65E-03	1.15E+00
Q9NQ84	G-protein coupled receptor family C group 5 member C	2.73E-03	1.51E+00
O60664	Perilipin-3	2.75E-03	9.06E-01
P98095	Fibulin-2	2.76E-03	9.47E-01
P16152	Carbonyl reductase [NADPH] 1	2.79E-03	1.27E+00
Q96GP6	Scavenger receptor class F member 2	2.79E-03	1.33E+00
Q8NC42	E3 ubiquitin-protein ligase RNF149	2.82E-03	1.07E+00
P09603	Macrophage colony-stimulating factor 1	2.92E-03	1.01E+00
Q6UVK1	Chondroitin sulfate proteoglycan 4	2.95E-03	1.01E+00
Q9Y2V2	Calcium-regulated heat-stable protein 1	2.95E-03	1.06E+00
Q13873	Bone morphogenetic protein receptor type-2	2.96E-03	1.25E+00
Q92945	Far upstream element-binding protein 2	2.96E-03	7.84E-01
O75976	Carboxypeptidase D	2.97E-03	1.12E+00
P39059	Collagen alpha-1(XV) chain	2.97E-03	9.58E-01
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein	2.99E-03	8.30E-01

P27487	Dipeptidyl peptidase 4	2.99E-03	1.38E+00
Q9NZZ3	Charged multivesicular body protein 5	2.99E-03	1.01E+00
P25025	C-X-C chemokine receptor type 2	3.08E-03	1.60E+00
Q9HCM3	UPF0606 protein KIAA1549	3.20E-03	1.15E+00
P16870	Carboxypeptidase E	3.21E-03	1.90E+00
Q8N114	Protein shisa-5	3.21E-03	9.07E-01
P24821	Tenascin	3.23E-03	1.01E+00
O75340	Programmed cell death protein 6	3.23E-03	1.30E+00
Q92686	Neurogranin	3.29E-03	2.02E+00
O00462	Beta-mannosidase	3.36E-03	1.04E+00
Q14508	WAP four-disulfide core domain protein 2	3.58E-03	9.15E-01
Q8WUM4	Programmed cell death 6-interacting protein	3.59E-03	1.17E+00
P52272	Heterogeneous nuclear ribonucleoprotein M	3.60E-03	1.60E+00
P40121	Macrophage-capping protein	3.63E-03	1.06E+00
P25205	DNA replication licensing factor MCM3	3.75E-03	1.40E+00
Q9NZV1	Cysteine-rich motor neuron 1 protein	3.82E-03	8.36E-01
Q9UN36	Protein NDRG2	3.89E-03	1.36E+00
Q9NPF0	CD320 antigen	3.92E-03	8.32E-01
P08473	Neprilysin	3.93E-03	1.52E+00
Q12805	EGF-containing fibulin-like extracellular matrix protein 1	3.96E-03	1.02E+00
P17643	5,6-dihydroxyindole-2-carboxylic acid oxidase	3.98E-03	9.73E-01
P02452	Collagen alpha-1(I) chain	3.98E-03	9.66E-01
P40189	Interleukin-6 receptor subunit beta	3.99E-03	1.18E+00
P43115	Prostaglandin E2 receptor EP3 subtype	4.01E-03	9.71E-01
Q99941	Cyclic AMP-dependent transcription factor ATF-6 beta	4.01E-03	1.34E+00
Q7Z5L7	Podocan	4.09E-03	8.21E-01
P41217	OX-2 membrane glycoprotein	4.09E-03	-1.10E+00
P28799	Progranulin	4.11E-03	8.46E-01
Q8NB14	Golgi membrane protein 1	4.15E-03	7.77E-01
Q8IWU5	Extracellular sulfatase Sulf-2	4.20E-03	1.10E+00
Q5IJ48	Protein crumbs homolog 2	4.22E-03	7.94E-01
Q7Z3B1	Neuronal growth regulator 1	4.29E-03	8.88E-01

Q99733	Nucleosome assembly protein 1-like 4	4.32E-03	8.44E-01
P16112	Aggrecan core protein	4.39E-03	9.22E-01
P02749	Beta-2-glycoprotein 1	4.56E-03	1.21E+00
O43490	Prominin-1	4.60E-03	1.12E+00
Q9H461	Frizzled-8	4.61E-03	7.18E-01
Q13177	Serine/threonine-protein kinase PAK 2	4.64E-03	8.95E-01
Q08334	Interleukin-10 receptor subunit beta	4.65E-03	6.56E-01
O43278	Kunitz-type protease inhibitor 1	4.70E-03	9.56E-01
P36871	Phosphoglucomutase-1	4.73E-03	9.33E-01
Q13201	Multimerin-1	4.75E-03	8.45E-01
P05106	Integrin beta-3	4.76E-03	1.30E+00
P20827	Ephrin-A1	4.77E-03	1.32E+00
P45985	Dual specificity mitogen-activated protein kinase 4	4.83E-03	1.76E+00
P22352	Glutathione peroxidase 3	4.86E-03	1.32E+00
Q969Z4	Tumor necrosis factor receptor superfamily member 19L	4.89E-03	9.54E-01
P04004	Vitronectin	4.91E-03	1.10E+00
P17813	Endoglin	4.96E-03	1.15E+00
Q9HBB8	Cadherin-related family member 5	4.97E-03	7.48E-01
P02649	Apolipoprotein E	4.97E-03	1.10E+00
O94772	Lymphocyte antigen 6H	5.00E-03	1.34E+00
Q9BSG0	Protease-associated domain-containing protein 1	5.05E-03	1.64E+00
P12319	High affinity immunoglobulin epsilon receptor subunit alpha	5.30E-03	1.29E+00
O75071	EF-hand calcium-binding domain-containing protein 14	5.30E-03	1.17E+00
P16444	Dipeptidase 1	5.33E-03	9.95E-01
Q13263	Transcription intermediary factor 1-beta	5.53E-03	8.88E-01
Q96FE5	Leucine-rich repeat domain receptor-interacting protein 1	5.53E-03	1.84E+00
P13686	Tartrate-resistant acid phosphatase type 5	5.54E-03	1.89E+00
P21980	Protein-glutamine gamma-glutamyltransferase 2	5.54E-03	1.43E+00
Q96J84	Kin of IRRE-like protein 1	5.56E-03	7.58E-01
Q9BQ51	Programmed cell death 1 ligand 2	5.56E-03	9.87E-01
P07204	Thrombomodulin	5.58E-03	5.62E-01
O43865	S-adenosylhomocysteine hydrolase-like protein 1	5.59E-03	1.81E+00

P33908	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	5.59E-03	1.01E+00
Q7Z7G0	Target of Nesh-SH3	5.60E-03	7.07E-01
P16284	Platelet endothelial cell adhesion molecule	5.62E-03	1.29E+00
P58107	Epiplakin	5.64E-03	1.35E+00
O15354	Prosaposin receptor GPR37	5.71E-03	9.41E-01
Q96JY6	PDZ and LIM domain protein 2	5.81E-03	1.02E+00
P15291	Beta-1,4-galactosyltransferase 1	5.84E-03	1.10E+00
P08833	Insulin-like growth factor-binding protein 1	5.86E-03	1.65E+00
Q9UGN4	CMRF35-like molecule 8	5.87E-03	8.82E-01
Q9H665	IGF-like family receptor 1	5.88E-03	7.59E-01
Q9HCU0	Endosialin	6.03E-03	1.07E+00
Q96DA0	Zymogen granule protein 16 homolog B	6.08E-03	1.49E+00
Q9H305	Cell death-inducing p53-target protein 1	6.23E-03	1.14E+00
Q96RW7	Hemicentin-1	6.25E-03	7.66E-01
P16070	CD44 antigen	6.67E-03	1.47E+00
P07307	Asialoglycoprotein receptor 2	6.77E-03	7.02E-01
Q68D85	Natural cytotoxicity triggering receptor 3 ligand 1	6.78E-03	7.18E-01
Q9H251	Cadherin-23	6.79E-03	1.11E+00
P01133	Pro-epidermal growth factor	6.80E-03	9.40E-01
P22304	Iduronate 2-sulfatase	7.07E-03	8.67E-01
P05156	Complement factor I	7.10E-03	9.12E-01
P16422	Epithelial cell adhesion molecule	7.11E-03	1.05E+00
O14498	Immunoglobulin superfamily containing leucine-rich repeat protein	7.13E-03	1.69E+00
O75882	Attractin	7.14E-03	6.04E-01
Q06481	Amyloid beta precursor like protein 2	7.14E-03	7.85E-01
P47756	F-actin-capping protein subunit beta	7.15E-03	1.54E+00
Q9UKZ9	Procollagen C-endopeptidase enhancer 2	7.17E-03	1.07E+00
P00742	Coagulation factor X	7.18E-03	1.37E+00
P05090	Apolipoprotein D	7.18E-03	2.03E+00
Q6UY11	Protein delta homolog 2	7.19E-03	9.21E-01
P31997	Carcinoembryonic antigen-related cell adhesion molecule 8	7.20E-03	1.70E+00
P63027	Vesicle-associated membrane protein 2	7.20E-03	8.16E-01

Q9UIB8	SLAM family member 5	7.21E-03	7.30E-01
P13598	Intercellular adhesion molecule 2	7.23E-03	1.61E+00
Q14393	Growth arrest-specific protein 6	7.27E-03	1.12E+00
Q9H3U7	SPARC-related modular calcium-binding protein 2	7.42E-03	9.72E-01
P31025	Lipocalin-1	7.42E-03	1.65E+00
O95998	Interleukin-18-binding protein	7.44E-03	8.37E-01
P05155	Plasma protease C1 inhibitor	7.45E-03	9.56E-01
Q92859	Neogenin	7.48E-03	1.15E+00
P0DOX2	Immunoglobulin alpha-2 heavy chain	7.51E-03	1.18E+00
P18428	Lipopolysaccharide-binding protein	7.53E-03	1.96E+00
P16403	Histone H1.2	7.54E-03	1.28E+00
P24347	Stromelysin-3	7.54E-03	1.32E+00
O94826	Mitochondrial import receptor subunit TOM70	7.55E-03	9.61E-01
Q9NZP8	Complement C1r subcomponent-like protein	7.56E-03	9.46E-01
Q9Y6R7	IgGFc-binding protein	7.56E-03	1.89E+00
Q9UHG2	ProSAAS	7.57E-03	9.33E-01
O95197	Reticulon-3	7.58E-03	1.24E+00
P23142	Fibulin-1	7.58E-03	9.64E-01
Q8NHP8	Putative phospholipase B-like 2	7.58E-03	1.41E+00
O75891	Cytosolic 10-formyltetrahydrofolate dehydrogenase	7.60E-03	-1.93E+00
P31946	14-3-3 protein beta/alpha	7.68E-03	-1.05E+00
P50395	Rab GDP dissociation inhibitor beta	7.78E-03	1.07E+00
P54826	Growth arrest-specific protein 1	7.78E-03	7.03E-01
P07195	L-lactate dehydrogenase B chain	7.79E-03	1.01E+00
Q7L0X0	TLR4 interactor with leucine rich repeats	7.81E-03	1.17E+00
Q9UM47	Neurogenic locus notch homolog protein 3	7.81E-03	6.51E-01
Q495M3	Proton-coupled amino acid transporter 2	7.82E-03	1.87E+00
P42785	Lysosomal Pro-X carboxypeptidase	7.83E-03	8.65E-01
P98164	Low-density lipoprotein receptor-related protein 2	7.83E-03	7.55E-01
Q16769	Glutaminyl-peptide cyclotransferase	7.84E-03	9.58E-01
Q9Y6U3	Scinderin	8.02E-03	1.70E+00
P55259	Pancreatic secretory granule membrane major glycoprotein GP2	8.06E-03	1.78E+00

Q9UHF0	Tachykinin-3	8.07E-03	1.34E+00
P12111	Collagen alpha-3(VI) chain	8.09E-03	6.96E-01
P09619	Platelet-derived growth factor receptor beta	8.10E-03	7.63E-01
P26927	Hepatocyte growth factor-like protein	8.12E-03	6.98E-01
O94813	Slit homolog 2 protein	8.13E-03	6.88E-01
P09758	Tumor-associated calcium signal transducer 2	8.19E-03	9.84E-01
P53801	Pituitary tumor-transforming gene 1 protein-interacting protein	8.30E-03	1.01E+00
Q6UX73	UPF0764 protein C16orf89	8.33E-03	1.20E+00
P41222	Prostaglandin-H2 D-isomerase	8.40E-03	1.21E+00
Q6EMK4	Vasorin	8.40E-03	1.21E+00
P01589	Interleukin-2 receptor subunit alpha	8.42E-03	7.30E-01
P14543	Nidogen-1	8.46E-03	8.42E-01
P28908	Tumor necrosis factor receptor superfamily member 8	8.47E-03	8.67E-01
Q8WVN6	Secreted and transmembrane protein 1	8.47E-03	1.23E+00
P81605	Dermcidin	8.48E-03	1.37E+00
Q15043	Metal cation symporter ZIP14	8.48E-03	1.05E+00
P11362	Fibroblast growth factor receptor 1	8.74E-03	1.20E+00
P80297	Metallothionein-1X	8.90E-03	1.31E+00
O00299	Chloride intracellular channel protein 1	9.05E-03	1.12E+00
Q03591	Complement factor H-related protein 1	9.08E-03	1.23E+00
P04406	Glyceraldehyde-3-phosphate dehydrogenase	9.22E-03	1.05E+00
O43399	Tumor protein D54	9.24E-03	1.09E+00
P54760	Ephrin type-B receptor 4	9.24E-03	1.06E+00
Q08380	Galectin-3-binding protein	9.27E-03	1.17E+00
P00558	Phosphoglycerate kinase 1	9.30E-03	1.17E+00
O14791	Apolipoprotein L1	9.33E-03	8.82E-01
P22732	Solute carrier family 2, facilitated glucose transporter member 5	9.41E-03	9.74E-01
P01876	Immunoglobulin heavy constant alpha 1	9.44E-03	1.36E+00
Q13145	BMP and activin membrane-bound inhibitor homolog	9.48E-03	1.08E+00
Q8WWW6	High affinity immunoglobulin alpha and immunoglobulin mu Fc receptor	9.52E-03	9.81E-01
P05556	Integrin beta-1	9.54E-03	8.29E-01
P15586	N-acetylglucosamine-6-sulfatase	9.61E-03	1.27E+00

P04196	Histidine-rich glycoprotein	9.63E-03	1.48E+00
P17936	Insulin-like growth factor-binding protein 3	9.69E-03	8.11E-01
Q92954	Proteoglycan 4	9.71E-03	1.33E+00
Q9UKY0	Prion-like protein doppel	9.72E-03	1.96E+00
P06454	Prothymosin alpha	9.73E-03	1.11E+00
Q13790	Apolipoprotein F	9.75E-03	1.07E+00
Q6UX06	Olfactomedin-4	9.78E-03	1.19E+00
P10586	Receptor-type tyrosine-protein phosphatase F	9.82E-03	8.35E-01
P35556	Fibrillin-2	9.92E-03	1.03E+00
Q969P0	Immunoglobulin superfamily member 8	9.93E-03	7.69E-01

5.4 Differentially changed urinary proteins at baseline using 40 cm C18-column

The first study was repeated to determine the repeatability and robustness of the assay, and to identify any peptides or protein groups that may not have been separated or recovered on the Exploris 480 orbitrap-mass spectrometer. The urinary proteome prepared from same 39 people with MND, and 26 age-matched healthy individuals was analysed for a second time on a Thermo Fusion Lumos orbitrap-mass spectrometer. 1 µg of urinary peptides were eluted onto a C18 trap-column, followed by a C18 analytical column that was now 40 cm in length to improve separation of the peptides. The same 45 variable m/z windows were analysed over an extended gradient of 210-minutes, and Spectronaut was used to analyse the MS1 and MS2 spectra against the generated spectral library to identify an average of 1,380 protein groups per sample ($n=65$). Spectronaut applied a TIC normalisation factor, and 1,950 differentially expressed protein groups were identified in people with MND. Peptide precursor filtering was applied in Spectronaut ($<50\%$ of samples, $q<0.05$, $-\text{Log}_{10} p$ value ≥ 1.30), reporting a final number of 1,418 protein groups in the volcano plot, highlighting 437 significantly different candidates in Spectronaut. The principal component analysis (PCA) plot displayed the clustering of samples from people with MND compared to healthy individuals based on their protein profiles. All 1,418 protein groups identified in Spectronaut were normalised to creatinine using MatLab. The 2-way T-tests of unique proteins between 39 people with MND and 26 healthy individuals identified 303 significantly changed proteins normalised to creatinine ($q<0.01$, $-\text{Log}_{10} q$ value ≥ 2.00 , **Appendix 8.7**). Of the 1,418 total proteins, 303 proteins were significantly changed.

5.4.1 Impact of analytical column length and gradient duration on urinary protein detection

The results of the second study translated to the high confidence discovery of peptides belonging to 156 protein groups that were otherwise undiscoverable in *section 5.3.7*. The extended analytical column length provided the necessary conditions to separate and identify these proteins which were summarized in **Appendix 8.8**, highlighting 11 protein groups in green (●, $q<0.01$, $-\text{Log}_{10} q$ value ≥ 2.00) that were significantly up-regulated in 39 people with MND compared to 26 healthy individuals at baseline.

5.5 Differentially changed urinary proteins of interest in people with MND

5.5.1 Significant urinary proteins at baseline by DIA-mass spectrometry

Down-stream analysis and further discussion of significant proteins in people with MND at baseline was focused on the results of *section 5.3.7*, identifying 257 significantly changed proteins at baseline normalised to creatinine ($q < 0.01$, $-\text{Log}_{10} q$ value ≥ 2.00 , **Figure 5.6**) in 39 people with MND. These significantly changed proteins have been associated with disease processes, including neurodegeneration (i.e., urinary p75^{ECD}, Shephard et al., 2017), muscle degeneration, and the inflammatory response. The three major subtypes of protein classifications involved in MND pathology has been characterized as; microglial activation and neuroinflammatory proteins, mitochondrial dysfunction and oxidative stress proteins, and dense TDP-43 and associated transposable element de-silencing proteins (O'Neill et al., 2024a). Hence, this study and future validation studies are focused on protein groups and gene functions relating to microglial activation and neuroinflammatory proteins, while showing interest in proteins related to neuro- and muscular degeneration (i.e., tumor necrosis factor receptors, elastic fibers, etc.), alongside proteins that have been previously explored in the literature as candidate biomarkers of MND prognosis.

Urinary p75^{ECD}, or tumour necrosis factor receptor superfamily member 16, UniProt ID; P08138, has been previously found by mass spectrometry (Shephard et al., 2014) and was amongst the 257 significant proteins that were differentially up-regulated in people with MND. Ranked at number 50 (**Table 5.3**) when normalised to creatinine, urinary p75^{ECD} demonstrated a significant difference in 39 people with MND compared to 26 healthy individuals (HI) (**Figure 5.7A**, $q = 2.51 \times 10^{-3}$). This agreed with significant changes at baseline for urinary p75^{ECD} quantified by ELISA (**Figure 5.7B**, $p < 0.0001$). The relationship of p75^{ECD} measured by DIA-mass spectrometry and ELISA was assessed by Pearson's correlation, reporting a significant correlation between both analytical techniques in **Figure 5.7C** ($r = 0.37$, $p = 0.022$) in 39 people with MND. However, there was not a strong correlation between these two values, which may be due to a single mass spectrometry injection compared to 5-6 replicates performed by ELISA (Shephard et al., 2017, Shephard et al., 2022).

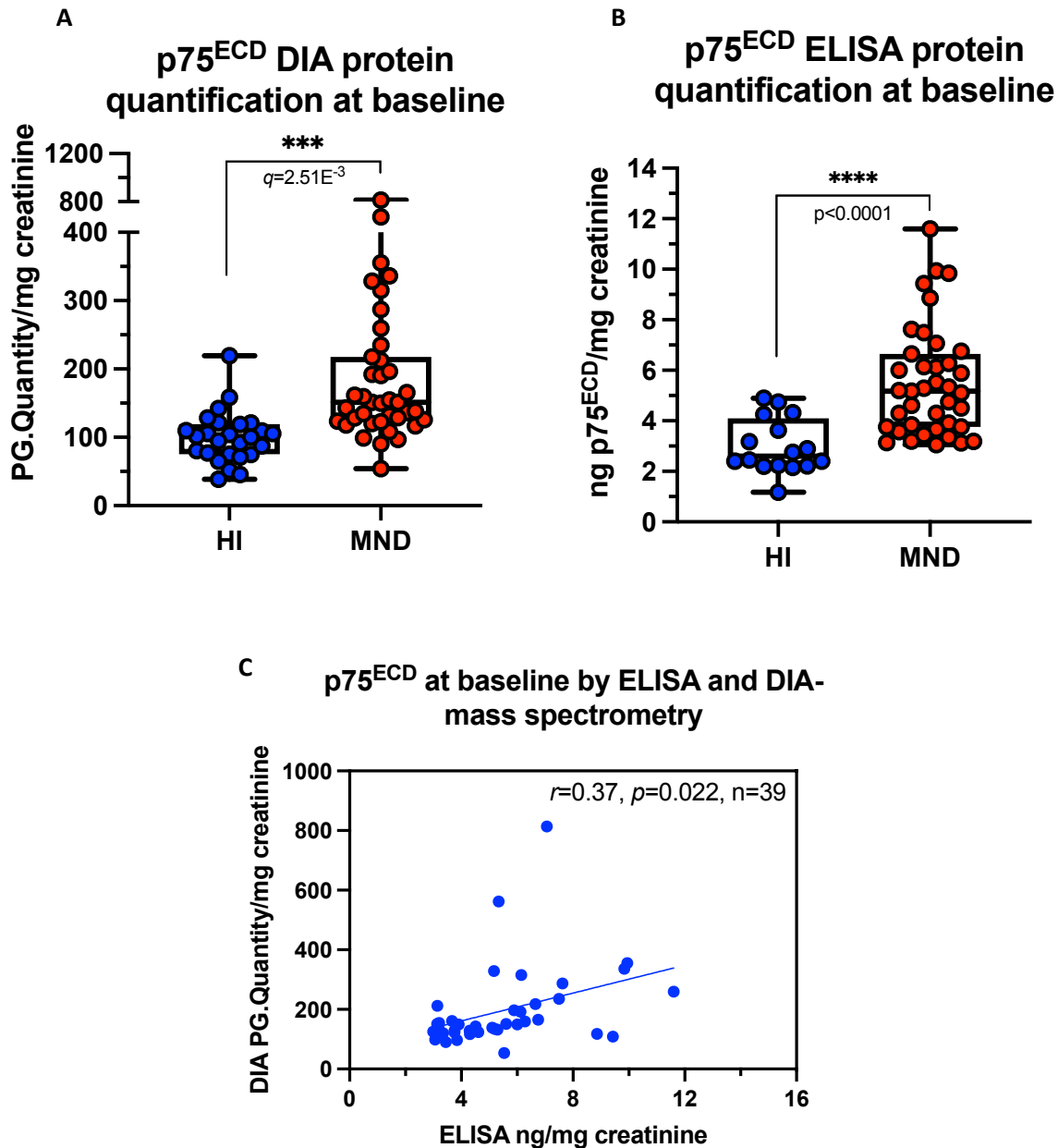


Figure 5.7: Relationship between urinary p75^{ECD} quantified by DIA and in-house ELISA

The relationship between urinary p75^{ECD} quantitation methods were explored by 2-sample T-test. **(A)** When normalised to creatinine, urinary p75^{ECD} was significantly different in 39 people with MND compared to 26 HI when quantified by DIA-mass spectrometry at baseline ($q=2.51E^{-3}$, Welch's T-test). This was performed by a single measurement. **(B)** Urinary p75^{ECD} detected by in-house ELISA was similarly significantly different in the same cohort of people with MND and healthy individuals ($p<0.0001$, Welch's T-test). These values are the average of ELISA assays performed with 3 technical replicates, across 3-5 repeat ELISA measurements. These methods were compared in **(C)** by Pearson's correlation, reporting a significant correlation between both analytical techniques ($r=0.37, p=0.022$). Statistical analyses performed in Prism 10.

5.5.2 Significant urinary proteins in MND related to immune dysfunction

The DIA study from *section 5.3.9* identified 257 significantly changed proteins in MND using a 25 cm analytical column. It was not feasible to analytically validate all proteins due to practical limitations, so a subset of proteins was selected for further investigation. Twelve inflammatory proteins (with accession numbers listed) selected from **Table 5.3** were graphed in **Figure 5.8A-F** to **Figure 5.9A-F** in 39 people with MND and 26 healthy individuals. Proteins were selected based on a statistical significance of $q < 0.01$ that was proportional to a > 1.5 fold-change in people with MND, most likely to reflect notable changes in disease pathology.

Complement factor I (P05156) in **Figure 5.8A** was significantly changed in people with MND ($q = 7.10 \times 10^{-3}$, fold-change=1.9) by processes of complement activation, and C-X-C chemokine receptor type 2 (P25025) observed significant change in **Figure 5.8B** ($q = 3.08 \times 10^{-3}$, fold-change=3.0) related to pro-inflammatory cytokine activation. Interleukin 18 binding protein (O95998) was explored and reported significant change in **Figure 5.8C** ($q = 7.44 \times 10^{-3}$, fold-change=1.8) that is an inhibitor of pro-inflammatory cytokine interleukin 18, and macrophage colony-stimulating factor-1 (P09603) reported significant change in MND in **Figure 5.8D** ($q = 2.92 \times 10^{-3}$, fold-change=2.0) as a cytokine involved in the regulation of macrophages and monocytes. There were two signal-transducing receptor components for interleukin 6 (P40189) in **Figure 5.8E** ($q = 3.99 \times 10^{-3}$, fold-change=2.2) and for interleukin 10 (Q08334) in **Figure 5.8F** ($q = 4.65 \times 10^{-3}$, fold-change=1.6) that reported significant change in people with MND.

Insulin-like growth factor binding protein 5 (P24593) was significantly changed in **Figure 5.9A** ($q = 1.97 \times 10^{-3}$, fold-change=2.0) as a binding protein involved in cell growth and regeneration, alongside CD44 antigen (P16070) as a cell surface that was increased in MND in **Figure 5.9B** ($q = 6.67 \times 10^{-3}$, fold-change=2.8). CD320 antigen (Q9NPF0) was significantly changed in MND in **Figure 5.9C** ($q = 3.92 \times 10^{-3}$, fold-change=1.8), with immune inhibitor CD59 glycoprotein (P13987) in **Figure 5.9D** ($q = 1.06 \times 10^{-3}$, fold-change=2.1), membrane glycoprotein lysosome associated membrane glycoprotein-1 (P11279) in **Figure 5.9E** ($q = 1.98 \times 10^{-3}$, fold-change=2.4), and lymphocyte function-associated antigen 3 (P19256, $q = 8.80 \times 10^{-4}$, fold-change=1.8) in **Figure 5.9F**.

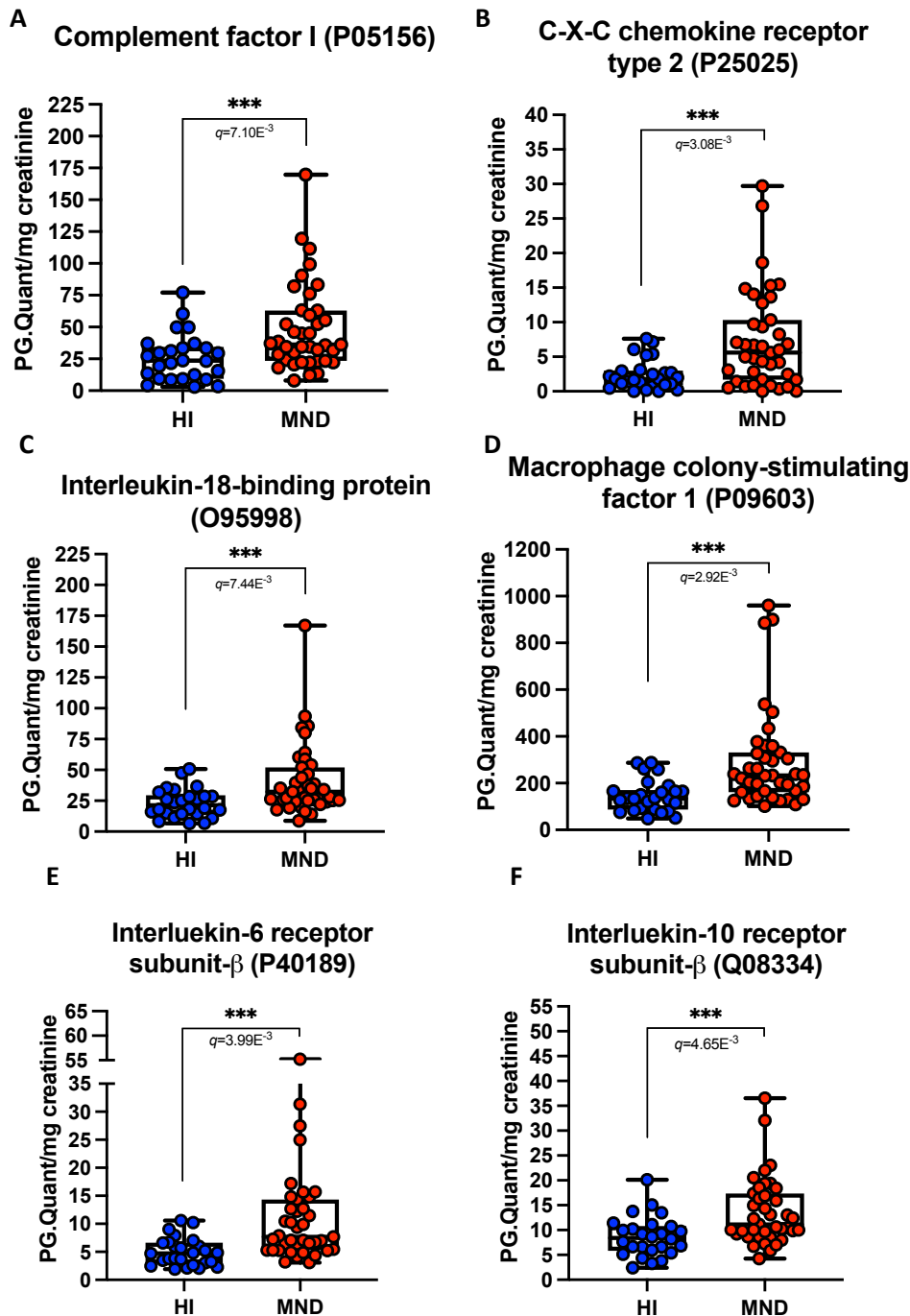


Figure 5.8: Significant proteins related to innate immune activation and inflammatory signalling pathways

The relationship between significant proteins of inflammatory processes was explored by 2-sample T-test in 39 people with MND and 26 healthy individuals. All protein candidates were significantly higher in people with MND compared to HI in (A) Complement factor I (P05156, $q=7.10E^{-3}$, fold-change=1.9), (B) C-X-C chemokine receptor type 2 (P25025, $q=3.08E^{-3}$, fold-change=3.0), (C) Interleukin 18 binding protein (O95998, $q=7.44E^{-3}$, fold-change=1.8), (D) macrophage colony-stimulating factor-1 (P09603, $q=2.92E^{-3}$, fold change=2.0), (E) interleukin 6 receptor subunit beta (P40189, $q=3.99E^{-3}$, fold-change=2.2), and (F) interleukin 10 receptor subunit beta (Q08334, $q=4.65E^{-3}$, fold-change=1.6). Statistical significance was determined using MatLab.

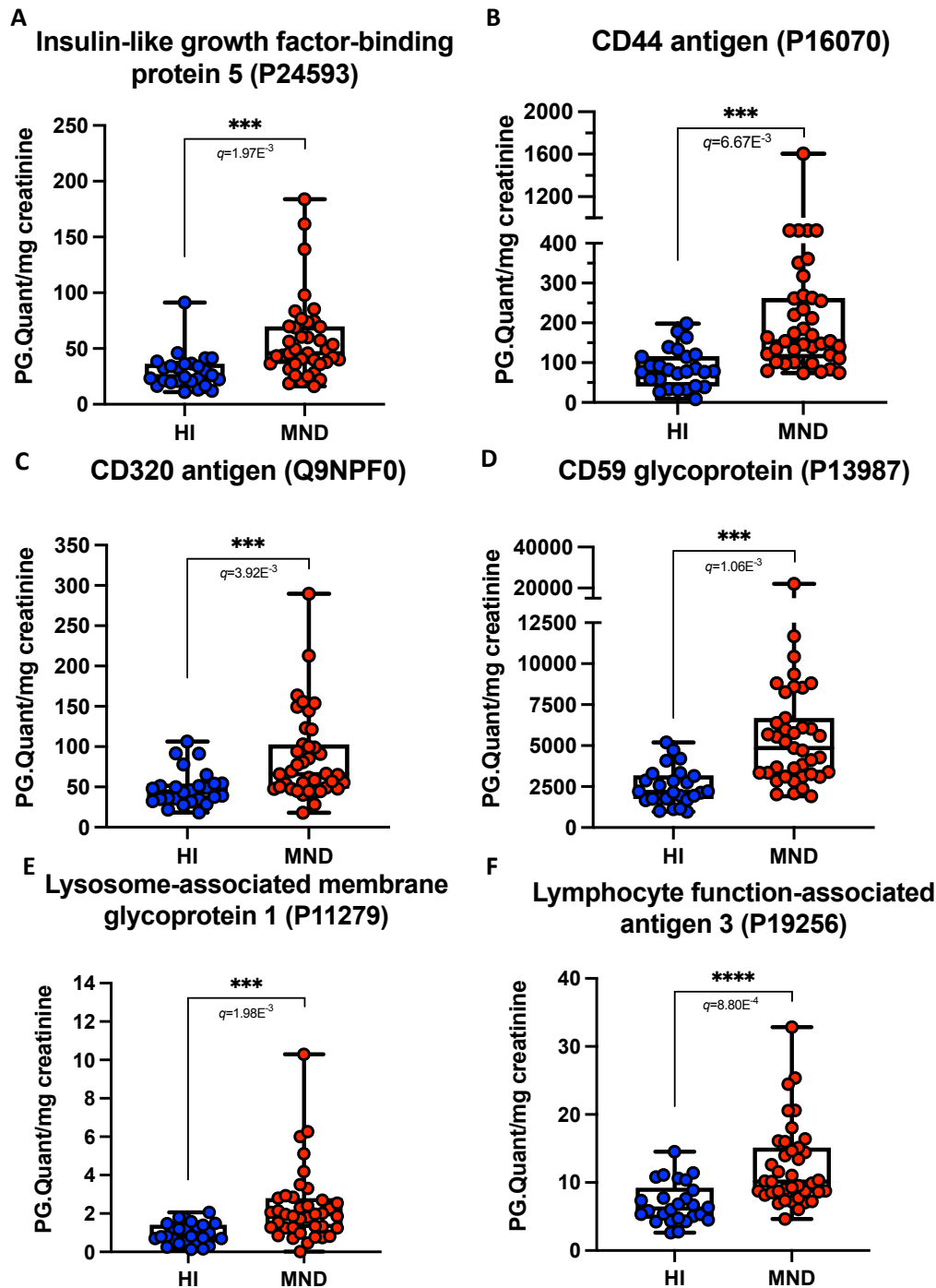


Figure 5.9: Significant proteins related to immune regulation and cellular interactions

The relationship between significant proteins of inflammatory processes was explored by 2-sample T-test in 39 people with MND and 26 HI. Five protein candidates were significantly higher in people with MND compared to healthy individuals in (A) insulin-like growth factor binding protein 5 (P24953, $q=1.97E^{-3}$, fold-change=2.0), (B) CD44 antigen (P16070, $q=6.67E^{-3}$, fold-change=2.8), (C) CD320 antigen (Q9NPF0, $q=3.92E^{-3}$, fold-change=1.8), (D) CD59 glycoprotein (P13987, $q=1.06E^{-3}$, fold-change=2.1), (E) lysosome associated membrane glycoprotein-1 (P11279, $q=1.98E^{-3}$, fold-change=2.4), and (F) lymphocyte function-associated antigen 3 (P19256, $q=8.80E^{-4}$, fold-change=1.8). Statistical significance was determined using MatLab.

5.5.3 Significant urinary proteins in MND related to other disease processes

In addition to inflammatory proteins relating, twelve proteins related to broader aspects of MND pathology of neurodegeneration, muscle degeneration, and lysosomal autophagy were investigated in 39 people with MND and 26 HI by 2-way T-test in Prism 10.

Pro-cathepsin H (P09668) (**Figure 5.10A**) was significantly changed in people with MND ($q=8.78E^{-4}$, fold-change=3.1), that is a key degradation lysosomal protein. Lysosomal protease cathepsin D (P07339) was significantly increased (**Figure 5.10B**, $q=1.43E^{-3}$, fold-change=2.6), as was extracellular matrix (ECM) protein collagen alpha-1(XV) chain (P39059) (**Figure 5.10C**, $q=2.97E^{-3}$, fold-change=1.9). Amyloid beta precursor protein (P05067) was also significantly increased in people with MND (**Figure 5.10D**, $q=1.47E^{-3}$, fold-change=2.1), alongside F-actin capping protein subunit beta (P47756) (**Figure 5.10E**, $q=7.15E^{-3}$, fold-change=2.9) and ECM protein Fibulin-5 (Q9UBX5) (**Figure 5.10F**, $q=1.20E^{-4}$, fold-change=2.4).

Prosaposin (P07602) was a significantly changed urinary protein in people with MND from this study, shown in **Figure 5.11A** ($q=1.46E^{-4}$, fold-change=2.0). Glycoprotein progranulin (P28799) was also significantly increased (**Figure 5.11B**, $q=4.11E^{-3}$, fold-change=1.8), and tumour necrosis factor receptor superfamily member 12A (Q9NP84) was significantly changed in MND (**Figure 5.11C**, $q=8.54E^{-5}$, fold-change=9.2), ranked 4th in differential abundance change (**Table 5.3**). Latent transforming growth factor beta binding protein-3 (Q9NS15) was significantly changed in MND (**Figure 5.11D**, $q=4.21E^{-4}$, fold-change=2.6) along with transcription factor zinc finger like protein-1 (O95159) (**Figure 5.11E**, $q=7.08E^{-5}$, fold-change=2.8) involved in neurogenesis. Receptor type tyrosine protein phosphatase-like N (Q16849) was one of the most outright significant proteins, ranked 6th in **Table 5.3** to exhibit higher fold-changes in healthy individuals compared to people with MND (**Figure 5.11F**, $q=8.00E^{-5}$, fold-change=-7.8). Tyrosine phosphatase may be involved in neuronal signaling.

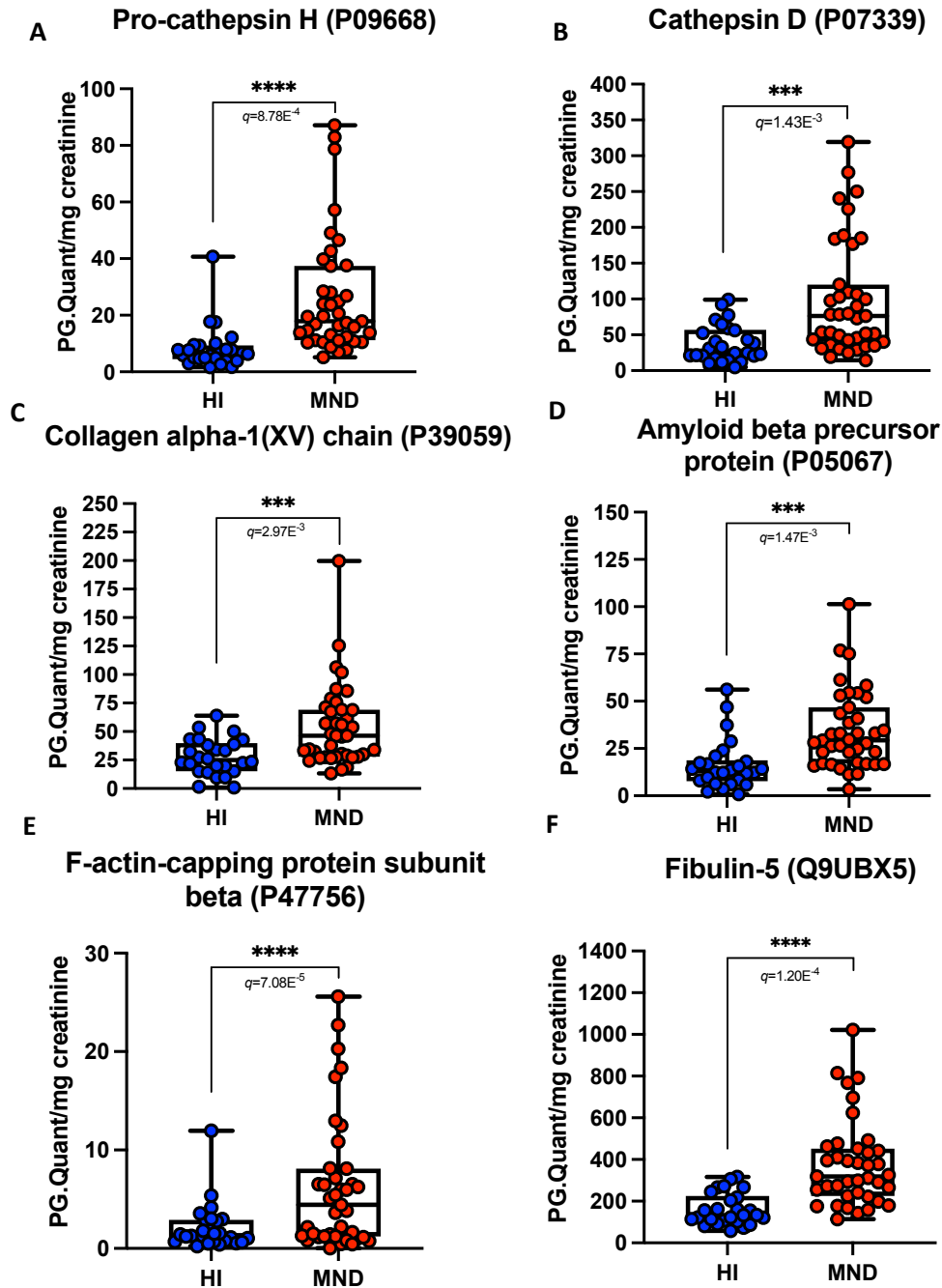


Figure 5.10: Significant proteins related to lysosomal and structural remodelling processes in MND

The relationship between significant proteins of other disease processes was explored by 2-sample T-test in 39 people with MND and 26 HI. All protein candidates were significantly higher in people with MND compared to healthy individuals in (A) Pro-cathepsin H (P09668, $q=8.78E^{-4}$, fold-change=3.1), (B) cathepsin D (P07339, $q=1.43E^{-3}$, fold-change=2.6), (C) collagen alpha-1(XV) chain (P39059, $q=2.97E^{-3}$, fold-change=1.9), (D) amyloid beta precursor protein (P05067, $q=1.47E^{-3}$, fold-change=2.1), (E) F-actin-capping protein subunit beta (P47756, $q=7.08E^{-5}$, fold-change=2.9), and (F) Fibulin-5 (Q9UBX5, $q=1.20E^{-4}$, fold-change=2.4). Statistical significance was determined using MatLab.

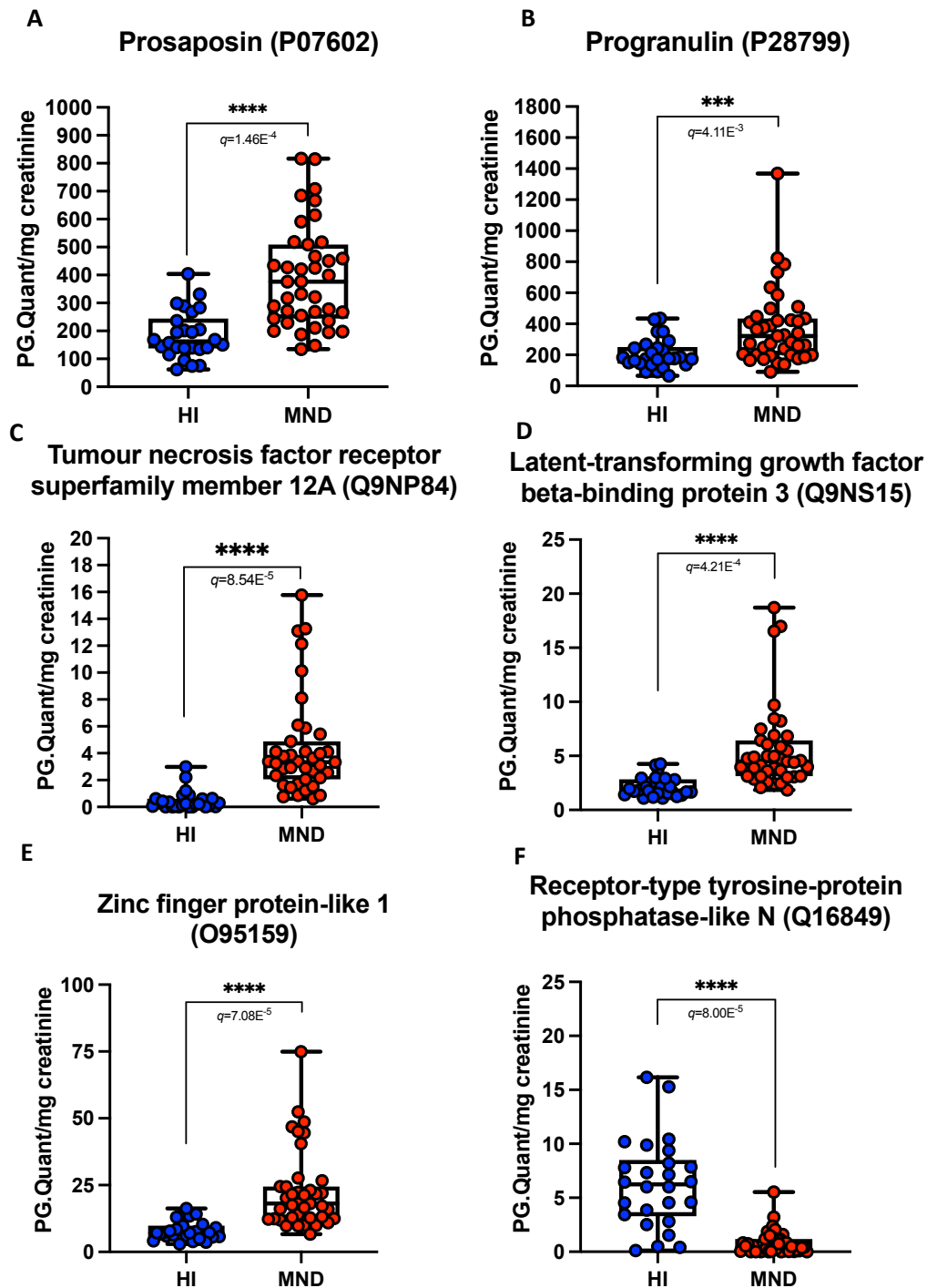


Figure 5.11: Significant proteins related to neurotrophic and signalling pathways in MND

The relationship between significant proteins of other disease processes was explored by 2-sample T-test in 39 people with MND and 26 HI. All protein candidates were significantly higher in people with MND compared to healthy individuals in (A) Prosaposin (P07602, $q=1.46E^{-4}$, fold-change=2.0), (B) progranulin (P28799, $q=4.11E^{-3}$, fold-change=1.8), (C) tumour necrosis factor receptor superfamily member 12A (Q9NP84, $q=8.54E^{-5}$, fold-change=9.2), (D) latent transforming growth factor beta binding protein-3 (Q9NS15, $q=4.21E^{-4}$, fold-change=2.6), (E) zinc finger like protein-1 (O95159, $q=7.08E^{-5}$, fold-change=2.8), and (F) receptor type tyrosine protein phosphatase-like N (Q16849, $q=8.00E^{-5}$, fold-change=-7.8). Statistical significance was determined using MatLab.

5.5.4 Interesting proteins that were not significantly changed

While the primary focus was on proteins that showed statistically significant changes ($q < 0.01$, **Table 5.3**) between people with MND and healthy individuals, a subset of proteins that did not meet the threshold for significance were found interesting. These proteins, due to previous studies investigating their involvement in MND and disease mechanisms, or their description for the first time in MND, may warrant further investigation.

Cystatin C (P01034) was unchanged in the urine of people with MND compared to healthy individuals ($q = 4.25E^2$, fold-change=2.9) that has been described previously as significant in the CSF of people with MND compared to healthy individuals (Pasinetti et al., 2006, Tsuji-Akimoto et al., 2009), alongside transthyretin (P02766) which has been previously described as significant in the CSF of people with MND ($q = 2.17E^1$, fold-change=1.3) (Ryberg et al., 2010). Urinary titin (Q8WZ42) involved in muscle function has been previously described as significant in MND urine compared to healthy individuals using targeted measurement (Yamada et al., 2021) but was unchanged in the current study ($q = 1.08E^2$, fold-change=3.7), as well as soluble monocyte differentiation antigen CD14 (P08571) that is expressed early in MND onset ($q = 3.16E^2$, fold-change=3.3) (Beers et al., 2020). Lastly, prolactin inducible protein (P12273) was identified with no significant change ($q = 1.25E^1$), however, this protein was amongst the largest fold-change differences at 26.3-fold higher in people with MND compared to healthy individuals. These proteins exhibited changes or implications in disease-related pathways that may warrant further investigation through targeted studies.

5.5.5 Normal urinary proteins

Outside of the proteins listed in **Table 5.3**, there were 1,093 proteins identified that were not differentially changed in people with MND. Two urinary proteins that were not expected to change between diseased and healthy urine were analysed by 2-way T-test in Prism 10. Albumin is a plasma protein that is highly abundant in serum, however, strictly regulated via the kidney under normal conditions. Albumin (P02768) was not significantly changed in people with MND compared to healthy individuals (**Figure 5.12A**, $q=2.50E^{-1}$, fold-change=0.32). This was expected as the glomerular filtration barrier prevents significant amounts of albumin from being excreted into the urine, and this process was not affected in people with MND with healthy kidney function.

Uromodulin is the most abundant protein in normal human urine, secreted by the thick ascending limb of the loop of Henle. Uromodulin (P07911) (**Figure 5.12B**) was as not significantly changed in people with MND compared to healthy individuals (HI, $q=9.30E^{-1}$, fold-change=0.96). Uromodulin secretion is an indication of normal renal tubular function and is strictly regulated by the kidney, such that changes in people with MND were not observed. This provided confidence in this discovery study using data independent acquisition (DIA) mass spectrometry techniques, demonstrating that biomarker discovery tools can differentiate between changes in protein abundance based on disease biology.

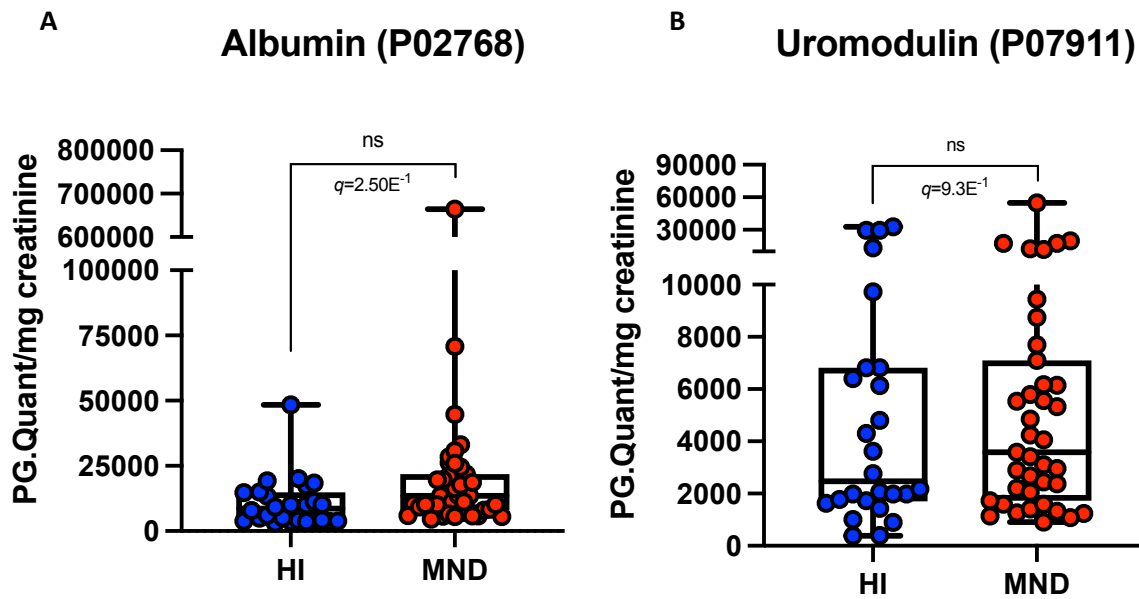


Figure 5.12: Normal urinary proteins not related to MND pathology

The relationship between non-disease specific proteins was explored by 2-sample T-test in 39 people with MND and 26 HI. Neither of two proteins were significantly higher in people with MND compared to healthy individuals in (A) albumin (P02768, $q=2.50E^{-1}$, fold-change=0.32) and (B) uromodulin (P07911, $q=9.30E^{-1}$, fold-change=0.96). Statistical significance was determined using Welch's T-test, Prism 10.

5.5.6 Proteins unique to people with MND or healthy individuals

Multiple proteins were discovered in one group, reported in only people with MND, or only healthy individuals. The quantity of these proteins was reported as PG.Quantity/mg creatinine by MatLab, then excluded from analysis as no 2-way T-test could be performed on proteins from one single group. These proteins were found particularly interesting and reported for further discussion. In total, 21 unique protein groups were identified. 19 unique proteins belonged only to people with MND, and 2 unique proteins belonged only to healthy individuals. These proteins were detected in a small number of individuals, on average 3 per condition, limiting the scope of these findings. However, their group-specific discovery is noteworthy. Secretoglobin family 3A member 1 (Q96QR1) was notably found in the highest number of samples, found in 12 people with MND. However, the remaining protein groups were found between 2 and 5 people with MND or healthy individuals. The quantity of each protein normalised to creatinine, was summarized in **Table 5.4**, with null values labeled as NaN that were not present in that group of individuals.

Table 5.4: Unique proteins discovered only in people with MND or healthy individuals by DIA mass spectrometry

Outside of the 257 significantly changed urinary proteins, a subset of unique proteins was found in one condition group only. These proteins were not expressed in both groups, such that MatLab could not perform a two-tailed T-test on protein quantities normalised to creatinine. This statistical analysis revealed 19 unique protein groups belonging to people with MND only, and 2 unique protein groups belonging to HI only. These proteins were reported ascending order of quantity, normalised to creatinine (PG.Quantity/mg creatinine), null values were reported as NaN.

PROTEIN ACCESSIONS	PROTEIN DESCRIPTIONS	MND PG.Quantity/mg creatinine	HI PG.Quantity/mg creatinine	Number of people found in
Q99999	Galactosyl ceramide sulfotransferase	0.07064447	NaN	2
Q9UN74	Protocadherin alpha-4	0.26585306	NaN	2
Q92743	Serine protease HTRA1	0.39423559	NaN	3
Q92747	Actin-related protein 2/3 complex subunit 1A	0.46414509	NaN	4
P05019	Insulin-like growth factor I	1.54671966	NaN	3
P20930	Filaggrin	1.69591608	NaN	2
Q16627	C-C motif chemokine 14	1.82783177	NaN	5
Q8NI32	Ly6/PLAUR domain-containing protein 6B	1.84529444	NaN	2
Q9Y5X9	Endothelial lipase	1.9309692	NaN	2
P05091	Aldehyde dehydrogenase, mitochondrial	2.04950855	NaN	2
Q00872	Myosin-binding protein C, slow-type	2.39292358	NaN	5
P48723	Heat shock 70 kDa protein 13	2.95385417	NaN	3
P62249	40S ribosomal protein S16	3.11954908	NaN	2
Q96QR1	Secretoglobin family 3A member 1	4.97402653	NaN	12
Q13231	Chitotriosidase-1	5.33006295	NaN	3
Q9H173	Nucleotide exchange factor SIL1	5.64497775	NaN	3
P41439	Folate receptor gamma	6.49650341	NaN	4
P14555	Phospholipase A2, membrane associated	18.5852722	NaN	4
P02747	Complement C1q subcomponent subunit C	39.8415421	NaN	5
P05120	Plasminogen activator inhibitor 2	NaN	0.09609189	2
P00505	Aspartate aminotransferase, mitochondrial	NaN	0.24061769	3

5.6 Gene ontology over-enrichment analysis

Gene ontology (GO) over-enrichment analysis (ORA) was carried out using the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system (Thomas et al., 2022). This analysis was performed to complete a comprehensive and high-throughput classification of the proteins discovered by DIA-mass spectrometry and their genes, to determine the relationship between proteins that were significant in people with MND at baseline and disease processes. The 257 significantly changed proteins in 39 people with MND were subject to the statistical ORA to identify which genes from the GO: cellular component, GO: biological processes, and GO: molecular function were over or under-expressed in people with MND compared to healthy individuals. The results were adjusted for multiple comparisons using the Benjamini-Hochberg method for false discovery rate (FDR, q -value). The scale of significance was subsequently applied to the group of differentially expressed proteins by Log10 q value, with a cut-off of $q < 0.01$. The gene ontology over-representation analysis was treated with an FDR at the $q < 0.05$ level, and the results were plotted in **Figure 5.8-5.11**. The threshold of differential abundance was applied via Log2-fold difference in protein abundance between people with MND and healthy individuals from the 50th percentile cut-off analysis (i.e., $q < 0.01$ passing in 50% or more of samples, as reported by Spectronaut).

The statistically enriched biological processes ($q < 0.05$, **Figure 5.13**) reflected larger processes including the inflammatory response and regulation of proteolysis. Within this, genes were over-enriched from pathways regulating immune and glial cell activation including astrocyte, neutrophil, transforming growth factor- β , and macrophage activation. Further, lysosomal, apoptotic, chemotaxis, and immune cell development pathways were enriched in people with MND. Some examples of proteins that are encoded for by the genes up-regulated in these pathways include pro-cathepsin H (**Figure 5.10A**), cathepsin D (**Figure 5.10B**), TGF- β receptors (**Figure 5.11D**), macrophage inhibitory factors and stimulating factors (**Figure 5.8D**), C-X-C type 2 chemokine receptors (**Figure 5.8B**), complement factors D and C1q, lysosome associated membrane glycoprotein 1 (**Figure 5.9E**) and 2, T-cell activation by CD44 antigen (**Figure 5.9B**), CD59 glycoprotein (**Figure 5.9D**), and CD320 antigen (**Figure 5.9E**).

Statistically enriched genes from the group of molecular pathways ($q < 0.05$, **Figure 5.14**) were representative of molecular-level activities performed by gene products regulating processes like death receptor activity and growth factor binding. Genes from the collagen binding, extracellular matrix elasticity, and receptor activity pathways (i.e., TNF, IGF, ACh, TGF- β) were statistically over-enriched. Some examples of proteins encoded by genes from these pathways included TGF- β receptors (**Figure 5.11D**) and binding proteins, tumor necrosis factor (TNF) receptor proteins like superfamily member 16 (p75^{ECD}, **Figure 5.7A**), and 12A (**Figure 5.11C**), alongside interleukin receptor subunits like IL-6 beta (**Figure 5.8E**), and IL-10 beta (**Figure 5.8F**).

Lastly, statistically enriched pathways from the cellular component analysis ($q < 0.05$, **Figure 5.15**) involved pathways that captured locations within the cellular compartment where molecular functions take place. Within this, genes were over-enriched from pathways involved in elastic fiber, extracellular matrix (ECM), and basement membrane proteins, myofibril constituents (i.e., reflective of muscular degeneration), lipoprotein particles of low- and intermediate densities, tumour necrosis factor superfamily complexes, and proteins involved in forming neuronal cell bodies, synapses, and the synaptic cleft. Some examples of proteins encoded by genes from these pathways included elastic fibre proteins like fibulin-1, Fibulin-5 (**Figure 5.10F**), and cadherin-8. Tumor necrosis factor (TNF) receptor proteins like superfamily member 16 (p75^{ECD}, **Figure 5.7A**), and 12A (**Figure 5.11C**), alongside ECM proteins and those involved in synapse formation including amyloid beta precursor protein (**Figure 5.10D**).

Some strategies to assess the intricacies of MND pathology and its heterogeneity have been proposed by analyzing the biological, molecular, and cellular foundation of applied ‘-omics’ methodologies in combination with gene clustering for identifying proteins and their genetic pathways that may be prognostic or predictive of disease pathology to improve sub-grouping. The gene ontology pathways of all 257 differentially changed proteins in people with MND were assessed for their significance and percent enrichment from each gene ontology classification term to identify proteins that are most likely to change because of disease pathology for ongoing and targeted validation techniques.

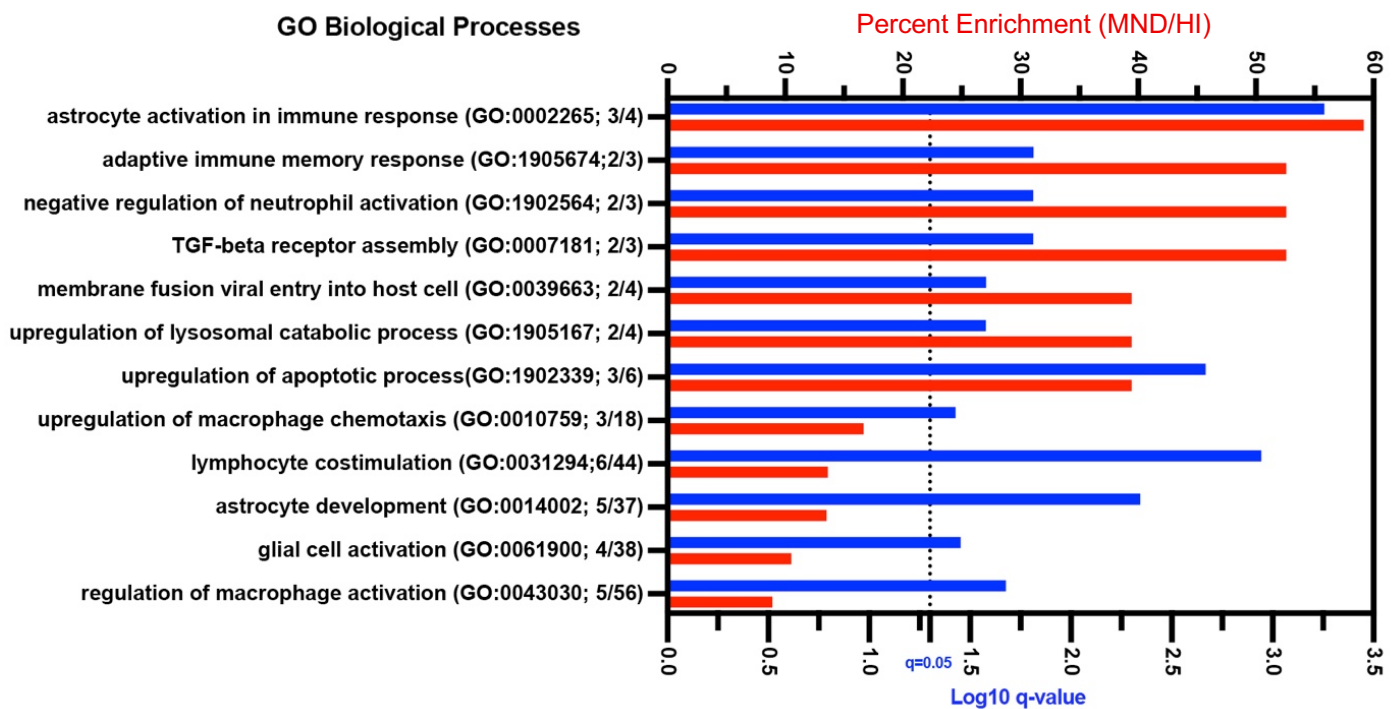


Figure 5.13: Gene ontology analysis of biological processes at baseline

Enriched pathways from 257 significantly changed proteins in people with MND and HI from the biological processes component of the gene ontology analysis statistical over-enrichment analysis. The Log10 q value in blue (●) and percent fold enrichment in red (●) of the significantly enriched biological processes were reported, revealing the number of genes related to pathways of astrocyte activation (GO:0002255, 3/4) and development (GO:0014002, 5/37), glial cell (GO:0061900, 4/38) and macrophage activation (GO:0043030, 5/56), amongst others. The top 12 pathways that are enriched (●) are shown, with FDR Log10 q value of < 0.05 , compared to the entire human genome.

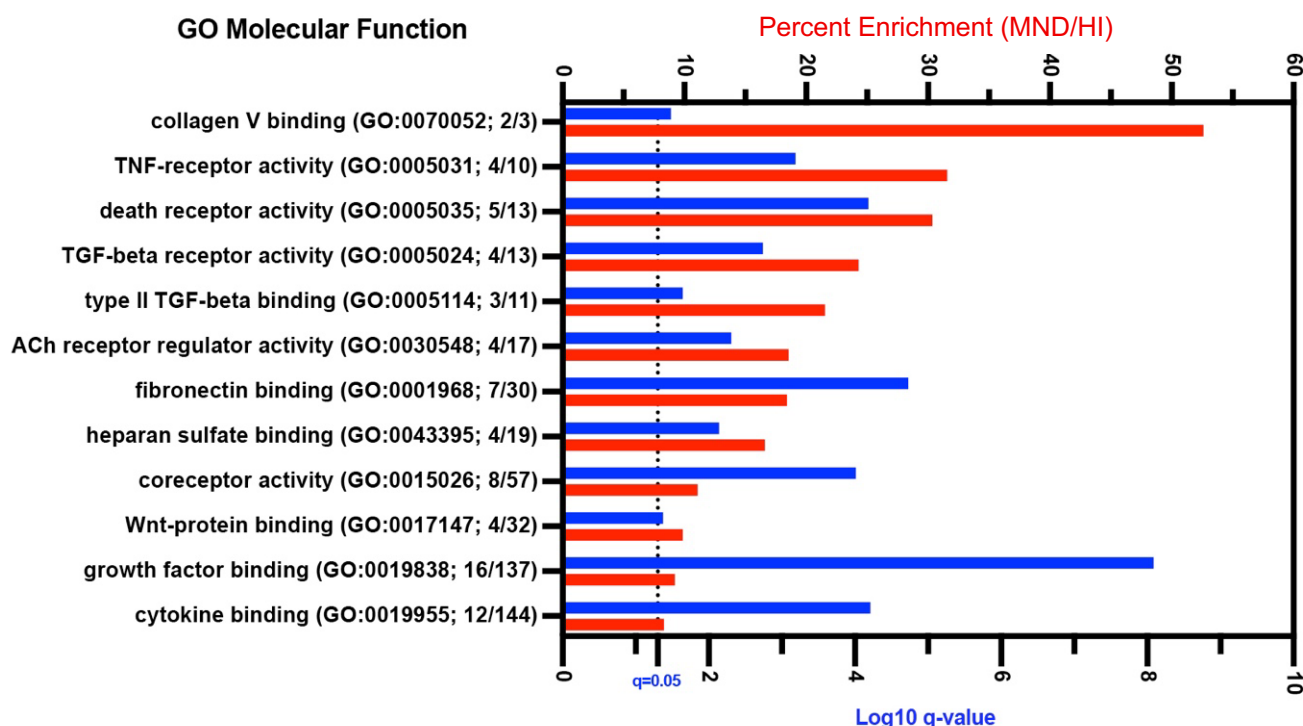


Figure 5.14: Gene ontology analysis of molecular functions at baseline

Enriched pathways from 257 significantly changed proteins in people with MND and HI from the molecular function component of the gene ontology analysis statistical over-enrichment analysis. The Log10 q value in blue (●) and percent fold enrichment in red (●) of the significantly enriched molecular functions are reported, revealing the number of genes related to pathways of collagen V (GO:0070052, 2/3) and fibronectin elastic fibre binding (GO:0001968, 7/30), death receptor (GO:0005035, 5/13) and TGF-beta receptor activity (GO:0005024, 4/13), amongst others. The top 12 pathways that are enriched (●) are shown, with FDR Log10 q value of < 0.05 , compared to the entire human genome.

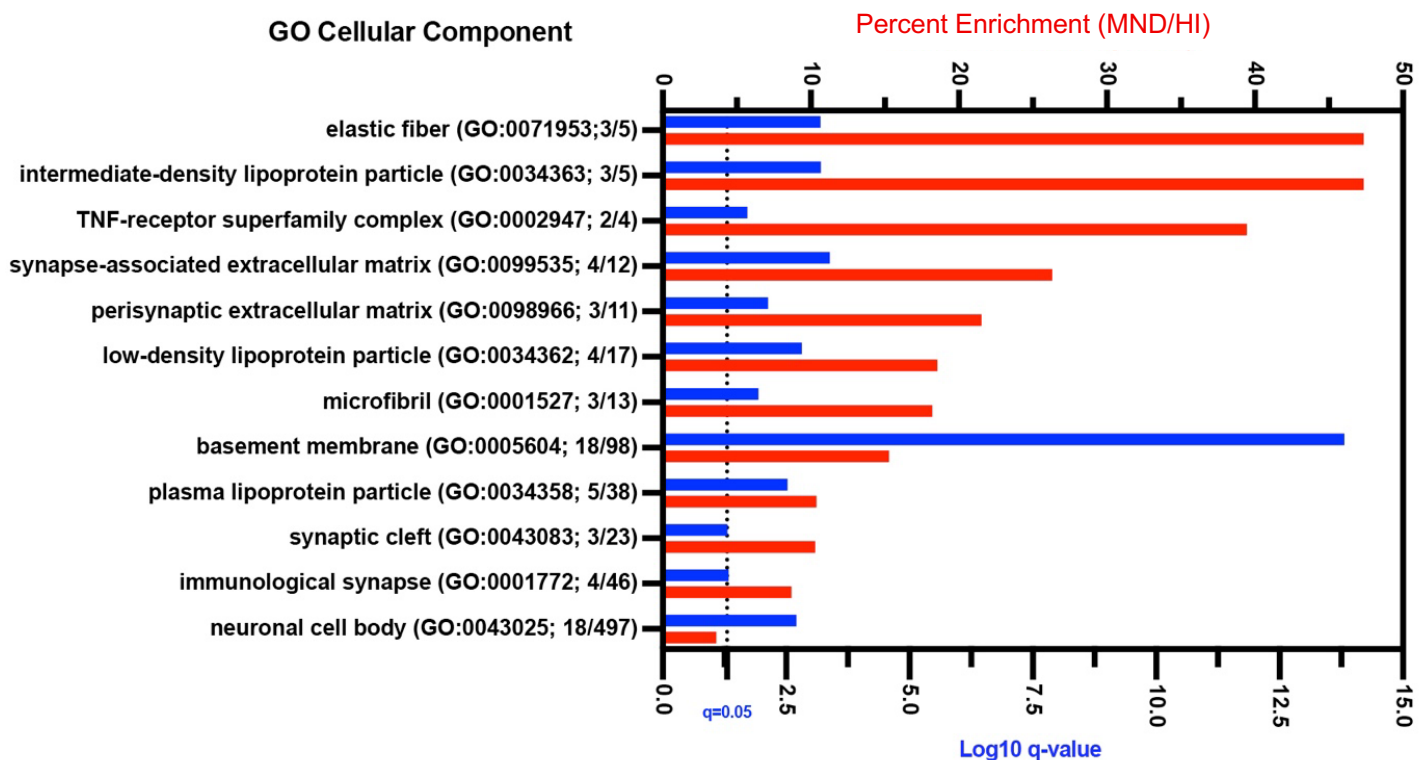


Figure 5.15: Gene ontology analysis of cellular components at baseline

Enriched pathways from 257 significantly changed proteins in people with MND and HI from the cellular component of the gene ontology analysis statistical over-enrichment analysis. The Log10 q value in blue (●) and percent fold enrichment in red (●) of the significantly enriched cellular components are reported, revealing the number of genes related to pathways of elastic fibres (GO:0071953, 3/5), TNF receptor complexes (GO:0002947, 2/4), immunological synapses (GO:0001772, 4/46) and neuronal cell bodies (GO:0043025, 18/497), amongst others. The top 12 pathways that are enriched (●) are shown, with FDR Log10 q value of < 0.05, compared to the entire human genome.

5.7 Candidate proteins for analytical validation by PRM

Based on the results obtained from the DIA mass spectrometry analysis, a set of potential protein candidates have been identified that warrant further investigation. This DIA study provided a comprehensive screening of the urinary proteome in people with MND, compared to healthy individuals at baseline. This revealed 257 significantly changed proteins, however, these candidates require validation to ensure their accurate identification, and precise quantitation to be candidate biomarkers that not only change with disease or predict pathology (i.e., predictive biomarkers of pro-inflammation) at baseline but can also correlate with disease by ALSFRS-R score and predict survival to indicate prognosis at baseline. To increase the confidence in the presence and quantitative accuracy of these proteins, ten candidates will be selected for further validation using parallel reaction monitoring (PRM) by orbitrap-mass spectrometry. Because the instrument spends all its time analyzing the target protein only, this provides greater sensitivity and quantitative accuracy as this method provides more data points across the elution peak. PRM, known for its higher specificity and sensitivity, will provide a more rigorous confirmation of the DIA results produced from this study. By employing PRM techniques, the aim is to achieve a higher level of confidence in the presence of these proteins in the urine of people with MND and healthy individuals, to improve the specificity of unique peptide detection and refine protein quantitation with greater precision.

The ten candidates were selected from twelve proteins significantly changed relating to processes of immune dysfunction within increased receptor activity pathways (i.e., TNF, IGF, ACh, TGF- β), macrophage, microglia, and astrocyte activity, amongst others, and twelve proteins relating to broad-spread disease processes like motor neuron death by mechanisms of programmed cell death, muscular degeneration by mechanisms of elastic fibre release, and axon degeneration. Ten candidates would be selected based on factors including signal intensity, peptide uniqueness, and their biological relevance to disease by gene over-representation analysis; ensuring that they are suitable targets for a more detailed analysis. The results of the DIA study and GO analysis provide robust evidence in the context of their roles in the urine of 39 people with MND, where PRM validation will look to further communicate their significance by increasing confidence in unique peptide detection and quantitation.

5.8 Discussion

MND is a progressive disease affecting multiple systems with a highly heterogeneous clinical presentation. Objective biomarker candidates that are detectable in the urine of people with MND may be prognostic at baseline by predicting expected survival outcomes. This may improve the design and analysis of future clinical trials by stratifying individuals by their expected survival (Benatar et al., 2024). In addition to serum NfL, other protein biomarkers of MND prognosis need to be identified (Benatar et al., 2024). The aim of this study was to use an un-targeted technique to detect novel proteins that were differentially changed in MND urine at baseline compared to healthy individuals, and to then identify significant candidates that could be further validated as biomarkers of MND prognosis or predictive of immune dysfunction. This aims to help in the construction of a prognostic biomarker panel alongside validated serum neurofilament light (NfL) (Benatar et al., 2020) and candidate urinary p75^{ECD} (Shepherd et al., 2017) to improve phase 2 clinical trial outcomes.

To describe the baseline clinical factors in this cohort, Cox proportional hazards regression analyses were performed based on clinical and neurological characteristics to evaluate survival outcomes. The age at baseline, gender, and site of disease onset were not prognostic covariates for survival in this cohort, reinforcing that survival outcomes and differential protein changes were not driven by age (<79 years) at baseline. However, both Δ FRS and urinary p75^{ECD} were independent prognosticators by univariate analysis, and by multivariate analysis after adjusting for confounding factors. This is aligned with previous publications by our group (Shepherd et al., 2017, Shepherd et al., 2022, Benatar et al., 2024b).

The focus of this investigation was on proteins related to activation and dysfunction of the immune response, identifying 257 significantly changed proteins in 39 people with MND compared to 26 healthy individuals normalised to creatinine. There are three major subtypes of proteins reported in people with MND, including those related to microglial activation and neuroinflammation, mitochondrial dysfunction and oxidative stress, and dense TDP-43 and associated transposable element de-silencing proteins (O'Neill et al., 2024a). This study identified proteins that were significantly changed from gene ontology pathways of astrocyte (GO:0002265), glial cell (GO:0061900), and macrophage (GO:0043030) activation, upregulation of apoptotic processes (GO:1902339) and death receptor activity (GO:0005035), cytokine binding (GO:0019955) and TGF-beta activity (GO:0005024), and elastic fiber (GO:0071953), basement membrane (GO:0005604), and collagen binding (GO:0070052) functions. These are involved with key processes of neuroinflammation,

neurodegeneration, and muscle degeneration, alongside other processes involving the structure of the extracellular matrix (ECM), growth factor activity receptors, programmed cell death receptors, and lysosomal glycoproteins and enzymes. The outcomes of this investigation supported the aims of identifying significantly changed proteins in people with MND, using gene ontology pathway analysis to relate significant candidates to processes of immune dysfunction for future validation.

The tool of choice for this investigation was orbitrap mass spectrometry in data dependent acquisition (DDA) mode to generate a spectral library of 1,970 protein groups, and data independent acquisition (DIA) mode for the relative quantitation analysis of all urinary proteins in people with MND. It was made sure that differentially expressed protein groups were present in at least 50% of all samples to reduce bias, as previously shown for CSF (Dellar et al., 2024). DIA mass spectrometry was used as a screening tool for protein discovery, collecting a broad range of precursor ions without peptide ion specificity (Lou and Shui, 2024). This approach analysed thousands of peptides from a single gradient, using an un-targeted technique to find novel biomarker candidates. To the best of our knowledge, this is the first study that has used shotgun proteomics to investigate how the urinary proteome may change in people with MND at baseline compared to healthy individuals. Compared to other studies, our spectral library of 1,970 protein groups aligned well with ~2000 previously discoverable proteins in urine (Muntel et al., 2015, Swensen et al., 2021) from other conditions, and CSF from people with MND (Trautwig et al., 2025, Dellar et al., 2024). Further, our demonstration of 257 significantly changed proteins between disease state and healthy individuals was similar to studies that have been performed in gastric cancer finding 177 significantly changed proteins with >1.5-fold change (Zhang et al., 2023), 237 significantly changed proteins in Parkinson's disease (Virreira Winter et al., 2021), and other diseases (Wu et al., 2025, Qin et al., 2024).

To design a robust and repeatable proteomics study, preparation of the urinary proteome by diafiltration was developed and refined by our laboratory (Shepherd et al., 2014). This was advantageous in protein yield compared to methods of protein extraction like chloroform and methanol precipitation (Saito et al., 2019b) by concentrating urinary proteins in-solution, rather than by resuspension. With an average spot urine protein content of 0.18 mg protein/mL, diafiltration concentrated the proteome into a detectable range and removed any interfering matrix below 3 kDa that otherwise clouded detection. The methodology was deemed valid, demonstrating the detection of urinary p75^{ECD}, biomarker candidate of MND progression (Shepherd et al., 2017), consistent with a well-established ELISA technique.

The measurement of urinary p75^{ECD} by DIA mass spectrometry and the in-house ELISA were significantly correlated, reinforcing the quantitative power of orbitrap-mass spectrometry under circumstances of low quantitative resolution by shotgun proteomics. Multiple studies have shown that the relative quantitation values obtained for proteins by DIA mass spectrometry as an un-targeted technique were comparable to targeted methods (Shi et al., 2018, He et al., 2019, Nakamura et al., 2016, Vildhede et al., 2018), although the precise quantification of each protein by PRM analysis will allow us to investigate the prognostic value of candidate proteins compared to the ALSFRS-R and urinary p75^{ECD} in future studies.

Performing a study to investigate the urinary proteome using un-targeted mass spectrometry techniques comes with some disadvantages. In data-dependent acquisition (DDA), peptides are selected for fragmentation based on their relative signal intensity (i.e., top 20) (Gillet et al., 2012). Inherently, this process is limited by the sensitivity and speed that the mass spectrometer can acquire the data (Michalski et al., 2011), resulting the lack of data reproducibility (Tabb et al., 2010). Data-independent acquisition (DIA) provides greater run to run reproducibility compared to DDA, and improved proteome coverage within a large dynamic range (Bruderer et al., 2016). However, DIA is still limited by performing label-free quantitative proteomics studies (Liu et al., 2025). When comparing proteins discovered from label free DIA mass spectrometry, the in-house urinary p75^{ECD} ELISA measures the quantity of p75^{ECD} in human urine across a minimum of six technical and five biological replicates (Shepherd et al., 2017), compared to one injection by orbitrap-mass spectrometry. One injection does not capture the full variability of urinary p75^{ECD}, or other proteins, and under these circumstances the validated ELISA is considered the gold-standard for urinary p75^{ECD} quantitation. To display assay repeatability using DIA, repeat injections demanded a considerable amount of instrument time over a 150-minute gradient. Future studies should incorporate multiple technical replicates and tandem mass tags (TMT), as performed by (Muntel et al., 2019) to capture low abundance peptides. The quantitation of urinary p75^{ECD} peptides by targeted mass spectrometry techniques like PRM with SIS peptides on a triple quadrupole (QQQ) mass spectrometer (Gerber et al., 2003), across multiple testing replicates, is a well-established technique to achieve results with greater accuracy, sensitivity, and specificity that could be comparable to an ELISA and should be explored in future studies. This has been performed in the literature before in other diseases (Kononikhin et al., 2023), though to our knowledge, this would be the first time a method like this would be applied to the urine of people with MND.

Spectronaut and MatLab were used to identify differentially changed in proteins people with MND, correcting them for the multiple testing hypothesis and renal normalisation by urinary creatinine. Spectronaut used a statistical cut off for Log2 fold-change values of ≥ 0.58 for up-regulated proteins, and ≤ 0.58 for down-regulated protein candidates, at significance level $q < 0.05$, and looked to find changed q values in $> 50\%$ of study participants to avoid influence from sparsely detected outliers. For biomarker detection techniques, it is understood that prognostic and predictive biomarkers must be present in at least 50% of a pilot population to exhibit change between two groups. Spectronaut tested the differential abundance of 1,350 unique protein groups, and MatLab was used to conduct multiple hypothesis testing, using a stricter q value ($q < 0.01$) adjusted for false discovery rate (FDR) to statistically correct for the multiple comparisons tests that represented the proportion of false positives that were expected (Lee and Lee, 2018). To account for changes in biological variation when examining spot-urine, a biofluid that contains a hyper-variable protein source (Nagaraj and Mann, 2011) including protein abundance and contaminants (Su et al., 2013, Mischak et al., 2011) depending on hydration status, MatLab was used to normalise protein groups to creatinine and apply the Benjamini Hochberg technique to control for FDR (Lee and Lee, 2018). Normal total protein concentration in spot-urine specimens is very low and does not exceed 10 mg/100 mL in people with healthy kidney function (Adachi et al., 2006). The variability of the urinary proteome is thought to be higher than serum or CSF because most excreted proteins do not have a functional role. Because there is no physiological need for precise homeostatic control, the quantitative degree of variation within the urinary proteome has not been studied (Nagaraj and Mann, 2011). However, the concentration and composition of urinary proteins can be influenced by physiological factors such as age, sex hormones, diet, hydration status, and exercise (Shao et al., 2019), and therefore, to control for hydration status creatinine was used to normalise the PG. Quantity of protein groups after accounting for the concentration factor, injection volume, and mg/mL of creatinine for each sample.

The outcome of this investigation was 257 significantly changed proteins in people with MND normalised to creatinine. From the total of 1,350 proteins identified in, 22% of those proteins were significantly changed in people with MND at baseline. It is documented that 20-30% of systemic serum proteins are discoverable in human urine that may be representative of biological functions (Eaton and Pooler, 2009). In assuming a 30% systemic reflection, this would attribute 6% of the total 1,350 proteins, or 77 out of 257 significantly changed proteins that may reflect systemic changes occurring in people with MND at baseline. The principal

component analysis demonstrated the distribution of protein abundance across both groups, with each data point representing the protein abundance profile of one sample. We observed that the two groups, 'MND' and 'CTRL' formed distinct clusters with a degree of overlap, which was expected given that not all proteins are changed in response to disease. The PC1 component captured 9.71% of the variation, and the PC2 component accounted for 7.62% of the variations. The biological variability in each group contributed to this overlap, suggesting that individual sample differences can influence the overall data distribution, and showing that a portion of the proteome is altered by biological change, but much remains un-altered.

Identifying statistical differences in proteins that were changed people with MND may not necessarily reflect processes of biological change, hence gene ontology analysis was required. PANTHER was used to perform a gene ontology over-representation analysis (Thomas et al., 2022) on 257 significantly changed proteins in people with MND to further clarify if the changes observed are a product of biological or pathological response to disease from the small percentage of proteins that may be reflective of systemic processes. Gene ontology terms of the significant proteins were investigated in the biological processes, molecular mechanisms, and cellular compartment of gene pathways encoding for those proteins. Enriched pathways within these processes were reported as by percent enrichment in people with MND, and by their Log10 q value, as a reflection of the number of genes found in this investigation compared to the human genome (percent enrichment), and their significance value ($q < 0.05$). Gene pathways from biological processes identified enriched genes from astrocyte activation (GO:0002255, 3/4) and development (GO:0014002, 5/37), glial cell (GO:0061900, 4/38) activation, and macrophage activation (GO:0043030, 5/56) pathways relating to immune processes. Molecular function pathways included those relating to muscle degeneration by collagen (GO:0070052, 2/3) and fibronectin elastic fibre binding (GO:0001968, 7/30) pathways, alongside mechanisms of programmed cell death by death receptor (GO:0005035, 5/13) activity, and the cellular component reported genes relating to pathways of change to synapses (GO:0001772, 4/46), neuronal cell bodies (GO:0043025, 18/497), and elastic fibres (GO:0071953, 3/5).

In addition to urinary p75^{ECD}, 24 proteins were candidates for investigation. The rationale for exploring candidate proteins was based on their significance ($q < 0.01$), and fold change (> 1.5). Evidence to support the aim of this study was provided in twelve protein candidates related to immune regulatory systems, pro- and anti-inflammation, and cell signalling pathways that may increase microglial activation and the interferon response. CD44 antigen

(81 kDa) was significantly increased in people with MND at baseline. CD44 has previously been associated with MND progression in mice models (Matsumoto et al., 2012, Pinner et al., 2017), found to be expressed in astrocytes and microglia, and associated with microglial activation by interferon-gamma (IFN- γ) (Matsumoto et al., 2012). In addition, interleukin-10 receptor subunit beta (37 kDa) was significantly increased, and its ligand interleukin-10 has been studied previously and found to be up-regulated in MND serum (Lu et al., 2016) alongside interleukin-6 (IL-6) receptor subunit beta (106 kDa) that has previously demonstrated a mild but significant increase in serum of slow MND progressors, which undergoes a late surge up-regulation (Lu et al., 2016) and was correlated with ALSFRS-R and manual muscle testing scores (Tortelli et al., 2020). IL-6 was also reported in MND serum by (Pronto-Laborinho et al., 2019) and (Devos et al., 2019). IL-6 together with the cytokine TGF-beta are known to promote the development of Th17 cells (Korn et al., 2009) that support chronic inflammation in auto-immune diseases (Miossec et al., 2009).

Interleukin-18 binding protein (IL-18BP, 52 kDa) was significantly increased at baseline in this cohort. IL-18BP is an endogenous inhibitor of its ligand, pro-inflammatory cytokine IL-18, which has previously shown to increase in sporadic MND serum at baseline, but was not detectable in CSF (Italiani et al., 2014). C-X-C motif chemokine receptor 2 (CXCR2, 41 kDa) is a G-protein coupled receptor that has been shown to increase in people with MND at the mRNA and protein level in cortical neurons of sporadic MND patients and SOD1^{G93A} transgenic mice. However, CXCR2 and its ligands need to be explored further for their impact on signalling pathways and selective motor neuron degeneration (La Cognata et al., 2023). Tocilizumab is an example of an experimental therapeutic previously explored for its ability to inhibit the transcription of these cytokines and chemokines in PBMCs of MND patients *in vitro* to regulate the level of inflammation (Mizwicki et al., 2012), however this never translated to the clinic. Other proteins like CD59 glycoprotein (14 kDa) and complement factor 1 (66 kDa) increased at baseline have been studied in post-mortem MND tissue and SOD1^{G93A} mice. CD59 plays a role in inhibiting the membrane attack complex (MAC) in the complement system (Iwasa et al., 2023) and complement factor 1 inactivates the complement system, which has shown in mice models to deposit C1q, C3, and C3b on motor endplates of the neuromuscular junction (Bahia El Idrissi et al., 2016a).

Insulin-like growth factors (IGFs) and their receptors are potent survival factors for motor neurons that have been investigated by immunohistochemistry to identify immunoreactivity. In spinal cord neurons, IGF-binding protein-5 (31 kDa) has shown to increase in the ventral horn (Wilczak et al., 2003). Macrophage colony stimulating factor-1 has been shown to

accelerate disease progression in SOD1^{G37R} mice and upregulate pro-inflammatory cytokine expression (Gowing et al., 2009), and the lysosomal membrane protein LAMP-1 (45 kDa) has been investigated as a biomarker of lysosomal defects in familial MND-linked HsSOD1^{G93A} mice models. This reported no detectable change (Cheng et al., 2018b) without lysosomal deficit in vivo in familial MND-linked spinal motor neurons (Cheng et al., 2018a).

This discussion was focused on the proteins related to immune dysfunction; however, some proteins were reported related to other parts of disease pathology to understand how processes of neurodegeneration and muscle degeneration are involved in MND. Cathepsin D (53 kDa) is a lysosomal protease involved in maintaining lysosomal homeostasis in neurons. Cathepsin D has been found to increase in the spinal cord of SOD1^{G93A} MND mice models (Wootz et al., 2006). Recently, several non-coding variants of genes, or long non-coding RNAs were found to be down-regulated in MND, leading to increased cathepsin D expression (Huang et al., 2024). Gene ontology analysis revealed significant enrichment in lysosomal catabolic processes (GO:1905176), supporting further investigation into cathepsin D and its role in MND. Tumor necrosis factor receptor superfamily member 12A (TNF12A) is not well established in MND but is a receptor for the cytokine TWEAK (receptor Fn14) that is a mild inducer of apoptosis, or motor neuron cell death (Petrovita et al., 2004). Biomarkers of neurodegeneration in MND warrant further exploration, belonging to the same family of receptors as urinary p75^{ECD} that is a candidate biomarker of MND progression by mechanism of mediated cell death (Shepherd et al., 2017).

Amyloid beta precursor protein (APP, 130 kDa) has increased in the CSF of people with MND (Steinacker et al., 2009) and may be a candidate prognostic biomarker (Illán-Gala et al., 2018). In contrast, studies in SOD1^{G93A} mice models have shown that the APP cleavage product, amyloid beta, can associate with SOD1 aggregates, suggesting a potential role in MND pathogenesis (Li et al., 2006). The physiological roles of other proteins like receptor-type tyrosine-protein phosphatase-like N (106 kDa), zinc finger protein-like-1 (34 kDa), collagen alpha-1(XV) chain (141 kDa), and fibulin-5 (50 kDa) are not well described in MND from the literature. Some of these proteins reported interesting fold-change (i.e., down-regulated in MND), and interesting mechanisms. Collagen and fibulin-5 are ECM and elastic fibre proteins which were significantly represented by GO analysis from GO:0070052 in molecular functions and GO:0071953 from the cellular component. In a predominantly muscle wastage disease, proteins from these pathways warrant investigation.

Lastly, six interesting proteins were reported in *section 5.4.4* that did not meet the significance threshold for this study ($q < 0.01$). These proteins included monocyte differentiation antigen CD14 (soluble CD14, 40 kDa) that has shown to increase in MND urine by ELISA (Beers et al., 2020), alongside studies exploring CD14 expression over disease progression (McGill et al., 2022), CD14⁺⁺ monocytes from MND patients with documented IFN responses (Quek et al., 2022), and an experimental therapeutic anti-CD14 antibody IC14 (atibuclimac) has been used in a phase 1 clinical trial (Gelevski et al., 2023), where urinary p75^{ECD} has been used as an exploratory biomarker (Henderson et al., 2021). Cystatin C (13.3 kDa) levels have been shown to decrease in the CSF of people with MND (Pasinetti et al., 2006, Tsuji-Akimoto et al., 2009) but is a common degenerative protein not specific to MND with poor prognostic value (Vijayakumar et al., 2019). Urinary n-terminal titin (23 kDa) has been suggested as a candidate biomarker with prognostic potential as a candidate marker of muscle degeneration (Yamada et al., 2021). TGF-beta receptor type-2 (65 kDa) shares a relationship to the mechanisms of its ligand TGF-beta, and this has been previously detected in serum of people with MND by ELISA and increased from symptom onset (Liu et al., 2012). Prolactin inducible protein (17 kDa) was used in this study for technical validation purposes. Although its urinary concentration was 26.3-fold higher in people with MND at baseline, this difference did not reach statistical significance, likely reflecting variability among samples. However, this protein was used for ongoing targeted method development for unique peptide identification and fragmentation. Prolactin inducible protein has been studied once previously MND. However, that study was inconclusive and no association was found between the ALSFRS-R or survival outcomes (Chiodini et al., 1990).

These candidate biomarkers must be related to functional disease decline, disease survival, or clinical outcome measures. As shown in **Figure 5.16**, adapted from (Kiernan et al., 2021), these proteins are indicated in MND pathology from their GO pathways and relationship to disease. Proteins representing the nervous system and inflammation were the focus, however, other proteins relating to neuronal cell death (p75^{ECD}, TNFRS12A, IGFBP-5), elastic fibers and the ECM (FIB-5, collagen chains), and lysosomal autophagy (cathepsin-D, LAMP-1) were explored. This supports the aim of identifying candidate biomarkers involved in immune dysfunction amongst other pathways, for future validation by PRM techniques to understand their relationship to functional disease decline by correlation to ALSFRS-R scores and disease survival by Cox proportional hazard models using a technique with more quantitative precision, sensitivity, and specificity in people with MND.

Figure 5.16 has been removed due to Copyright restrictions

Figure 5.16: How protein-based biomarker candidates are involved in the pathophysiology of MND

The pathophysiology of MND involves mechanisms of neurodegeneration, muscle degeneration, lysosomal autophagy, and others in central and peripheral pathways. Candidate proteins that were discovered by DIA mass spectrometry were related to their GO pathways in disease. Candidate proteins were identified in the nervous system, contributing towards neuroinflammation and disruption of axonal transport mechanisms. These proteins included interleukin-6 and -10 receptors, interleukin-18 binding protein, glycoproteins CD44, CD14, CD58, CD59, CD320, TGF-beta receptor-2, macrophage colony stimulating factor-1, C-X-C motif chemokine receptor-2 (CXCR2) and complement factor I (CFI). Other mechanisms of disease include programmed neuronal cell death, which was attributed to proteins p75^{ECD}, tumour necrosis factors and their receptors (TNFRS12A), and IGF-binding proteins (IGFBP5). Defects in protein aggregation and lysosome autophagy are a characteristic of disease, with proteins pro-cathepsin H (CTSH), cathepsin D (CTSD), and LAMP-1, alongside defects in elastic fibers and myelination by fibulin-5 and ECM proteins like collagen alpha(IV) chain involved in muscular degeneration. These are candidate proteins that were significantly changed in people with MND that are involved with mechanisms of MND dysfunction. *Adapted from (Kiernan et al., 2021).*

CHAPTER 6.
VALIDATING URINARY PROTEIN
CANDIDATES OF IMMUNE
DYSUNCTION IN MOTOR NEURON
DISEASE BY MASS
SPECTROMETRY

6.1 Introduction

Motor Neuron Disease (MND) is limited in the way of biomarkers that are validated for use in clinical trials. Biomarkers that are prognostic, or predictive at baseline, are important tools to mitigate this issue. Prognostic biomarkers are used to predict survival from baseline and to select an eligibility criterion for clinical trials to appropriately stratify individuals into groups by survival probability. In addition, predictive biomarkers are used at baseline to identify individuals who are most likely to experience an effect from an experimental drug (Group, 2001, Benatar et al., 2024b). This pilot study aimed to analytically verify ten urinary protein candidates that were discovered in *Chapter 5* that are related to immune dysfunction, and other characteristics of disease, using parallel reaction monitoring (PRM) and orbitrap-mass spectrometry (Ronsein et al., 2015). This pilot study was focused, but not limited to candidate proteins of immune dysfunction, amongst proteins relating to neurodegeneration and muscle degeneration as viable candidates that were previously discovered in people with MND using data independent acquisition (DIA) and orbitrap-mass spectrometry.

The results from *Chapter 5* used DIA and orbitrap-mass spectrometry to conduct an extensive, unbiased, and non-targeted early discovery phase of proteins that were significantly changed in the urine of people with MND. This study discovered 257 significantly changed urinary proteins, identifying significant changes in gene ontology pathways and proteins that related to disease. To perform analytical validation, this study focused on improving the quantitative resolution and precision of quantitation for ten biomarker candidates using PRM and orbitrap-mass spectrometry. The PRM technique is based on the data dependent acquisition (DDA) technique, using peptide mass-to-charge (m/z) and charge (z) scheduling to pinpoint candidates amongst the noise of full-scan MS1 data to benefit the specificity and precision of protein quantification. Their fragmentation b^+ and y^+ ions are monitored with high resolution, improving peak detection and mass accuracy of specific peptides in complex protein groups. This meant that their relationship to disease by the ALSFRS-R, Δ FRS (Kimura et al., 2006), and survival outcomes could be explored at baseline to establish their possible utility as predictive or prognostic biomarkers of MND.

To focus on protein biomarker validation by PRM, a strict set of criteria was applied to narrow down the list of proteins for further investigation from *Chapter 5*, section 5.3.6. In short, these criteria included the presence of at least one unique peptide that was detectable with sufficient signal for precise quantification, a minimum peptide length of 8 amino acids to ensure specificity, and a high number of peptide-specific matches (PSMs) in Proteome Discoverer to indicate strong ionization efficiency using nano-flow liquid chromatography.

Additional considerations included the absence of missed or non-specific cleavages and variable post-translational modifications that could affect quantification accuracy. Instances of this included identifying unique peptide sequences containing the amino acid proline; the cyclic structure of proline introduces higher susceptibility to fragmentation under collision-induced dissociation (CID) conditions resulting in non-ideal fragmentation patterns (Schwartz and Bursey, 1992). Low energy CID conditions lead to cleavages at the peptide backbone and produce sequence-informative *a*, *b*, and *y* product ions (Roepstorff and Fohlman, 1984). Theoretical *m/z* data of peptides are matched to their product ions to identify the protein, and Skyline was used as the matching and sequencing algorithm software in this study.

Tumour necrosis factor receptor superfamily member 16, or p75, and 12A are implicated in regulating programmed neuronal cell death and were top candidates for validation. Urinary p75^{ECD} has been extensively explored in the past by ELISA (Shepherd et al., 2017), and peptides from the extracellular domain (ECD) of the p75 neurotrophin receptor (p75) by mass spectrometry (Shepherd et al., 2014), and in this study by DIA using orbitrap-mass spectrometry. Validation by PRM would provide another analytical technique for the detection and quantitation of urinary p75 in MND urine. Tumour necrosis factor receptor superfamily member 12A is involved with immune system regulation and cellular responses to stress signals as a TWEAK receptor (Dwyer et al., 2021), regulating NF- κ B pathways that are critical in the pathogenesis of MND and regulating the immune response, and programmed cell death (Wajant, 2013).

Fibulin-5 is an extracellular matrix (ECM) protein that has been demonstrated to colocalize with elastic fibers (Nakamura et al., 2002) with a potential role in a predominantly muscle wastage disease. Changes in expression could reflect some degree of muscle degeneration. Remaining candidates include the cytokine receptor subunits of interleukin-6; a pro-inflammatory cytokine (Garbers et al., 2012), and interleukin-10; an anti-inflammatory cytokine (Ahlers et al., 2022), alongside macrophage colony stimulating factor-1; involved in activation of macrophages in the periphery (Müller et al., 2007) and microglia in the CNS of mice (Yamamoto et al., 2010), as central candidate modulators of the immune response. Cell adhesion molecule of CD44 antigen (Matsumoto et al., 2012) plays a role in microglial activation and tissue repair, and lysosomal protease cathepsin D may contribute towards existing immune activation and failure to clear cellular debris and misfolded proteins (Hossain et al., 2021). Monocyte differentiation antigen CD14 (Janova et al., 2016) was not significantly changed in *Chapter 5* but warranted further investigation at baseline due to

soluble CD14 being identified in MND urine (Beers et al., 2020) and involvement in clinical trials (Gelevski et al., 2023). Prolactin inducible protein (Chiodini et al., 1990) was investigated in this study for its high signal, unique fragmentation pattern, and great ionization efficiency as a technical design control. However, it has not been previously implicated in MND. This protein was fragmented to optimize the method design for the simultaneous detection of 18 selected peptides from 10 proteins in MND urine.

Nine of ten protein candidates shared a common involvement in immune modulation, neuro inflammation, programmed neuronal cell death, and tissue repair, with a strong biological relevance to MND. However, their exact roles in MND need further validation through quantitative methods like PRM to confirm their prognostic and predictive potential of these processes involved in MND that can reliably be used in clinical settings. This chapter is a pilot study for the quantitative and simultaneous analytical verification of these candidate peptides and proteins in the urine of people with MND, exploring their relationship to disease by correlation to ALSFRS-R scores, and to prognosis by Cox-regression survival analyses.

Table 6.1: Overview of candidate proteins selected for PRM verification

Ten candidate proteins that were differentially expressed in people with MND from DIA and gene ontology analysis were selected based on disease relevance and detectability by LCMS. Each protein is presented with a unique accession number, brief functional description, and detectable peptides considered for targeting.

Protein name	Accession number	Protein description	Unique peptide sequences in Proteome Discoverer
Tumour necrosis factor receptor superfamily member 16 (p75)	P08138	p75; candidate biomarker of neuronal cell death that mediates apoptosis; neurodegeneration (Shepherd et al., 2017)	<u>C</u> AYGYYQDETTGR V <u>C</u> EAGSGLVFS <u>C</u> QDK WADAE <u>C</u> EEIPGR
Cathepsin D	P07339	Lysosomal protease; maintains lysosomal-dependent protein homeostasis in the brain (Hossain et al., 2021)	TMSEVGGSVEDLIAK YSQAVPAVTEGPIPEVLK LSDPEDYTLK
Tumour necrosis factor receptor superfamily member 12A	Q9NP84	TWEAK receptor, associated with neuronal cell death (apoptosis); neurodegeneration (Dwyer et al., 2021)	GSSWSADLDK GSSWSADLDK <u>C</u> MD <u>C</u> ASCR (missed 1) ARPHSDFCLG <u>C</u> AAAPPAPFR
Interleukin-6 receptor subunit beta	P40189	Pro-inflammatory cytokine receptor associated with chronic inflammation (Garbers et al., 2012)	LTWTNPSIK VTSDHINFDPVYK SDAAVLTI <u>P</u> A <u>C</u> DFQATHPVMDLK
Interleukin-10 receptor subunit beta	Q08334	Anti-inflammatory cytokine receptor, inhibits pro-inflammatory TH-17 cell response (Ahlers et al., 2022)	NILQWESPAFAK FQITPQYDFEVLK IENEYETWTMK
Fibulin-5	Q9UBX5	ECM elastin-binding protein involved in elastic fibre assembly (Nakamura et al., 2002).	GDMM <u>C</u> VNQNGGYL <u>C</u> IPR DQPFTILYR QSGQ <u>C</u> LDIDE <u>C</u> R
CD44 antigen	P16070	Mobilises T-cells and monocytes to area of inflammation (Matsumoto et al., 2012). Increased in FTD in CSF (van der Ende et al., 2022)	LTVGAAQVPAQLLVGALR AFPALTSLDLSDNPGLGER ITGTMPPLPLEATGLALSSLR
Macrophage colony-stimulating factor 1	P09603	Cytokine; regulates peripheral macrophages (Müller et al., 2007) and central monocytes and microglia (Yamamoto et al., 2010) in mice	AFLLVQDIMEDTMR SHSSGSVLPLGELEGR TFYETPLQLEK
Prolactin-inducible protein	P12273	Not significantly changed in MND (Chiodini et al., 1990). Used as a technical control.	ELGI <u>C</u> PDDAAVPIK TVQIAAVVDVIR NFDIPK
CD14	P08571	Organiser of microglial response to infection and injury to CNS (Janova et al., 2016)	LTVGAAQVPAQLLVGALR ITGTMPPLPLEATGLALSSLR AFPALTSLDLSDNPGLGER

C: carbamidomethyl modification. Fixed modification mass is +57 Da
(missed 1): missed cleavage

6.2 Validating proteins by parallel reaction monitoring

Both DIA and DDA data was manually interrogated in Proteome Discoverer, Freestyle, and Skyline to assess the abundance and fragmentation patterns of unique peptides, to identify ten protein candidates that were discussed in *Chapter 5*. Proteins were chosen based on their relationship to disease by gene ontology over-representation analysis, fold change, and significance in people with MND at baseline. The final list of 10 protein candidates and their discoverable unique peptides are discussed in **Table 6.1**. These proteins, and their unique peptides, met the criteria for PRM validation and were selected due to their involvement in key pathways relating to neurodegeneration, muscle degeneration, neuroinflammation, or as a technical control (PIP).

6.2.1 Clinical characteristics of people with MND and healthy individuals

The same thirty-nine people with MND and twenty-six healthy individuals from *Chapter 5* were utilised for this study. Their clinical characteristics have been previously described in *section 5.3.1*.

6.2.2 Targeted identification of urinary p75 by PRM

The targeted peptide PRM method was applied to the urine of sample MND 166 to identify Tumour necrosis factor receptor superfamily member 16, or otherwise the p75 neurotrophin receptor (p75^{NTR}), as a protein group with unique peptides in the extracellular region (AA: 29-250) previously established by (Shepherd et al., 2014). Sample MND #166 was treated in the same way as samples from **Table 5.1**, undergoing diafiltration, reduction, alkylation, digestion with Trypsin, and analysis by orbitrap-mass spectrometry. Proteome Discoverer was used to generate a report, identifying the sequence of 427 amino acids for urinary p75 in **Figure 6.1**, confirmed by checking the match against the UniProt entry for accession number P08138 and highlighting the ECD region (—). Three unique peptides for urinary p75 were highlighted in green (●, **Figure 6.1**) as CAYGYQDETTGR, VCEAGSGLVFSCQDK, WADAECEEIPGR, in order of appearance in the protein sequence.

Table 6.2 highlighted the details of each unique peptide found for urinary p75 all found within the ECD (ECD; AA:29-250). CAYGYQDETTGR was 12 amino acids in length located between 109- and -121 of the sequence. CAYGYQDETTGR, any many other peptides in this study, contained a homogenous carbamidomethyl post-translational modification on the cysteine (labelled, C) residue at the N-terminal, adding +57 Da to the peptide mass. This was a result of alkylation treatment to prevent cysteines from forming disulfide bonds, promoting the identification of the peptide by mass spectrometry. The monoisotopic mass of CAYGYQDETTGR was identified as 1583.64 Da with a charge state of +2, generating a discoverable precursor mass of 792.33 m/z. Unique peptide VCEAGSGLVFSCQDK was 15 amino acids in length located between 127- and -141 of the sequence. Two cysteine (C) residues were contained in the amino acid sequence VCEAGSGLVFSCQDK in position 2 and 11 with carbamidomethyl modifications, resulting in a monoisotopic mass of 1656.74 Da with a charge state of +2, generating a discoverable precursor mass of 828.87 m/z. Lastly, unique peptide WADAECEEIPGR was 12 amino acids in length located between 183- and -194 of the 427 amino acid sequence. One cysteine (C) residue was reported in position 5 with carbamidomethyl modification, resulting in a monoisotopic mass of 1432.62 Da with a charge state of +2, generating a discoverable precursor mass of 716.40 m/z. Each peptide had high ionisation efficiency, with 26, 30, and 35 PSMs reported, respectively. Unique peptides CAYGYQDETTGR and WADAECEEIPGR highlighted in green in **Table 6.2** were pursued for further targeting by PRM and were within the ECD region of the sequence. VCEAGSGLVFSCQDK was excluded due to ongoing detectability issues.

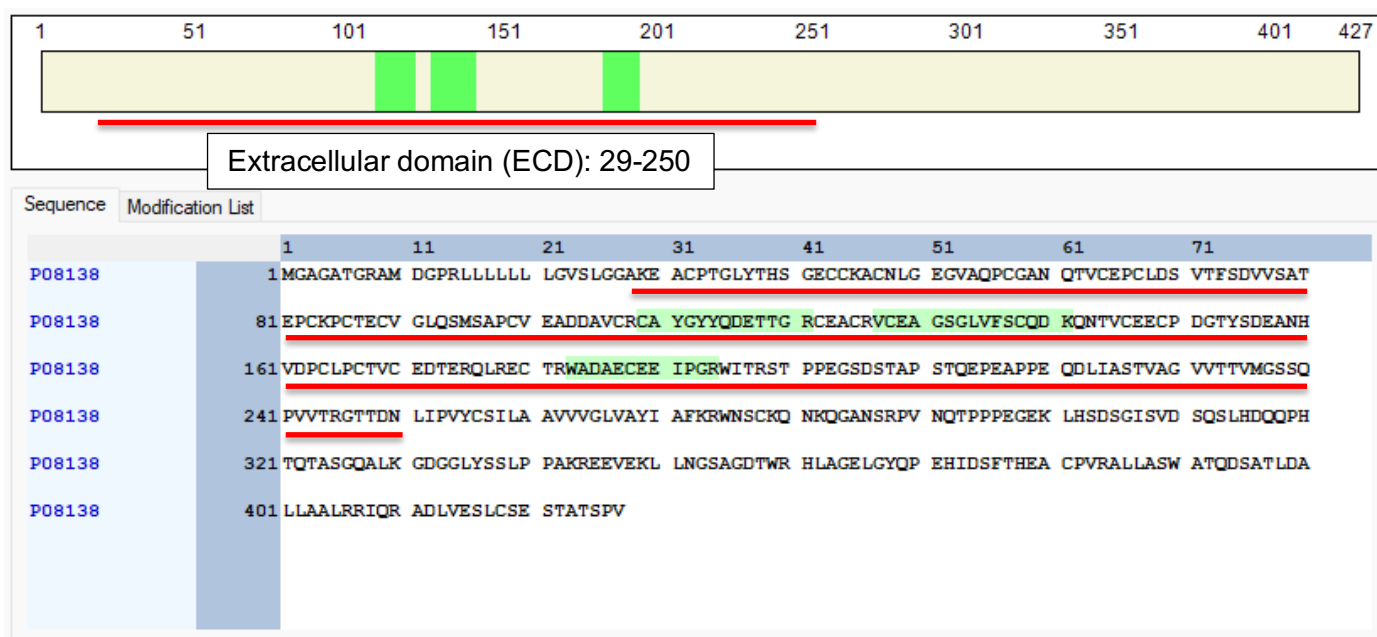


Figure 6.1: Unique peptide sequence coverage of tumour necrosis factor receptor superfamily member 16

Sequence coverage of the p75 neurotrophin receptor, also known as tumor necrosis factor receptor superfamily 16 (TNFRSF16; UniProt ID: P08138), was detected in the urine of person MND166 after diafiltration, reduction, alkylation, digestion with Trypsin, and analysis using orbitrap-mass spectrometry. Proteome Discoverer reported a sequence length of 427 amino acids. The extracellular domain (ECD) of the p75 neurotrophin receptor is described between 29-250 of the amino acid sequence, highlighted in red (—). Highlighted in green are three unique peptides: CAYGYQDETTGR, VCEAGSGLVFSQDK, and WADAECEEIPGR which all reside in the ECD. Adapted from *Proteome Discoverer*.

Table 6.2: Summary of unique peptides from tumour necrosis factor receptor superfamily member 16

Orbitrap-mass spectrometry identified three unique peptides using Proteome Discoverer to report their amino acid sequence, number of peptide specific matches, monoisotopic mass (Da), charge state (z), and precursor mass m/z ratio. Each peptide reported cysteine residues (C) with carbamidomethyl modifications within their unique sequence, adding a modified mass of +57 Da to each peptide. Peptides CAYGYQDETTGR and WADAECEEIPGR, highlighted in green, were selected to undergo further validation by PRM.

TNR16, p75 (P08138)	Peptide sequence	PSMs	Monoisotopic mass (Da)	Charge state (z)	Precursor mass (m/z)
109-121 (12 AA)	<u>C</u> AYGYQDETTGR	26	1583.64	2	792.33
127-141 (15 AA)	V <u>C</u> EAGSGLVFS <u>C</u> QDK	30	1656.74	2	828.87
183-194 (12 AA)	WADAE <u>C</u> EEIPGR	35	1432.62	2	716.40

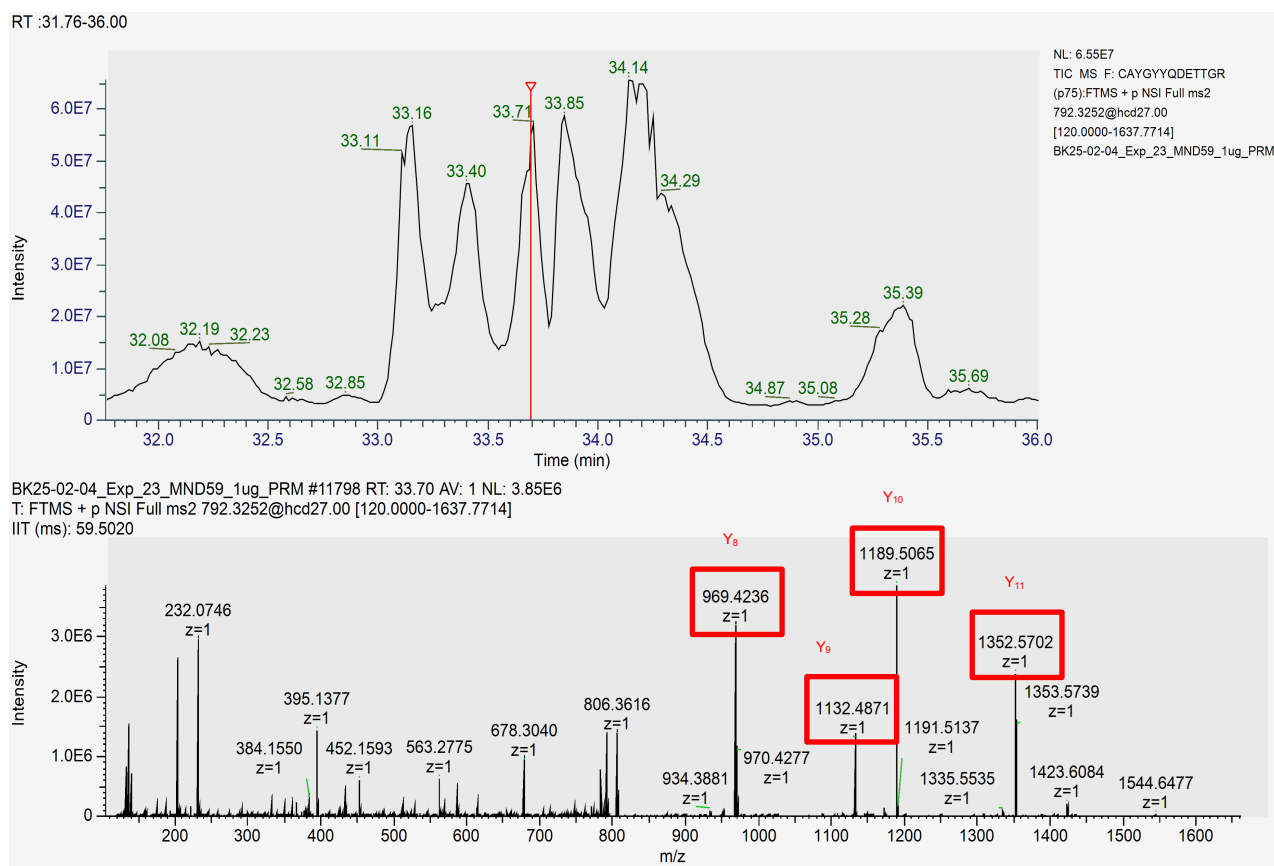
C: carbamidomethyl modification, added mass +57 Da.

AA: amino acid. Peptides highlighted in green undergoing further validation by PRM.

6.2.3 Targeted identification of unique peptides belonging to urinary p75 by PRM

Once the extracellular domain (ECD) of the p75 neurotrophin receptor (TNFRSF16; UniProt ID: P08138) was confirmed, two unique peptides derived from the ECD of the p75^{NTR}, CAYGYYQDETTGR and WADAECEEIPGR, were targeted by PRM analysis to determine the sensitivity of peptide quantification. Using orbitrap-mass spectrometry, the precursor mass (m/z) and charge (z) was used to schedule the isolation of the peptide precursor in an initial MS1 scan, such that the instrument could target and fragment each unique peptide into their characterizing b and y ions in a secondary product MS2 scan. For unique peptide CAYGYYQDETTGR, the precursor m/z of 792.33 and z of 2⁺ was used to generate targeted quantitation data in **Figure 6.2A-B**. Before the data was applied to Skyline for quantitative analysis, the .raw file of each peptide was manually interrogated in FreeStyle (**Figure 6.2A-B**) to confirm the presence of both the precursor m/z , and transition y ions belonging to each peptide (**Figure 6.2B**), their intensity (y -axis, **Figure 6.2A-B**), and their retention times within the 80-minute gradient (x -axis, **Figure 6.2A-B**). The MS1 precursor scan of peptide CAYGYYQDETTGR, m/z 792.33⁺⁺, was identified at a retention time of 33.71 minutes with an intensity of 6.6E⁷ (**Figure 6.2A**). The peptide sequence was confirmed by the MS2 fragmentation pattern in **Figure 6.2B**, finding product ions y^8 ; 969.42⁺, y^9 ; 1132.49⁺, y^{10} ; 1189.51⁺, and y^{11} ; 1352.57⁺ in high abundance. **Figure 6.2B** represents the fragmented data from the MS2 scan, generated by Proteome Discoverer. Column #1 represents the order of b^+ and b^{2+} ion fragments in red (●, discovered) derived from the N-terminal side of the peptide bond, and column #2 represents y^+ and y^{2+} ion fragments in blue (●, discovered) from the C-terminal side. The fragment ion data reconstructs the amino acid sequence of peptide CAYGYYQDETTGR, confirming its presence, sequence, and discoverable ion coverage in MND urine.

Figure 6.2B reports the detection of every y^+ ion from the C-terminal, indicating that the peptide has undergone complete and accurate fragmentation in the MS2 scan. This indicates that the PRM method is highly optimized for robust coverage, with high sensitivity and resolution for the targeted peptides, providing high confidence in their identification and quantification as a validation technique.



#1	b ⁺	b ²⁺	Sequence	y ⁺	y ²⁺	#2
1	161.03793	81.02260	C- Carbamidomethyl			13
2	232.07504	116.54116	A	1423.61245	712.30986	12
3	395.13837	198.07282	Y	1352.57534	676.79131	11
4	452.15983	226.58355	G	1189.51201	595.25964	10
5	615.22316	308.11522	Y	1132.49054	566.74891	9
6	778.28649	389.64688	Y	969.42721	485.21725	8
7	906.34507	453.67617	Q	806.36389	403.68558	7
8	1021.37201	511.18964	D	678.30531	339.65629	6
9	1150.41460	575.71094	E	563.27837	282.14282	5
10	1251.46228	626.23478	T	434.23577	217.62152	4
11	1352.50996	676.75862	T	333.18809	167.09769	3
12	1409.53142	705.26935	G	232.14042	116.57385	2
13			R	175.11895	88.06311	1

Figure 6.2: CAYGYQDETTGR peptide detection from MND59 in MS1/MS2 scan

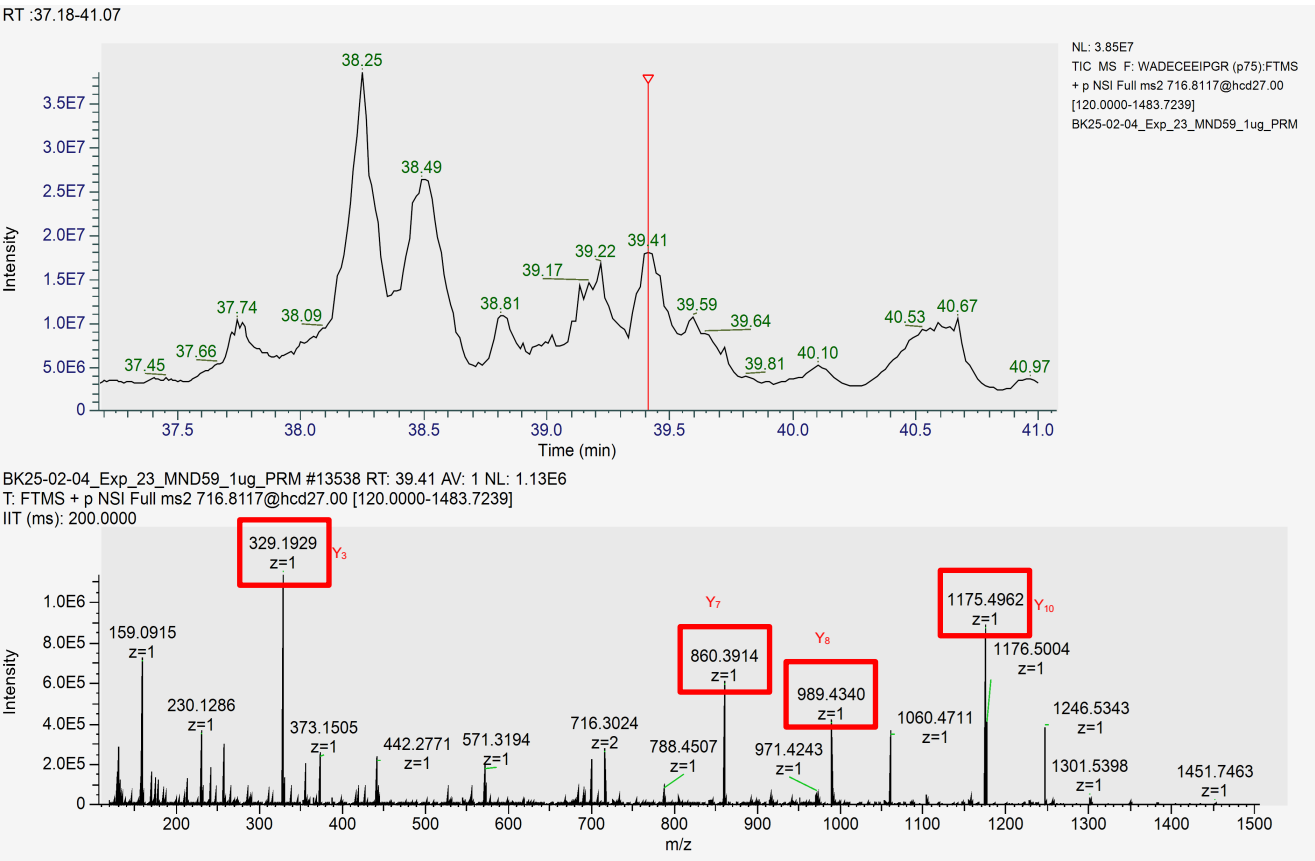
(A) Identification of unique peptide CAYGYQDETTGR for the protein group Tumour necrosis factor receptor superfamily member 16 (p75^{NTR}). For the PRM method, at retention time of 33.71 in sample MND 59, p75^{NTR} detection was optimized to monitor the precursor mass from the 792.33 ion and scan MS2 for transition ions; finding Y⁸; 969.42⁺, Y⁹; 1132.49⁺, Y¹⁰; 1189.51⁺, and Y¹¹; 1352.57⁺ in highest abundance. (B) highlights the number of b⁺ (red) and y⁺ (blue) ions identified in Spectronaut for peptide CAYGYQDETTGR belonging to the p75 neurotrophin receptor (TNFRSF16; UniProt ID: P08138).

The same interrogation process was completed for second unique peptide, WADAECEEIPGR, belonging to urinary p75 in MND urine. For unique peptide WADAECEEIPGR, the precursor m/z of 716.40 and z of 2^+ was used to generate targeted quantitation data in **Figure 6.3A-C**. The MS1 precursor scan of peptide WADAECEEIPGR, m/z 716.40 $^{2+}$, was identified at a retention time of 39.41 minutes with an intensity of $3.9E^7$ (**Figure 6.3A**). The peptide sequence was confirmed by the MS2 fragmentation pattern in **Figure 6.3B**, finding product ions y^3 ; 329.19 $^+$, y^7 ; 860.39 $^+$, y^8 ; 989.43 $^+$, and y^{10} ; 1175.50 $^+$ in high abundance. The signal-to-noise ratio of both peptides from **Figure 6.2A-B** and **Figure 6.3A-B** was determined as >201 in FreeStyle based on the intensity of their MS1 spectra (**Table 5**). **Figure 6.3B** represents fragment ions from the MS2 scan, generated by Proteome Discoverer. Column #1 represents the order of b^+ and b^{2+} ion fragments from the N-terminus in red (●, discovered), and column #2 represents y^+ and y^{2+} ion fragments from the C-terminus in blue (●, discovered). The fragment ion data reconstructs the amino acid sequence of peptide WADAECEEIPGR, confirming its presence, sequence, and ion coverage in MND urine. **Figure 6.3B** reports the detection of all y^+ ions, indicating that the peptide has undergone complete and accurate fragmentation in the MS2 scan using a robust method with high confidence and sensitivity.

6.2.4 Targeted ID of unique peptides belonging to urinary p75 by Skyline

Bioinformatics was performed using Skyline (v. 23.1.0.268), for the high-throughput and simultaneous analysis of candidate peptides in MND urine. PRM files were imported and analysed further in Skyline in **Figure 6.4A-B**. Representative extracted ion chromatograms (XICs) for the tryptic peptides CAYGYQDETTGR (**Figure 6.4A**) and WADAECEEIPGR (**Figure 6.4B**) derived from urinary p75 in the ECD are reported, with their respective precursor mass in neutral, protonated $[M+1]$ and doubly protonated $[M+2]$ ionized form of the peptide following electrospray ionization. Further, the top three fragment ions were reported for quantitation, along with their retention time and mass measurement accuracy shown above the chromatographic peak. For CAYGYQDETTGR, Skyline quantified from fragment ions y^8 – 969.4272 $^+$, y^{10} – 1189.5120 $^+$, y^{11} - 1352.5753 $^+$, and for WADAECEEIPGR from fragment ions y^3 – 329.1932 $^+$, y^6 – 860.3931 $^+$, and y^{10} - 1175.4997 $^+$. The retention time was consistent between FreeStyle .raw data, identified at 33.6 and 39.4 minutes, with a provided mass error of +3.1 and +2.6 ppm, respectively.

A



B

#1	b ⁺	b ²⁺	Seq.	y ⁺	y ²⁺	#2
1	187.08659	94.04693	W			12
2	258.12370	129.56549	A	1246.53684	623.77206	11
3	373.15065	187.07896	D	1175.49973	588.25350	10
4	444.18776	222.59752	A	1060.47278	530.74003	9
5	573.23035	287.11881	E	989.43567	495.22147	8
6	733.26100	367.13414	<u>C-</u> Carbamidomethyl	860.39308	430.70018	7
7	862.30359	431.65544	E	700.36243	350.68485	6
8	991.34619	496.17673	E	571.31984	286.16356	5
9	1104.43025	552.71876	I	442.27724	221.64226	4
10	1201.48302	601.24515	P	329.19318	165.10023	3
11	1258.50448	629.75588	G	232.14042	116.57385	2
12			R	175.11895	88.06311	1

Figure 6.3: WADAECEEIPGR peptide detection from MND59 in MS1/MS2 scan

(A) Identification of unique peptide WADAECEEIPGR for the protein group Tumour necrosis factor receptor superfamily member 16 (p75^{NTR}). For the PRM method, at retention time of 39.40 in sample MND 59, p75^{NTR} detection was optimized to monitor the precursor mass from the 716.40 ion and scan MS2 for transition ions; finding Y³; 329.19⁺, Y⁷; 860.39⁺, Y⁸; 989.43⁺, and Y¹⁰; 1175.50⁺ in highest abundance. (B) highlights the number of b⁺ (red) and y⁺ (blue) ions identified in Spectronaut for peptide WADAECEEIPGR belonging to the p75 neurotrophin receptor (TNFRSF16; UniProt ID: P08138).

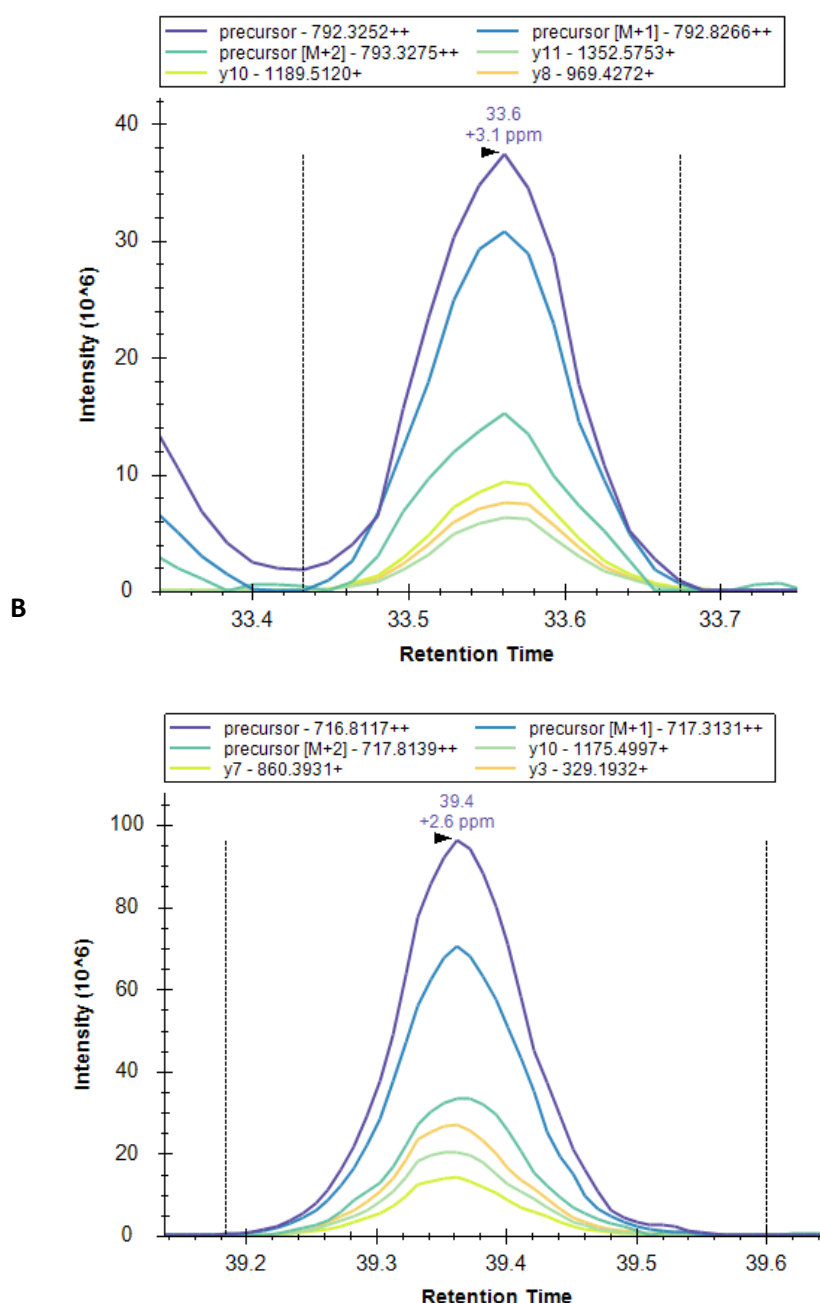


Figure 6.4: Extracted ion chromatogram in Skyline representative of tryptic peptides from urinary p75

Tryptic peptides CAYGYQDETTGR, and WADAECEEIPGR, were analysed in Skyline. The top 3 ranked fragment ions used for quantitation were highlighted, with retention time and mass measurement accuracy shown above the chromatogram peak. **(A)** The CAYGYQDETTGR peptide elutes with a retention time of 33.6 minutes across the 80-minute gradient. MS2 scan spectra shows the intensity and relative rank of the fragment ion intensities. Skyline performed quantification from MS2 fragment ions including y^8 – 969.4272⁺, y^{10} – 1189.5120⁺, y^{11} – 1352.5753⁺. **(B)** The WADAECEEIPGR peptide elutes with a retention time of 39.4 minutes across the 80-minute gradient. MS2 scan spectra for WADAECEEIPGR shows the intensity and relative rank of the fragment ion intensities. Skyline performed quantification from MS2 fragment ions including y^3 – 329.1932⁺, y^6 – 860.3931⁺, and y^{10} – 1175.4997⁺.

6.2.5 Optimized PRM assay parameters for p75 peptide quantification

The advantage of protein validation by PRM and orbitrap-mass spectrometry starts with collecting accurate, reproducible, and reliable data for the simultaneous and high-throughput detection of urinary peptides. The PRM was optimised for robustness using three transition ions, minimum injection time (ms) adjustments, automatic gain control (AGC) target gain (%) adjustments, and Q1 resolution windows. Improvements in quantitative precision were noted in both p75 peptides with a 1 µg injection volume, where **Table 6.3** reports a high minimum signal-to-noise ratio of >1,160 indicating a clean signal, and an average of 36 points per peak suggesting accurate peak integration by well resolved chromatography. The optimised longer minimum injection time (150 ms) for lower abundance peptide CAYGYYQDETTGR ensured the collection of sufficient ions, without over-sampling peptide WADAECEEIPGR (21.17 ms). An AGC target of up to 1000% was set to achieve a high S/N ratio without overloading the detector, and a Q1 resolution of 1.4 Da prevented isotopic overlap to maintain sufficient specificity for each target peptide. The PRM method was tuned to balance the sensitivity, specificity, and precision of peptide measurements in this study.

Table 6.3: Instrument performance and detection characteristics of tumour necrosis factor receptor superfamily member 16 (p75) peptides

Detailed PRM fragmentation data obtained for CAYGYQDETTGR and WADAECEEIPGR peptides belonging to urinary p75. Peptide fragmentation (y^+) ions are reported, scheduling their retention times, precursor masses, signal metrics, and optimised instrument parameters.

Peptide (ion)	Retention time (min)	m/z	Points per peak	Peak area	S/N ratio	Min Injection (ms)	AGC target %	Q1 resolution (Da)
<u>C</u> AYGYQDETTGR (MS1)	33.6	792.33	15	1.54E ⁸	5.69E ³	150.00	1000	1.4
<u>C</u> AYGYQDETTGR (y^8)	33.6	969.42	29	1.33E ⁷	3.50E ³	150.00	1000	1.4
<u>C</u> AYGYQDETTGR (y^{10})	33.6	1189.50	36	1.71E ⁷	4.98E ³	150.00	1000	1.4
<u>C</u> AYGYQDETTGR (y^{11})	33.6	1352.57	29	1.20E ⁶	1.16E ^{3*}	150.00	1000	1.4
WADAE <u>C</u> EEIPGR (MS1)	39.4	716.40	42	7.41E ⁸	1.20E ⁴	21.17	1000	1.4
WADAE <u>C</u> EEIPGR (y^3)	39.4	329.19	47	7.50E ⁷	3.36E ⁴	21.17	1000	1.4
WADAE <u>C</u> EEIPGR (y^6)	39.4	860.39	46	3.83E ⁷	4.78E ³	21.17	1000	1.4
WADAE <u>C</u> EEIPGR (y^{10})	39.4	1175.50	47	5.71E ⁷	3.01E ⁴	21.17	1000	1.4
C: Carbamidomethyl group [+57 Da]. RT = retention time, m/z = mass-to-charge ratio, S/N = signal to noise ratio, AGC = automatic gain control, Q1 = quadrupole 1, min = minutes, ms = milliseconds, Da = Daltons. *=minimum S/N ratio recorded.								

6.3 Simultaneous detection of 18 unique peptides in MND urine

The optimized PRM method parameters were applied to detect 18 unique peptides belonging to ten protein candidates subject to quantitative validation in MND urine. Retention time scheduling was used to maximise the sensitivity of simultaneous peptide detection over the 80-minute gradient, uniquely addressing the properties of each individual peptide to maximise their abundance and fragmentation. **Table 6.4** reports 18 unique peptides that were targeted and their corresponding protein groups, identifying parameters including their name and sequence, precursor mass and charge (m/z^z), product m/z , MS1 intensity, retention time (minutes), peak width (seconds), points per peak, and peptide specific matches. At minimum, two unique peptides with three product y ions were quantified by Skyline for adequate sensitivity and specificity. However, two peptides from protein groups cathepsin D (P07339) and tumour necrosis factor receptor superfamily member 12A (Q9NP84) were exempt from this prerequisite. Cathepsin D was initially discoverable with three unique peptides, TMSEVGGSVEDLIAK, YSQAVPAVTEGPIPEVL, and LSDPEDYTLK (**Table 6.1**), however, only LSDPEDYTLK had a signal high enough ($2.70E^7$, **Table 6.4**) to be reliably detected. Further, tumour necrosis factor receptor superfamily member 12A was not detected at a sufficient abundance level by ARPHSDFCLGCAAAPPAPFR for quantification. Peptides GSSWSADLDK and GSSWSADLDKCMDCASC were related, where the latter was a missed cleavage (missed 1) product of the former. Due to the nature of the missed cleavage, GSSWSADLDKCMDCASC was the only detectable peptide in human urine, albeit with the lowest abundance ($8.01E^6$, **Table 6.4**), resulting in 18 detectable peptides from 10 protein candidates.

Consideration of other variables, including retention time, peak width, and the number of points across the peak were noted to ensure minimal retention time overlap between peptides (i.e., VTSDHINFDPVYK RT: 44.00, GSSWSADLDKCMDCASC RT:44.20, **Table 6.4**) within the 80-minute gradient. Optimal peak width resolution was important for well-defined chromatography allowing for accurate quantification, ensuring the collection of a minimum of 7 data points across the peak for robust peak integration (Zeng and Bateman, 2023). The average points per peak reported across 18 peptides was 35.22, with a minimum of 7 found in the lowest abundance peptide GSSWSADLDKCMDCASC, and the highest reported with 57 found in LTVGAAQVPAQLLVGALR (**Table 6.4**).

Table 6.4: Summary of unique peptides and fragment ions for protein target validation using PRM mass spectrometry

Unique peptides selected for each target protein performed on specimen MND166, with precursor and fragment ion characteristic, MS1 intensity, retention time, peak widths, number of points per peak and peptide specific matches used to evaluate their suitability for PRM quantification using orbitrap mass spectrometry.

Protein name (accession)	Discoverable unique peptides	Precursor m/z ^z	Product m/z	MS1 Intensity	Retention time (min)	Peak width (s)	Points per peak	PSMs
Tumour necrosis factor receptor superfamily member 16 (p75, P08138)	CAYGYQDETTGR	792.33 ⁺⁺	969.43 1189.51 1352.58	1.90E ⁷	33.60	20	15	18
	WADAECEEIPGR	716.81 ⁺⁺	329.19 860.39 1175.50	1.20E ⁸	39.40	39	42	42
Cathepsin D (P07339)	LSPEDYTLK	533.28 ⁺⁺	433.22 768.37 865.42	2.70E ⁷	39.90	20	41	30
CD44 antigen (P16070)	FAGVFHVEK	517.28 ⁺⁺	659.34 815.43 886.47	5.51E ⁸	39.90	64	47	175
	YGFIEGHVVIPR	693.88 ⁺⁺	272.17 777.47 906.51	5.16E ⁸	51.50	39	45	196
CD14 (P08571)	LTVGAAQVPAQLLVGALR	889.04 ⁺⁺	1037.64 1136.71 1264.77	1.26E ⁸	71.60	30	57	75
	VLDLSCNR	488.75 ⁺⁺	536.22 649.31 764.34	1.44E ⁸	31.90	32	22	22
Macrophage colony stimulating factor-1 (P09603)	AFLLVQDIMEDTMR	841.42 ⁺⁺	1010.42 1138.48 1237.55	2.15E ⁸	73.20	27	43	22
	SHSSGSVLPLGELEGR	812.92 ⁺⁺	870.47 983.55 1400.73	8.32E ⁷	51.00	35	33	43
Prolactin inducible protein (P12273)	ELGICPDAAVPIK	805.93 ⁺⁺	357.24 1038.57 1198.61	2.73E ⁷	58.10	31	41	29
	YTACLQDDNPK	678.78 ⁺⁺	748.29 861.38 1092.44	2.48E ⁷	28.74	26	47	42
IL-6 receptor subunit	VTSDHINFDPVYK	512.26 ⁺⁺⁺	310.17 506.30 718.35	8.68E ⁷	44.00	22	13	13
	LTWTNPSIK	530.30 ⁺⁺	659.37	8.16E ⁷	43.70	47	26	10

beta (P40189)			845.45 946.50					
IL-10 receptor subunit beta (Q08334)	NILQWESPAFAK	702.37 ⁺⁺	935.46 1063.52 1176.60	2.10E ⁷	57.40	51	51	32
	IENEYETWTMK	722.33 ⁺⁺	795.37 958.43 1201.52	1.32E ⁷	44.40	32	17	10
Tumour necrosis factor receptor superfamily member 12A (Q9NP84)	GSSWSADLDK <u>C</u> MD <u>C</u> ASC (missed 1)	702.61 ⁺⁺⁺	765.79 801.31 844.83	8.01E ⁶	44.20	25	7	13
Fibulin-5 (Q9UBX5)	DQPFTILYR	576.81 ⁺⁺	455.26 665.39 909.51	1.67E ⁷	54.80	37	50	38
	QSGQ <u>C</u> LDIDE <u>C</u> R	740.81 ⁺⁺	807.33 920.41 1080.44	3.15E ⁸	31.30	55	38	85
<u>C</u> : carbamidomethyl modification [+57 Da]. Accession = UniProt ID, m/z = mass-to-charge ratio, z = charge, min = minutes, s = seconds, PSMs = peptide specific matches. (missed 1) = indicates 1 missed cleavage in peptide sequence.								

6.3.1 Retention time scheduling of target unique peptides in MND urine

With the goal to individually target all 18 unique peptides from **Table 6.4** from complex urine samples across an 80-minute gradient, various parameters for each peptide needed to be configured including their time range: including the AGC target, maximum injection time, and the Q1 resolution. Each of the 18 peptides was assigned a scheduled time window (minutes) within the gradient to ensure that each peptide was detected during its optimal retention window. This was reported in **Table 6.5**, ordering the peptides from their retention time windows from the start (i.e., YTACLCDDNPK: 26-32 minutes) to the end (i.e., AFLLVQDIMEDTMR: 71-75 minutes) of the 80-minute gradient. The AGC target percentage was used to control how many ions were accumulated during the scheduled retention time window. For lower abundance peptides, a higher AGC target % of up to 1,500 was reported to increase the number of ions for more sensitive detection, compared to a lower AGC target % of 600 for high abundance peptides to avoid over-saturation of the detector. The maximum injection time was proportional to this, allowing a longer injection time of up to 250 ms for lower abundant peptides to allow for ion accumulation. Quadrupole (Q1) resolution was fine-tuned between 0.7 and 1.4 Da based on the precursor m/z of each peptide. This achieved the highest inter-peptide resolution, ensuring that the precursor ion of each peptide was accurately isolated before fragmentation for the greatest specificity. This highlights the sensitive, specific, and robust detection of 18 unique peptides in a single 80-minute gradient.

Table 6.5: Targeted PRM acquisition parameters for mass spectrometry analysis

Targeted list of 18 unique peptides with precursor m/z , charge state, retention time in increasing order, automatic gain control (AGC) percentage, maximum injection time (ms), and quadrupole resolution for the scheduled mass scan for each unique peptide using orbitrap mass spectrometry on a Thermo Exploris 480.

Compound (protein abbreviation)	Precursor m/z^z	Time range (min) ↓	AGC target (%)	Max injection time (ms)	Q1 resolution (Da)
YTA <u>C</u> L <u>C</u> DDNPK (PIP)	678.78 ⁺⁺	26-32	600	150	0.7
QSGQ <u>C</u> LDIDE <u>C</u> R (FIB5)	740.81 ⁺⁺	30-34	1000	200	1.0
<u>C</u> AYGYQDETTGR (p75)	792.33 ⁺⁺	30-34	1000	200	1.4
VLDL <u>S</u> CNR (CD14)	488.75 ⁺⁺	30-34	1000	200	0.7
WADAE <u>C</u> EEIPGR (p75)	716.40 ⁺⁺	36-41	1000	200	1.4
FAGVFHVEK (CD44)	517.28 ⁺⁺	38-44	600	150	0.7
LSPEDYTLK (CTSD)	533.28 ⁺⁺	38-43	1000	200	0.7
LTWTNPSIK (IL6B)	530.30 ⁺⁺	42-50	1000	200	0.7
VTSDHINFDPVYK (IL6B)	512.26 ⁺⁺⁺	42-48	1500	250	0.7
GSSWSADLDKCMDCASC (TNF12A)	702.61 ⁺⁺⁺	42-48	1500	250	1.4
IENEYETWTK (IL10B)	722.33 ⁺⁺	43-49	1200	200	1.0
SHSSGSVLPLGELEGR (CSF1)	812.92 ⁺⁺	49-53	1200	200	1.0
YGFIEGHVVIPR (CD44)	693.88 ⁺⁺	50-54	600	150	1.0
DQPFTILYR (FIB5)	576.81 ⁺⁺	53-59	1200	200	0.7
NILQWESPAFAK (IL10B)	702.37 ⁺⁺	56-62	1000	200	1.4
ELGICPDAAVPIK (PIP)	805.93 ⁺⁺	57-61	1200	200	1.0
LTVGAAQVPAQLLVGALR (CD14)	889.04 ⁺⁺	58-75	600	150	1.4
AFLLVQDIMEDTMR (CSF1)	841.42 ⁺⁺	71-75	1200	200	1.0
Conditions: full scan 350-1400 m/z range.					

6.3.2 Detection and repeatability of target peptides in MND urine

Using this PRM method, 18 unique peptides from 10 protein groups were simultaneously detected in MND urine. **Figure 6.5A** provides the global overview of the chromatographic traces for each of the 18 peptides targeted across the 80-minute gradient. Further, the coefficient of variation (CV%) was reported in a histogram (**Figure 6.5B**) demonstrating the quantitative precision for three repeat measurements for each of the 18 peptide candidates. The median intra-assay CV% was reported as 3.4% across three technical replicate injections onto the Thermo Exploris 480 orbitrap-mass spectrometer using nano-electrospray ionisation (ESI) with a flow rate of 300 nL/min, which is a prerequisite for assay validation. Unique peptide WADAECEEIPGR (p75) reported the highest median intra-assay CV of 15% highlighted in red (●, CV 15%). This is higher than what is expected for intra-assay ELISA CV's, highlighting that further validation is required for this peptide. The average peptide peak area for both p75 peptides was calculated in FreeStyle, with CAYGYQDETTGR being $1.0\text{E}^9 \pm 2.9\text{E}^7$ with a CV of 2.9%, and WADAECEEIPGR being $3.7\text{E}^8 \pm 2.6\text{E}^7$ with a CV of 7.0%. Both peptides together produced an average area of $1.4\text{E}^9 \pm 5.0\text{E}^7$ with an intra-assay CV of 3.6% across three replicates. This was more comparable to ELISA intra-assay CV's of <10%. This study was completed once, and multiple biological replicates need to be measured in the future. However, the inter-assay variability by in-house ELISA was comparable. The average concentration in MND166 was reported as $6,639.4 \pm 327.1$ ng p75^{ECD}/mL, generating an inter-assay CV of 4.93% across three ELISA plate replicates. p75 detected by both assays produced an intra-assay CV of <10%, and the median reported CV for all remaining peptides was 3.4% which was deemed valid and acceptable for this assay (**Figure 6.5B**).

The quantitative results for p75 peptide CAYGYQDETTGR (**Figure 6.6A**), and WADAECEEIPGR (**Figure 6.6B**) were normalised to the TIC accounting for variances in injection load. This demonstrated high reproducibility for each p75 peptide across three technical replicates. The retention time reproducibility of each 18 peptide candidates was reported in **Figure 6.7A**, overlapping the retention time CV% across three technical replicates in the PRM analysis. The mass error of all peptide candidates was an average of $-3.0 \text{ ppm} \pm 1.3 \text{ ppm}$ (**Figure 6.7B**), and the run-to-run retention time (minutes) reproducibility was assessed by linear regression model in Skyline compared to an in-silico DDA library, reporting excellent linearity ($r=0.9803$) for all 18 targeted peptide candidates compared to the interpolated PRM library in **Figure 6.8**.

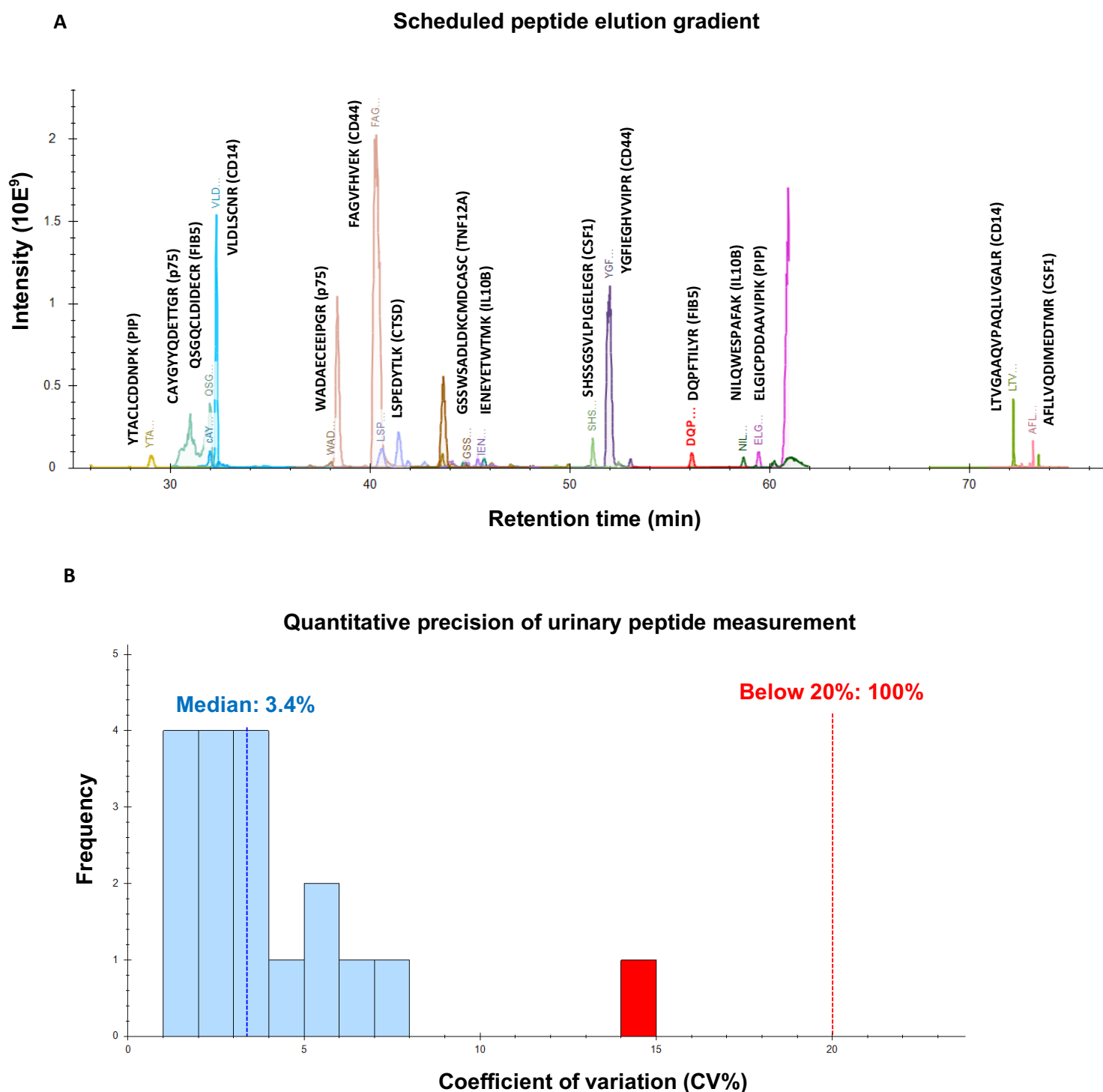


Figure 6.5: PRM results for the quantification of 10 candidate proteins, and 18 unique peptides in triplicate from MND166 urine

(A) provides a global overview of all 18 unique peptide targets showing their scheduled retention times and intensity profiles over the optimised 80-minute PRM method gradient. (B) Histogram that demonstrates the quantitative precision (CV) for three repeat measurements of each 18 unique peptides. The median CV was 3.4% across three technical replicate injections. Unique peptide WADAECEEIPGR, belonging to p75, had the highest CV of 15%. However, all targeted peptides were below the 20% CV threshold over three replicates. *Adapted from Skyline.*

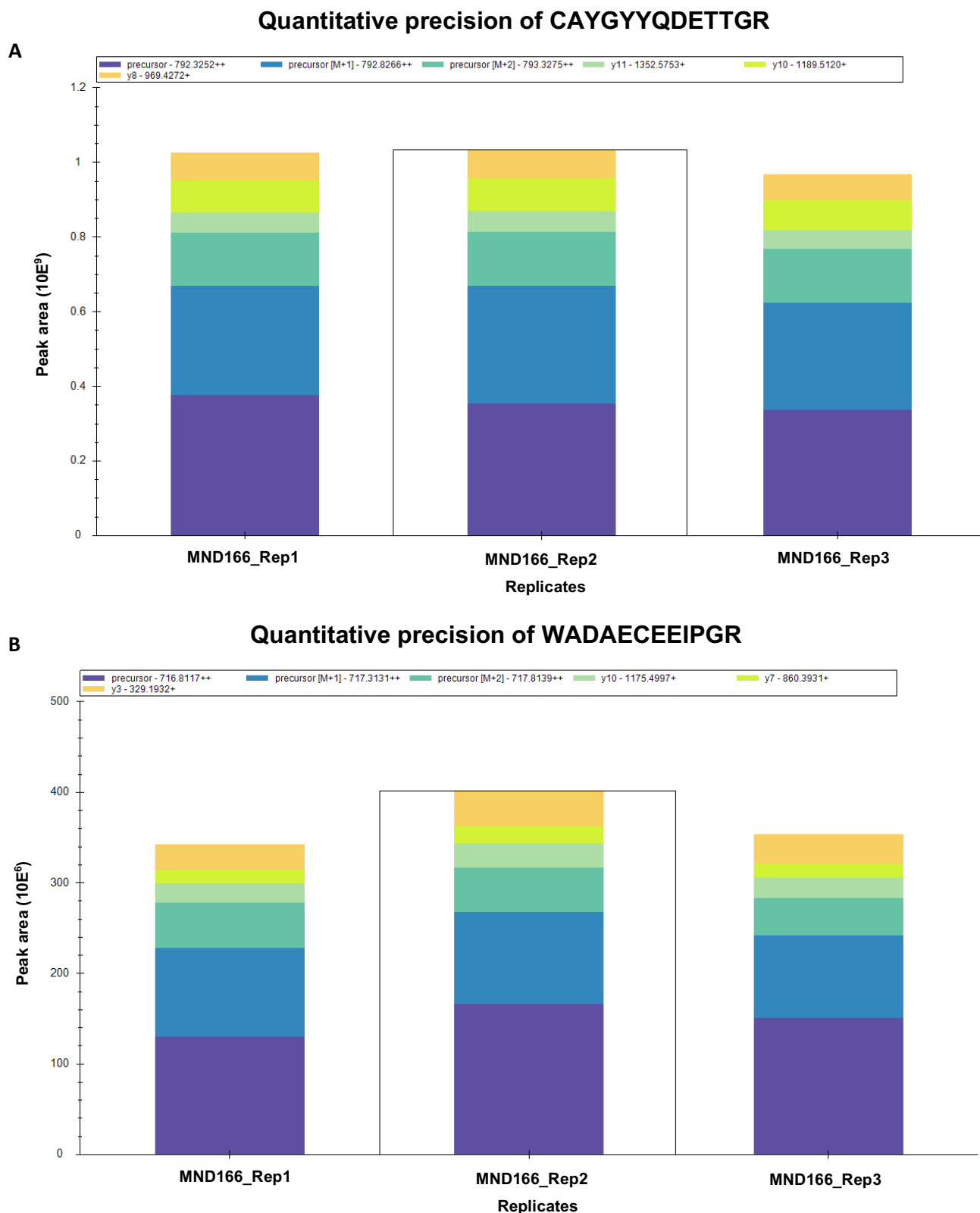


Figure 6.6: PRM results for the quantification of tumour necrosis factor receptor superfamily member 16 (p75) in triplicate from MND166 urine

(A) the normalised peak area of the precursor and product ions of CAYGYQDETTGR to the total TIC used for quantitation shows a reproducibly low CV of 2.9%% across three replicates. (B) the normalised peak area of precursor and product ions of WADAECEEIPGR to the total TIC used for quantitation shows a CV of 7.0%% across three replicates. This demonstrates a reproducibly high correlation across three technical replicates for unique peptides belonging to urinary p75 in human urine. *Adapted from Skyline.*

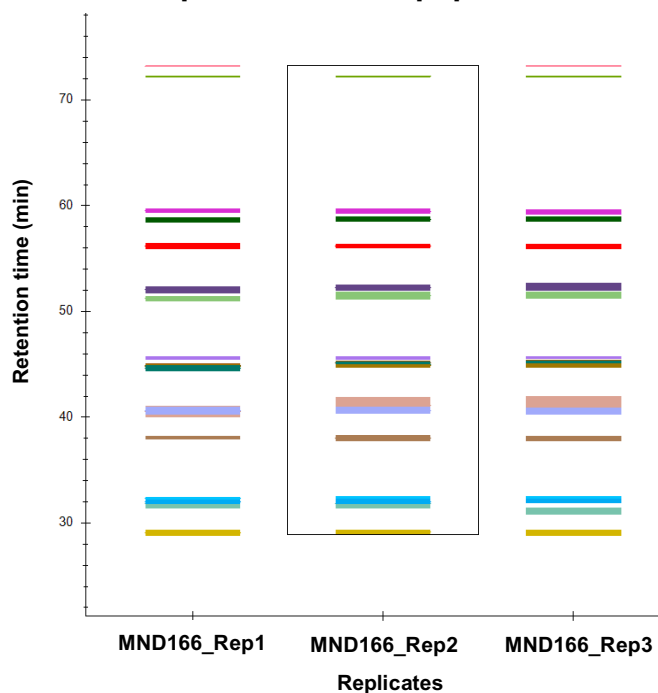
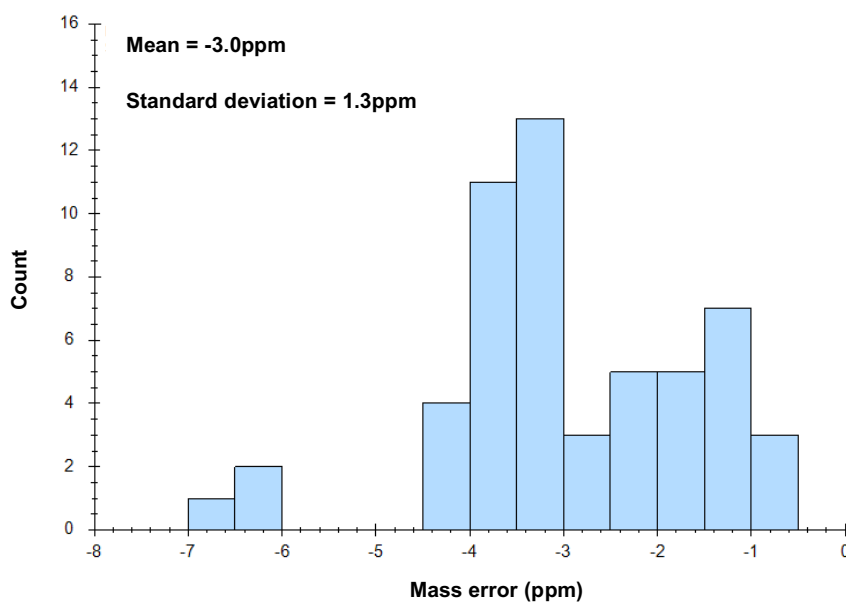
A Quantitative precision of all peptide retention times**B****Peptide mass accuracy**

Figure 6.7: Quantitative precision and accuracy of PRM results for the quantification of 10 candidate proteins, and 18 unique peptides in triplicate from MND166 urine

(A) demonstrates across three technical replicates the retention time reproducibility for all 18 targeted peptides. (B) the mean mass error (ppm) across all 18 targeted peptides was -3.0 ppm, with a standard deviation of 1.3 ppm. The maximum mass error threshold was set to ± 20 ppm. *Adapted from Skyline.*

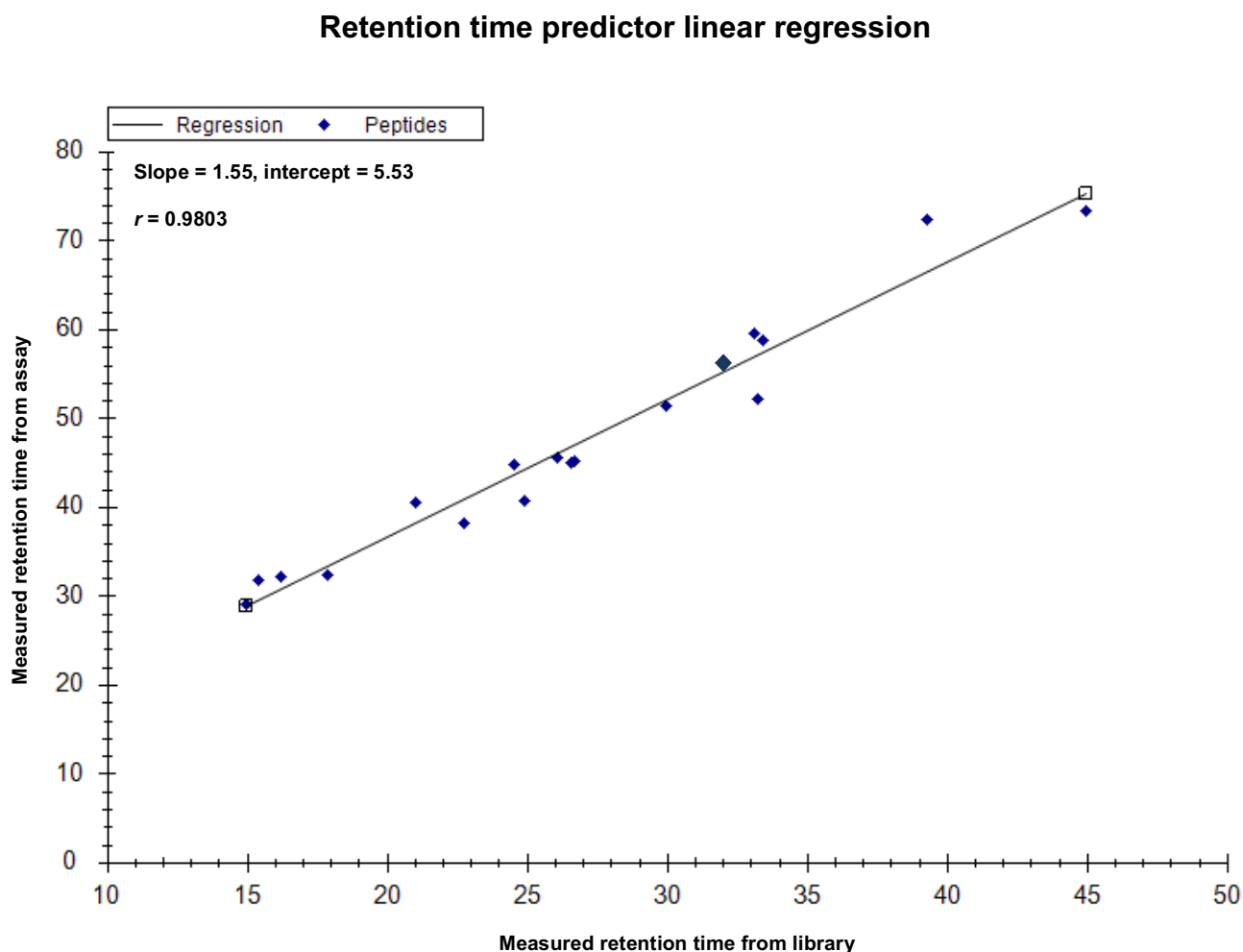


Figure 6.8: Retention time predictor linear regression model of 18 unique peptides

This linear regression model measured the retention time (minutes) obtained from the PRM assay for all 18 targeted peptides and compared them to an imported library containing data-dependent acquisition (DDA) data. This indicated that there was a strong correlation between the retention time of each peptide in each PRM assay ($r=0.9803$) when compared to the library data. In the future if stable isotope standard (SIS) peptides are used as internal standards, Skyline can perform a linear regression prediction for each peptide measured by PRM compared to the SIS peptide retention times. *Adapted from Skyline.*

6.3.3 Candidate proteins for validation quantified by DIA-mass spectrometry

Twelve proteins related to neuroinflammation, and twelve related to other disease mechanisms like neurodegeneration, muscle degeneration, and axonal degeneration were investigated in *section 5.4.2* and *5.4.3* of *Chapter 5* by DIA mass spectrometry in 39 people with MND and 26 healthy individuals. Ten of those proteins were chosen as candidates for analytical validation by the optimized PRM mass spectrometry method. Their result from analysis by 2-way T-test in MatLab after correction for false discovery rate (FDR) from the DIA mass spectrometry method was reported and summarized in **Table 6.6**, demonstrating their q value by 2-way T-test, and fold-change by differential analysis using MatLab. Proteins were selected on the premise of statistical significance ($q < 0.01$) by differential change in people with MND, that was proportional to a >1.5 fold-change to reflect notable change in disease pathology.

Table 6.6: Candidate proteins quantified by DIA mass spectrometry from *Chapter 5*

Ten protein groups were selected from *Chapter 5* for verification by PRM mass spectrometry. The relative abundance (PG.Quantity/mg creatinine) of ten protein groups quantified by DIA mass spectrometry using MatLab (*q* value) in 39 people with MND and 26 healthy individuals are reported. All statistical and fold change analyses (2-way T-tests) were performed using MatLab accounting for false discovery rate (FDR).

Protein name (accession)		Healthy individuals (n=26)	People with MND (n=39)	<i>q</i> value (<i>q</i> <0.01)	Fold change
Tumour necrosis factor receptor superfamily member 16 (p75, P08138)	Mean PG.Quantity/mg creatinine $\pm$ SD	99.9 $\pm$ 37.6	197.8 $\pm$ 139.2	2.51E ⁻⁰³	1.98
Cathepsin D (P07339)	Mean PG.Quantity/mg creatinine $\pm$ SD	38.5 $\pm$ 27.3	101.1 $\pm$ 81.7	1.43E ⁻⁰³	2.60
Macrophage colony stimulating factor-1 (P09603)	Mean PG.Quantity/mg creatinine $\pm$ SD	145.6 $\pm$ 68.7	293.0 $\pm$ 210.2	2.92E ⁻⁰³	2.01
CD44 antigen (P16070)	Mean PG.Quantity/mg creatinine $\pm$ SD	85.6 $\pm$ 48.7	237.4 $\pm$ 256.1	6.67E ⁻⁰³	2.77
IL-6 receptor subunit beta (P40189)	Mean PG.Quantity/mg creatinine $\pm$ SD	5.0 $\pm$ 2.5	11.3 $\pm$ 9.7	3.99E ⁻⁰³	2.27
Tumour necrosis factor receptor superfamily member 12A (Q9NP84)	Mean PG.Quantity/mg creatinine $\pm$ SD	0.48 $\pm$ 0.71	4.4 $\pm$ 3.7	8.54E ⁻⁰⁵	9.15
Fibulin-5 (Q9UBX5)	Mean PG.Quantity/mg creatinine $\pm$ SD	153.6 $\pm$ 71.8	399.3 $\pm$ 228.7	1.20E ⁻⁰⁴	2.34
CD14 (P08571)	Mean PG.Quantity/mg creatinine $\pm$ SD	226.9 $\pm$ 253.3	740.2 $\pm$ 1154.0	3.16E ⁻⁰² NS	3.27
IL-10 receptor subunit beta (Q08334)	Mean PG.Quantity/mg creatinine $\pm$ SD	8.7 $\pm$ 4.0	13.7 $\pm$ 6.8	4.65E ⁻⁰³	1.58
Prolactin inducible protein (P12273)	Mean PG.Quantity/mg creatinine $\pm$ SD	43.8 $\pm$ 39.1	1152.0 $\pm$ 3798.0	1.25E ⁻⁰¹ NS	26.30
NS: not significant					

6.3.4 Simultaneous quantification of 18 unique peptides in MND urine at baseline

The urinary proteome prepared from 39 people with MND, and 26 age-matched healthy individuals was analysed by the PRM method on a Thermo Exploris 480 mass spectrometer. 1 µg of urinary peptides were directly injected onto an in-house made 3.0 µm C18 analytical column that was 25 cm in length from a Thermo Vanquish Neo uHPLC system. 18 scheduled retention time windows were analysed over a gradient of 80-minutes, simultaneously detecting 18 unique peptides from 10 protein candidates. Skyline was used to analyse the MS1 and MS2 spectra against a spectral library generated by build-up data dependent acquisition (DDA) data from *section 6.3.1* to identify all 18 peptides per sample ($n=65$). Given this PRM method was a relative abundance analysis, Skyline applied a normalisation factor to account for variances in injection load such that equal amounts of peptides were analysed. The target intensities during PRM were quantified by the total ion current (TIC), such that variations in overall sample ionization efficiency were accounted for. Resulting from this, Skyline exported a 'normalised area,' named 'peptide abundance' for each unique peptide.

6.3.5 Peptide quantity normalised to creatinine

The peptide quantities for 18 unique peptides discovered by PRM analysis and Skyline were adjusted for renal dilution by urinary creatinine to account for inter-individual variances in hydration status, time of day, and protein concentrations, ensuring changes in peptides, and protein biomarkers reflect true biological changes of disease rather than variances in urine dilution. In the same way as performed previously (*section 5.3.9*), the absolute quantity of peptides injected onto the mass spectrometer was calculated. This produced a 'hypothetical' injection volume that was loaded onto the mass spectrometer to achieve 1 µg of protein load for each sample. The 'peptide abundance' of each unique peptide was divided by the 'hypothetical' injection volume and creatinine, generating a 'peptide abundance/mg creatinine.' This produced a normalised peptide quantity to creatinine that reflected the neat concentration of each urinary peptide, prior to sample preparation, to avoid circumstances of a statistically incorrect 'double normalisation.' The PRM study was performed using the same samples digested with Trypsin and prepared for *Chapter 5 (section 5.3.6)*, such that peptide normalisation to TIC by Skyline was equal to that performed by Spectronaut, and the normalisation to creatinine was consistent with previous results.

Each unique peptide was grouped, normalised, and analysed in Prism 10. Two condition groups were generated unique to disease status, sorting into 'MND' in red ($n=39 \times 18$ unique peptides, ●) or 'HI' in blue ($n=26 \times 18$ unique peptides, ●) to perform unique two-tailed

unpaired T-tests for each unique peptide quantified by PRM. As a result, the two tailed T-tests of unique peptides between people with MND and healthy individuals identified 13 out of 18 significantly changed peptides normalised to creatinine at baseline between **Figure 6.9A-B** to **Figure 6.18A-B**. Summary **Table 6.7**, and all in-text values for the abundance of unique peptides by PRM was listed as the average 'peptide quantity/mg creatinine' $\pm$ SD in people with MND and healthy individuals, alongside the significance and fold difference of each unique peptide candidates at baseline.

Unique peptides from tumour necrosis factor receptor superfamily member 16 (p75, P08138) were significantly changed in people with MND, consistent with previous studies demonstrating significant change at baseline (Shepherd et al., 2017). Unique peptide CAYGYYQDETTGR was significantly higher in 39 people with MND; $2.63\text{E}^5 \pm 1.59\text{E}^5$ compared to 26 HI; $1.21\text{E}^5 \pm 9.46\text{E}^4$ ($p < 0.0001$, 2.18-fold change, **Figure 6.9A**), alongside WADAECEEIPGR in people with MND; $5.61\text{E}^5 \pm 6.31\text{E}^5$ compared to 26 HI; $1.27\text{E}^4 \pm 5.37\text{E}^3$ ($p < 0.0001$, 44.01-fold change, **Figure 6.9B**). The one unique peptide from cathepsin D (P07339), LSPEDYTLK, was significantly higher in 39 people with MND; $4.70\text{E}^5 \pm 3.37\text{E}^5$ compared to 26 HI; $1.86\text{E}^5 \pm 1.67\text{E}^5$ ($p < 0.0001$, 2.53-fold change, **Figure 6.10**). Macrophage colony stimulating factor-1 demonstrated both peptides AFLLVQDIMEDTMR, as significantly higher in 39 people with MND; $3.23\text{E}^5 \pm 6.11\text{E}^5$ compared to 26 HI; $3.14\text{E}^4 \pm 3.61\text{E}^4$ ($p = 0.0050$, 4.02-fold change, **Figure 6.11A**), and SHSSGSVLPLGELEGR, significantly higher in 39 people with MND; $7.92\text{E}^5 \pm 4.69\text{E}^5$ compared to 26 HI; $5.90\text{E}^5 \pm 2.42\text{E}^5$ ($p = 0.0265$, 1.34-fold change, **Figure 6.11B**). CD44 antigen (P16070) was consistent across both peptides, FAGVFHVEK, significantly higher in 39 people with MND; $1.90\text{E}^7 \pm 9.01\text{E}^6$ compared to 26 HI; $5.27\text{E}^6 \pm 4.46\text{E}^6$ ($p = 0.0015$, 2.07-fold change, **Figure 6.12A**), and YGFIEGHVVIPR was significantly higher in 39 people with MND; $4.77\text{E}^6 \pm 3.59\text{E}^6$ compared to 26 HI; $2.24\text{E}^6 \pm 1.87\text{E}^6$ ($p = 0.0005$, 2.13-fold change, **Figure 6.12B**).

Likewise, interleukin-6 receptor subunit beta (P40189) was significantly changed by VTSDHINFDPVYK, significantly higher in 39 people with MND; $1.01\text{E}^5 \pm 1.25\text{E}^5$ compared to 26 HI; $8.36\text{E}^4 \pm 4.26\text{E}^4$ ($p < 0.0001$, 2.41-fold change, **Figure 6.13A**), and LTWTNPSIK, significantly higher in 39 people with MND; $1.71\text{E}^5 \pm 9.55\text{E}^4$ compared to 26 HI; $1.00\text{E}^5 \pm 8.54\text{E}^4$ ($p = 0.0027$, 1.71-fold change, **Figure 6.13B**). The single peptide of tumour necrosis factor receptor superfamily member 12A (Q9NP84), GSSWSADLDKCMDASC, was significantly higher in 39 people with MND; $2.29\text{E}^5 \pm 4.66\text{E}^5$ compared to 26 HI; $5.00\text{E}^4 \pm 1.28\text{E}^5$ ($p = 0.0281$, 4.57-fold change, **Figure 6.14**).

However, some peptides belonging to the protein groups were not changed in MND compared to healthy individuals. The quantification of fibulin-5 (Q9UBX5) reported peptide DQPFTILYR as not significantly changed in 39 people with MND; $2.04E^5 \pm 1.75E^5$ compared to 26 HI; $1.41E^5 \pm 1.05E^5$ ($p=0.0741$, 1.45-fold change, **Figure 6.15A**), compared to its second peptide QSGQCLDIDECR, that was significantly higher in 39 people with MND; $2.34E^6 \pm 1.49E^6$ compared to 26 HI; $1.12E^6 \pm 4.44E^5$ at baseline ($p<0.0001$, 2.09-fold change, **Figure 6.15B**). Interleukin-10 receptor subunit beta (Q08334) peptide NILQWESPAFAK was not significantly changed in 39 people with MND; $1.23E^6 \pm 3.63E^6$ compared to 26 HI; $3.31E^5 \pm 8.84E^5$ ($p=0.1468$, 3.71-fold change, **Figure 6.18A**), however, IENEYETWTMK was significantly changed in 39 people with MND; $9.61E^4 \pm 8.71E^4$ compared to 26 HI; $3.79E^4 \pm 3.27E^4$ ($p=0.0004$, 2.54-fold change, **Figure 6.18B**).

Interestingly, monocyte differentiation antigen CD14 (P08571) reported both peptides, LTVGAAQVPAQLLVGALR, as not significantly changed in 39 people with MND; $2.10E^6 \pm 3.03E^6$ compared to 26 HI; $1.23E^6 \pm 1.26E^6$ ($p=0.1151$, 1.71-fold change, **Figure 6.16A**), and VLDLSCNR, not significantly changed in 39 people with MND; $3.01E^6 \pm 2.39E^6$ compared to 26 HI; $1.99E^6 \pm 2.17E^5$ ($p=0.0798$, 1.51-fold change, **Figure 6.16B**). Similarly, prolactin inducible protein (P12273) peptide ELGICPDAAVPIK was not significantly changed in 39 people with MND; $3.18E^6 \pm 1.02E^7$ compared to 26 HI; $3.53E^5 \pm 2.60E^5$ ($p=0.0923$, 8.99-fold change, **Figure 6.17A**), and YTACLCDNPK was not significantly changed in 39 people with MND; $2.67E^6 \pm 8.81E^6$ compared to 26 HI; $2.10E^5 \pm 1.90E^5$ ($p=0.0898$, 12.69-fold change, **Figure 6.17B**). These results were summarised in **Table 6.7**.

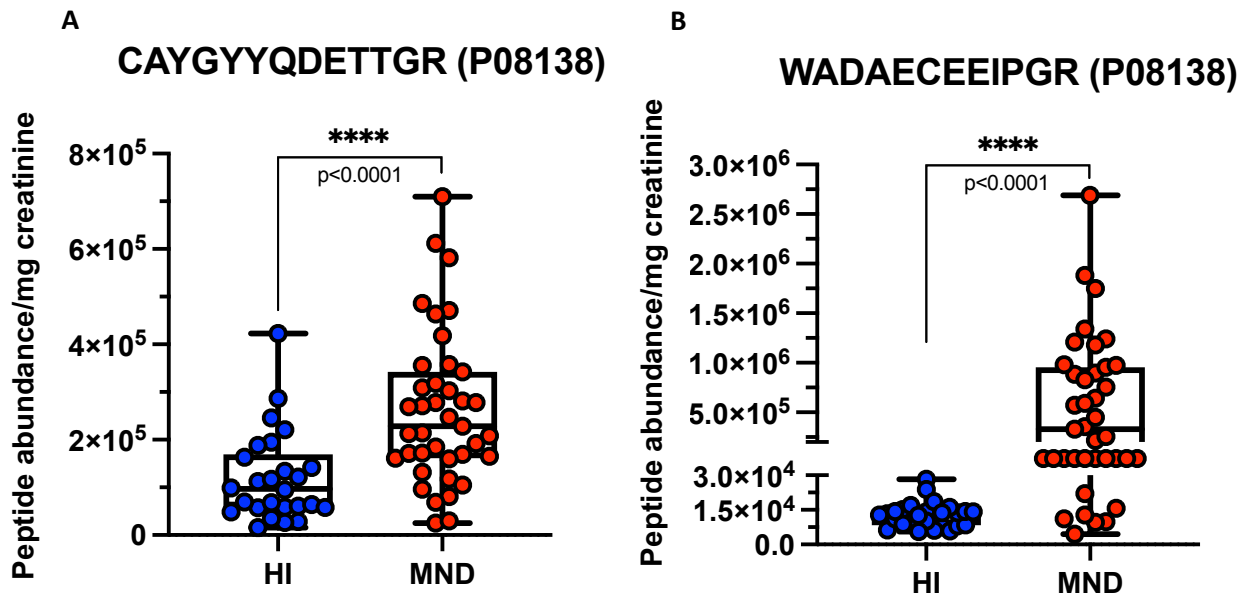


Figure 6.9: Quantification of unique peptides for tumour necrosis factor receptor superfamily member 16 (p75, P08138)

Results presented as average peptide abundance $\pm$ SD. **(A)** Unique peptide CAYGYQDETTGR was significantly higher in 39 people with MND; $2.63\text{E}^5 \pm 1.59\text{E}^5$ compared to 26 HI; $1.21\text{E}^5 \pm 9.46\text{E}^4$ ($p<0.0001$, 2.18-fold change, independent two tailed t-test). **(B)** Unique peptide WADAECEEIPGR was significantly higher in 39 people with MND; $5.61\text{E}^5 \pm 6.31\text{E}^5$ compared to 26 HI; $1.27\text{E}^4 \pm 5.37\text{E}^3$ ($p<0.0001$, 44.01-fold change, independent two tailed t-test). All statistical analysis was performed in Prism 10.

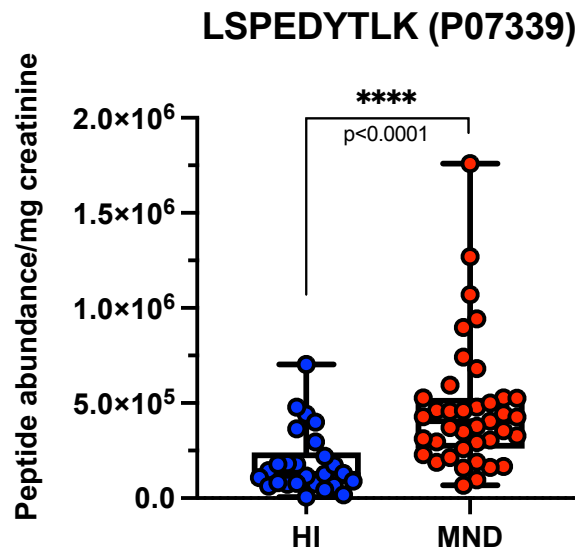


Figure 6.10: Quantification of unique peptide for Cathepsin D (P07339)

(A) Results presented as average peptide abundance $\pm$ SD. **(A)** Unique peptide LSPEDYTLK was significantly higher in 39 people with MND; $4.70 \times 10^5 \pm 3.37 \times 10^5$ compared to 26 HI; $1.86 \times 10^5 \pm 1.67 \times 10^5$ ($p < 0.0001$, 2.53-fold change, independent two tailed t-test). All statistical analysis was performed in Prism 10.

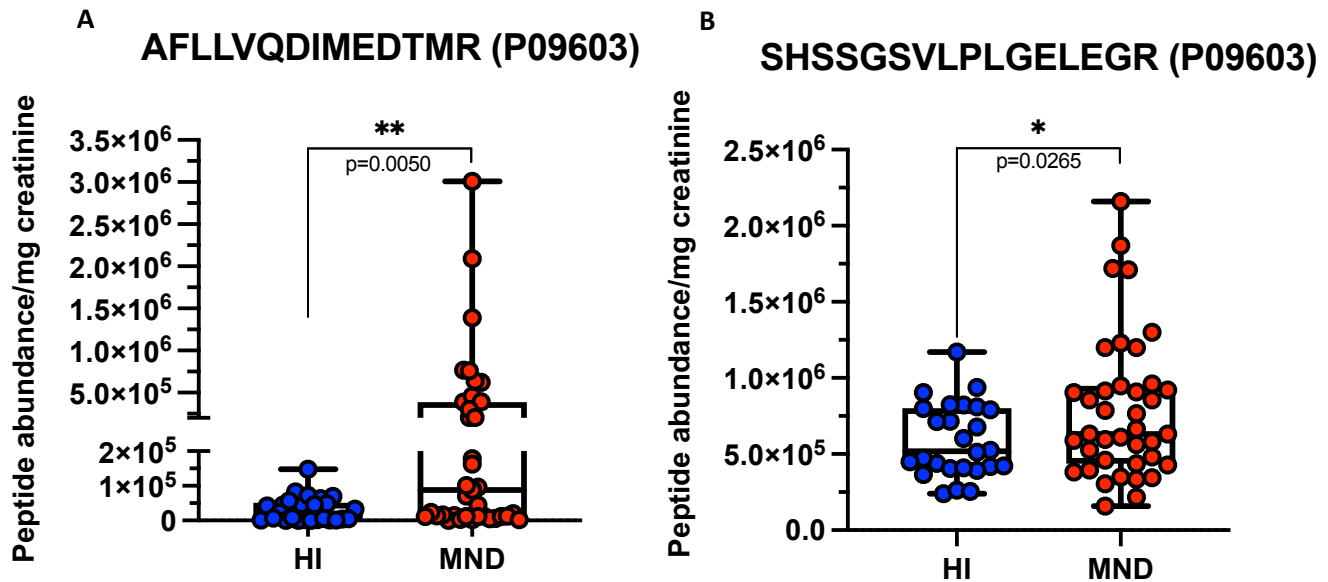


Figure 6.11: Quantification of unique peptides for macrophage colony stimulating factor-1 (P09603)

Results presented as average peptide abundance $\pm$ SD. **(A)** Unique peptide AFLLVQDIMEDTMR was significantly higher in 39 people with MND; $3.23E^5 \pm 6.11E^5$ compared to 26 HI; $3.14E^4 \pm 3.61E^4$ ($p=0.0050$, 4.02-fold change, independent two tailed t-test). **(B)** Unique peptide SHSSGSVLPLGELEGR was significantly higher in 39 people with MND; $7.92E^5 \pm 4.69E^5$ compared to 26 HI; $5.90E^5 \pm 2.42E^5$ ($p=0.0265$, 1.34-fold change, independent two tailed t-test). All statistical analysis was performed in Prism 10.

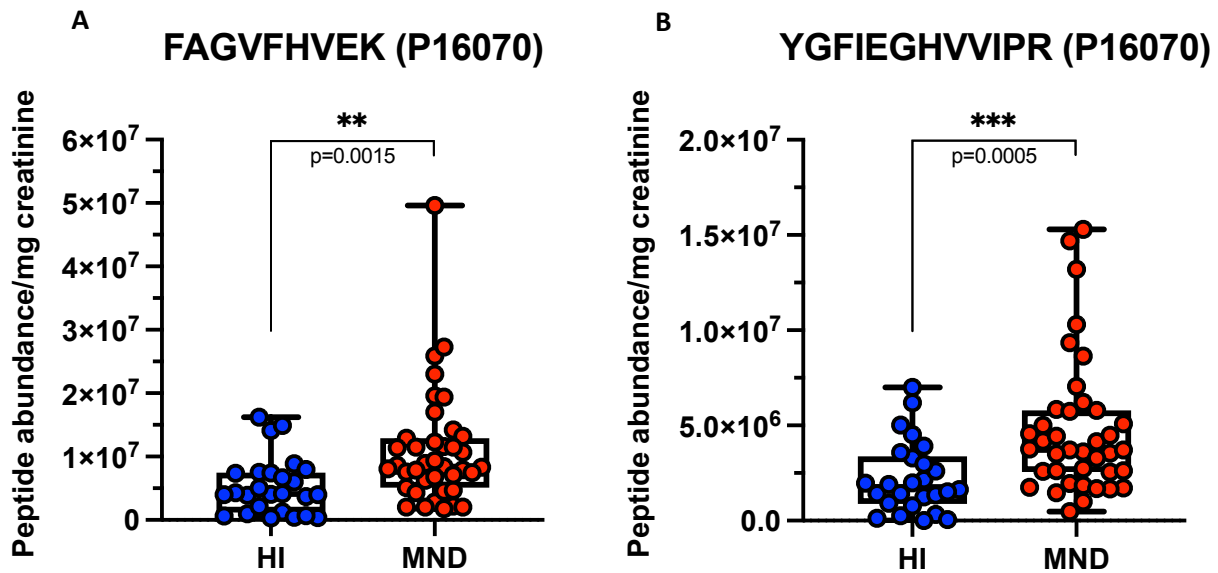


Figure 6.12: Quantification of unique peptides for CD44 antigen (P16070)

Results presented as average peptide abundance $\pm$ SD. **(A)** Unique peptide FAGVFHVEK was significantly higher in 39 people with MND; $1.90\text{E}^7 \pm 9.01\text{E}^6$ compared to 26 HI; $5.27\text{E}^6 \pm 4.46\text{E}^6$ ($p=0.0015$, 2.07-fold change, independent two tailed t-test). **(B)** Unique peptide YGFIEGHVVIPR was significantly higher in 39 people with MND; $4.77\text{E}^6 \pm 3.59\text{E}^6$ compared to 26 HI; $2.24\text{E}^6 \pm 1.87\text{E}^6$ ($p=0.0005$, 2.13-fold change, independent two tailed t-test). All statistical analysis was performed in Prism 10.

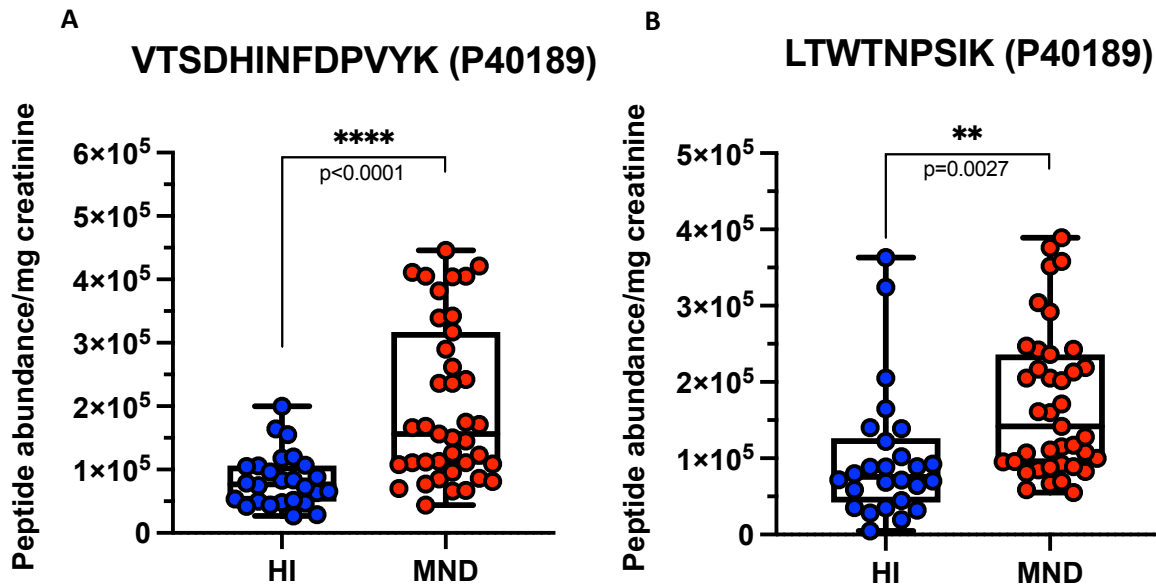


Figure 6.13: Quantification of unique peptides for interleukin-6 receptor subunit beta (P40189)

Results presented as average peptide abundance $\pm$ SD. **(A)** Unique peptide VTSDHINFDPVYK was significantly higher in 39 people with MND; $1.01 \times 10^5 \pm 1.25 \times 10^5$ compared to 26 HI; $8.36 \times 10^4 \pm 4.26 \times 10^4$ ($p < 0.0001$, 2.41-fold change, independent two tailed t-test). **(B)** Unique peptide LTWTNPSIK was significantly higher in 39 people with MND; $1.71 \times 10^5 \pm 9.55 \times 10^4$ compared to 26 HI; $1.00 \times 10^5 \pm 8.54 \times 10^4$ ($p = 0.0027$, 1.71-fold change, independent two tailed t-test). All statistical analysis was performed in Prism 10.

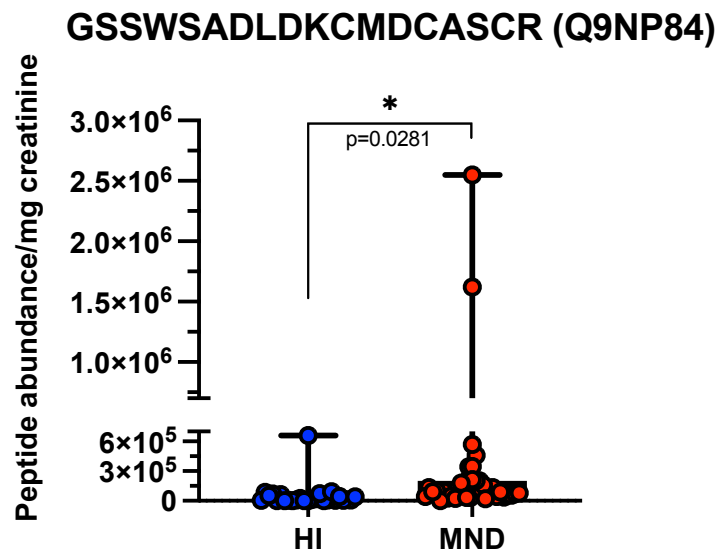


Figure 6.14: Quantification of unique peptide for tumour necrosis factor receptor superfamily member 12A (Q9NP84)

Results presented as average peptide abundance $\pm$ SD. Unique peptide GSSWSADLDKCMDASC was significantly higher in 39 people with MND; $2.29\text{E}^5 \pm 4.66\text{E}^5$ compared to 26 HI; $5.00\text{E}^4 \pm 1.28\text{E}^5$ ($p=0.0281$, 4.57-fold change, independent two tailed t-test).

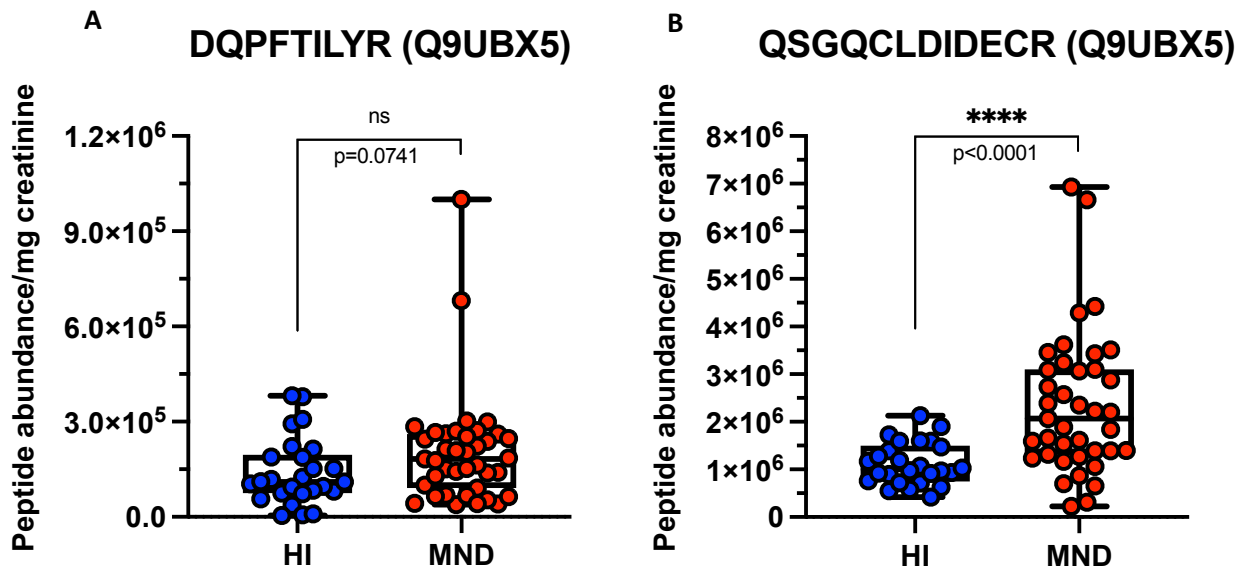


Figure 6.15: Quantification of unique peptides for fibulin-5 (Q9UBX5)

Results presented as average peptide abundance $\pm$ SD. **(A)** Unique peptide DQPFTILYR was not significantly different in 39 people with MND; $2.04 \times 10^5 \pm 1.75 \times 10^5$ compared to 26 HI; $1.41 \times 10^5 \pm 1.05 \times 10^5$ ($p=0.0741$, 1.45-fold change, independent two tailed t-test). **(B)** Unique peptide QSGQCLDIDECR was significantly higher in 39 people with MND; $2.34 \times 10^6 \pm 1.49 \times 10^6$ compared to 26 HI; $1.12 \times 10^6 \pm 4.44 \times 10^5$ ($p<0.0001$, 2.09-fold change, independent two tailed t-test). All statistical analysis was performed in Prism 10.

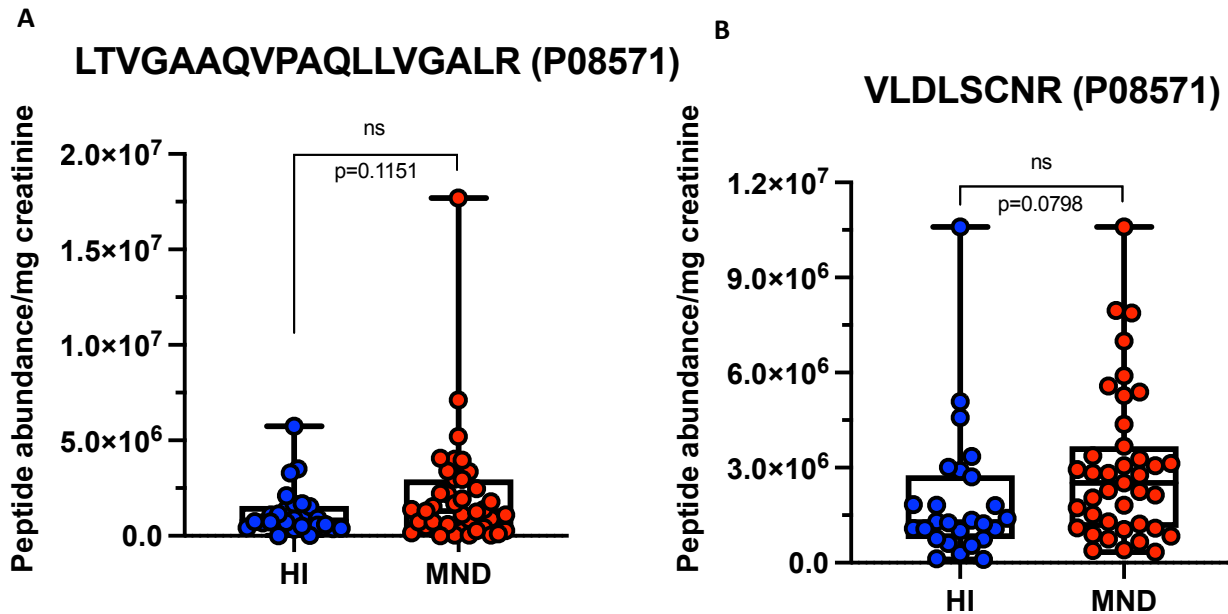


Figure 6.16: Quantification of unique peptides for monocyte differentiation antigen CD14 (P08571)

Results presented as average peptide abundance $\pm$ SD. **(A)** Unique peptide LTVGAAQVPAQLLVGALR was not significantly different in 39 people with MND; $2.10\text{E}^6 \pm 3.03\text{E}^6$ compared to 26 HI; $1.23\text{E}^6 \pm 1.26\text{E}^6$ ($p=0.1151$, 1.71-fold change, independent two tailed t-test). **(B)** Unique peptide VLDLSCNR was not significantly changed in 39 people with MND; $3.01\text{E}^6 \pm 2.39\text{E}^6$ compared to 26 HI; $1.99\text{E}^6 \pm 2.17\text{E}^5$ ($p=0.0798$, 1.51-fold change, independent two tailed t-test). All statistical analysis was performed in Prism 10.

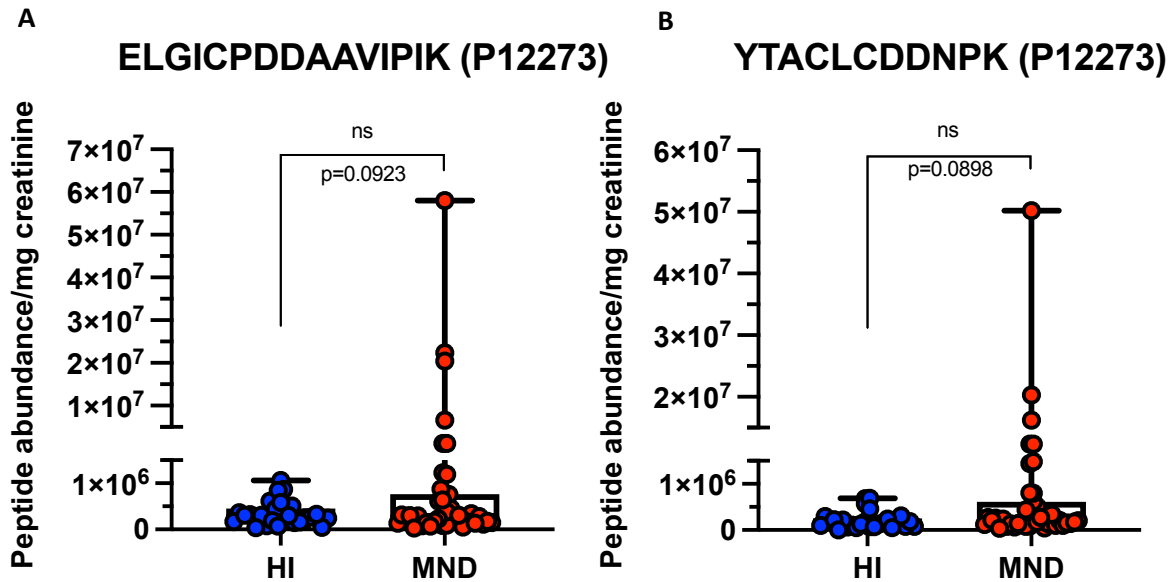


Figure 6.17: Quantification of unique peptides for prolactin inducible protein (P12273)

Results presented as average peptide abundance $\pm$ SD. **(A)** Unique peptide ELGICPDDAAVIPIK was not significantly changed in 39 people with MND; $3.18E^6 \pm 1.02E^7$ compared to 26 HI; $3.53E^5 \pm 2.60E^5$ ($p=0.0923$, 8.99-fold change, independent two tailed t-test). **(B)** Unique peptide YTACLCDDNPK was not significantly changed in 39 people with MND; $2.67E^6 \pm 8.81E^6$ compared to 26 HI; $2.10E^5 \pm 1.90E^5$ ($p=0.0898$, 12.69-fold change, independent two tailed t-test). All statistical analysis was performed in Prism 10.

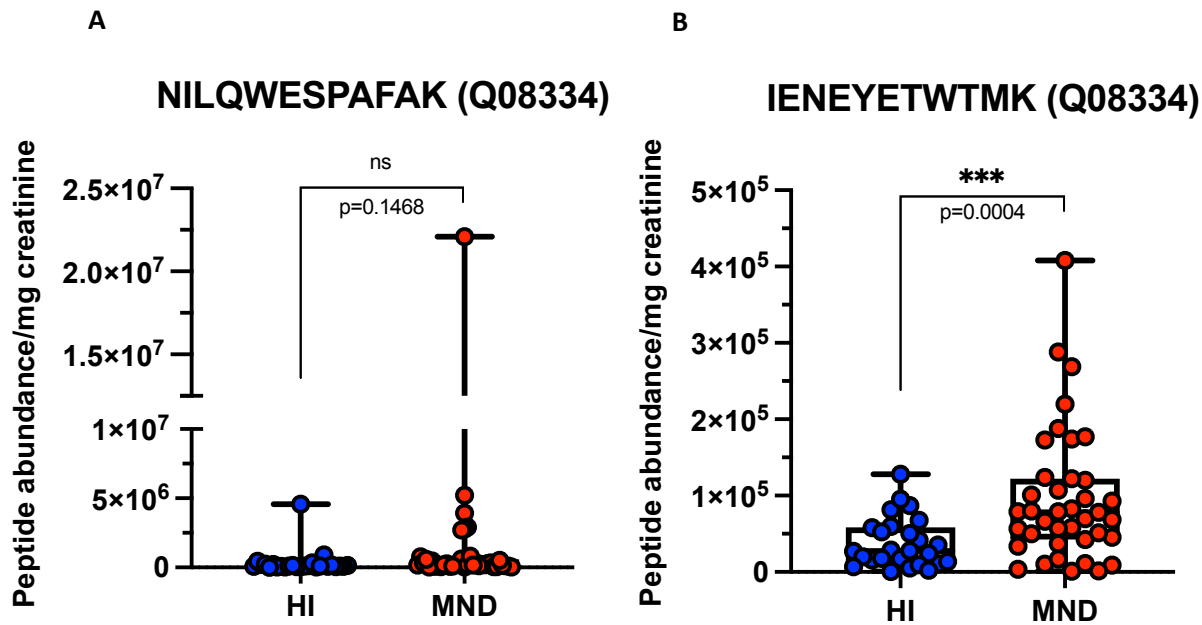


Figure 6.18: Quantification of unique peptides for interleukin-10 receptor subunit beta (Q08334)

Results presented as average peptide abundance $\pm$ SD. **(A)** Unique peptide NILQWESPAFAK was not significantly changed in 39 people with MND; $1.23\text{E}^6 \pm 3.63\text{E}^6$ compared to 26 HI; $3.31\text{E}^5 \pm 8.84\text{E}^5$ ($p=0.1468$, 3.71-fold change, independent two tailed t-test). **(B)** Unique peptide IENEYETWTMK was significantly changed in 39 people with MND; $9.61\text{E}^4 \pm 8.71\text{E}^4$ compared to 26 HI; $3.79\text{E}^4 \pm 3.27\text{E}^4$ ($p=0.0004$, 2.54-fold change, independent two tailed t-test). All statistical analysis was performed in Prism 10.

Table 6.7: Summary of significant urinary peptides by PRM at baseline

18 candidate peptides were quantified by PRM mass spectrometry (peptide abundance/mg creatinine) in 39 people with MND and 26 healthy individuals. Statistical analyses were performed by 2-way T-tests in Prism 10 (*p* value) and fold-change was calculated in Excel. Values presented as mean $\pm$ SD.

Protein name (accession)	Quantified from unique peptides	Healthy individuals (n=26)	People with MND (n=39)	<i>p</i> value	Fold difference in MND/Hi
Tumour necrosis factor receptor superfamily member 16 (p75, P08138)	<u>C</u> AYGYQDETTGR	$1.21\text{E}^5 \pm 9.46\text{E}^4$	$2.63\text{E}^5 \pm 1.59\text{E}^5$	<0.0001	2.18
	WADAE <u>C</u> EEIPGR	$1.27\text{E}^4 \pm 5.37\text{E}^3$	$5.61\text{E}^5 \pm 6.31\text{E}^5$	<0.0001	44.01
Cathepsin D (P07339)	LSPEDYTLK	$1.86\text{E}^5 \pm 1.67\text{E}^5$	$4.70\text{E}^5 \pm 3.37\text{E}^5$	<0.0001	2.53
Macrophage colony stimulating factor-1 (P09603)	AFLLVQDIMEDTMR	$3.14\text{E}^4 \pm 3.61\text{E}^4$	$3.23\text{E}^5 \pm 6.11\text{E}^5$	0.0050	4.02
	SHSSGSVLPLGELEGR	$5.90\text{E}^5 \pm 2.42\text{E}^5$	$7.92\text{E}^5 \pm 4.69\text{E}^5$	0.0265	1.34
CD44 antigen (P16070)	FAGVFHVEK	$5.27\text{E}^6 \pm 4.46\text{E}^6$	$1.90\text{E}^7 \pm 9.01\text{E}^6$	0.0015	2.07
	YGFIEGHVVIPR	$2.24\text{E}^6 \pm 1.87\text{E}^6$	$4.77\text{E}^6 \pm 3.59\text{E}^6$	0.0005	2.13
IL-6 receptor subunit beta (P40189)	VTSDHINFDPVYK	$8.36\text{E}^4 \pm 4.26\text{E}^4$	$1.01\text{E}^5 \pm 1.25\text{E}^5$	<0.0001	2.41
	LTWTNPSIK	$1.00\text{E}^5 \pm 8.54\text{E}^4$	$1.71\text{E}^5 \pm 9.55\text{E}^4$	0.0027	1.71
Tumour necrosis factor receptor superfamily member 12A (Q9NP84)	GSSWSADLDK <u>C</u> MDC <u>A</u> SC (missed 1)	$5.00\text{E}^4 \pm 1.28\text{E}^5$	$2.29\text{E}^5 \pm 4.66\text{E}^5$	0.0281	4.57
Fibulin-5 (Q9UBX5)	DQPFTILYR	$1.41\text{E}^5 \pm 1.05\text{E}^5$	$2.04\text{E}^5 \pm 1.75\text{E}^5$	0.0741	1.45
	QSGQ <u>C</u> LDIDE <u>C</u> R	$1.12\text{E}^6 \pm 4.44\text{E}^5$	$2.34\text{E}^6 \pm 1.49\text{E}^6$	<0.0001	2.09
CD14 (P08571)	LTVGAAQVPAQLLVGALR	$1.23\text{E}^6 \pm 1.26\text{E}^6$	$2.10\text{E}^6 \pm 3.03\text{E}^6$	0.1151	1.71
	VLDL <u>S</u> CNR	$1.99\text{E}^6 \pm 2.17\text{E}^5$	$3.01\text{E}^6 \pm 2.39\text{E}^6$	0.0798	1.51
Prolactin inducible protein (P12273)	ELGI <u>C</u> PPDAAVPIK	$3.53\text{E}^5 \pm 2.60\text{E}^5$	$3.18\text{E}^6 \pm 1.02\text{E}^7$	0.0923	8.99
	YTA <u>C</u> L <u>C</u> DDNPK	$2.10\text{E}^5 \pm 1.90\text{E}^5$	$2.67\text{E}^6 \pm 8.81\text{E}^6$	0.0898	12.69
IL-10 receptor subunit beta (Q08334)	NILQWESPAFAK	$3.31\text{E}^5 \pm 8.84\text{E}^5$	$1.23\text{E}^6 \pm 3.63\text{E}^6$	0.1468	3.71
	IENEYETWTMK	$3.79\text{E}^4 \pm 3.27\text{E}^4$	$9.61\text{E}^4 \pm 8.71\text{E}^4$	0.0004	2.54

6.3.6 Urinary proteins quantified by PRM at baseline

For each protein group, PRM analysis was achieved through quantification of three product y^+ ions from two unique peptides to ensure specificity, with exceptions to cathepsin D and tumour necrosis factor receptor superfamily member 12A. The quantity reported for each peptide, 'peptide abundance/mg creatinine,' was summed together in Skyline to generate a 'protein abundance/mg creatinine' value for each protein group, that was a product of the abundance of their corresponding unique peptides. Thereby, the relative amount of each protein group was quantified in the urine of 39 people with MND, and 26 healthy individuals at baseline. The quantity of 10 candidate proteins discovered by PRM analysis and Skyline were adjusted for renal dilution by urinary creatinine, and each protein group was analysed in Prism 10. Two condition groups were generated unique to disease status, sorting into 'MND' in red ($n=39 \times 10$ candidate proteins, ●) or 'HI' in blue ($n=26 \times 10$ candidate proteins, ●) to perform unique two-tailed unpaired T-tests for each protein group quantified by PRM. As a result, the two tailed T-tests of unique peptides between people with MND and healthy individuals identified 7 out of 10 significantly changed peptides normalised to creatinine at baseline (**Figure 6.18A-D**, **Figure 6.20A-C**). **Table 6.8** is a summary showing the abundance of each candidate protein group listed as the average 'protein abundance/mg creatinine' $\pm$ SD in people with MND and healthy individuals, alongside the significance and fold difference of each unique peptide candidates at baseline.

Tumour necrosis factor receptor superfamily member 16 (p75, P08138) was significantly changed in people with MND, consistent with (Shepherd et al., 2017) demonstrating significant change at baseline. Urinary p75 was significantly higher in people with MND; $8.24E^5 \pm 6.98E^5$ compared to HI; $1.33E^5 \pm 9.55E^4$ ($p<0.0001$, 6.18-fold change, **Figure 6.19A**). Likewise, lysosomal protein cathepsin D reported significant change in people with MND; $4.70E^5 \pm 3.36E^5$ compared to HI; $1.86E^5 \pm 1.67E^5$ ($p<0.0001$, 2.53-fold change, **Figure 6.19B**), as did macrophage colony stimulating factor-1, higher in people with MND; $1.12E^6 \pm 8.20E^5$ compared to HI; $6.20E^5 \pm 2.40E^5$ ($p=0.0008$, 1.80-fold change, **Figure 6.19C**), and CD44 antigen in people with MND; $1.57E^7 \pm 1.14 E^7$ compared to HI; $7.51E^6 \pm 6.14E^6$ ($p<0.0001$, 2.09-fold change, **Figure 6.19D**).

Both unique peptides from pro-inflammatory cytokine receptor interleukin-6 receptor subunit beta (P40189) reported significant change in people with MND; $3.73E^5 \pm 2.15E^5$ compared to HI; $1.84E^5 \pm 1.24E^5$ ($p<0.0001$, 2.03-fold change, **Figure 6.20A**), alongside candidate marker of neurodegeneration in tumour necrosis factor receptor superfamily member 12A

(Q9NP84) significantly changed in people with MND; $2.29\text{E}^5 \pm 4.66\text{E}^5$ compared to HI; $5.00\text{E}^4 \pm 1.28\text{E}^5$ ($p=0.0282$, 4.57-fold change, **Figure 6.20B**), and candidate marker of muscle degeneration, fibulin-5 (Q9UBX5), significantly changed in people with MND; $2.55\text{E}^6 \pm 1.58\text{E}^6$ compared to HI; $1.26\text{E}^6 \pm 4.99\text{E}^5$ ($p<0.0001$, 2.02-fold change, **Figure 6.20C**) at baseline.

However, the last three remaining protein candidates did not exhibit significant change upon validation by PRM analysis. Of these proteins, candidate immune marker monocyte differentiation antigen CD14 (P08571) did not significantly change in people with MND; $5.11\text{E}^6 \pm 5.00\text{E}^6$ compared to HI; $3.22\text{E}^6 \pm 3.19\text{E}^6$ ($p=0.0673$, 1.59-fold change, **Figure 6.21A**). Immune response and cell signalling candidate prolactin inducible protein (P12273) saw no change in either unique peptide, showing no significant change in people with MND; $8.54\text{E}^6 \pm 2.70\text{E}^7$ compared to HI; $7.62\text{E}^5 \pm 6.22\text{E}^5$ ($p=0.0795$, 11.19-fold change, **Figure 6.21B**), however elicited the highest fold change amongst all protein groups. Lastly, candidate anti-inflammatory cytokine interleukin-10 receptor subunit beta (Q08334) reported no significant change in people with MND; $1.32\text{E}^6 \pm 3.65\text{E}^6$ compared to HI; $3.69\text{E}^5 \pm 8.78\text{E}^5$ ($p=0.1252$, 3.59-fold change, **Figure 6.21C**) at baseline. The quantitative summary of each protein group was reported in **Table 6.8**.

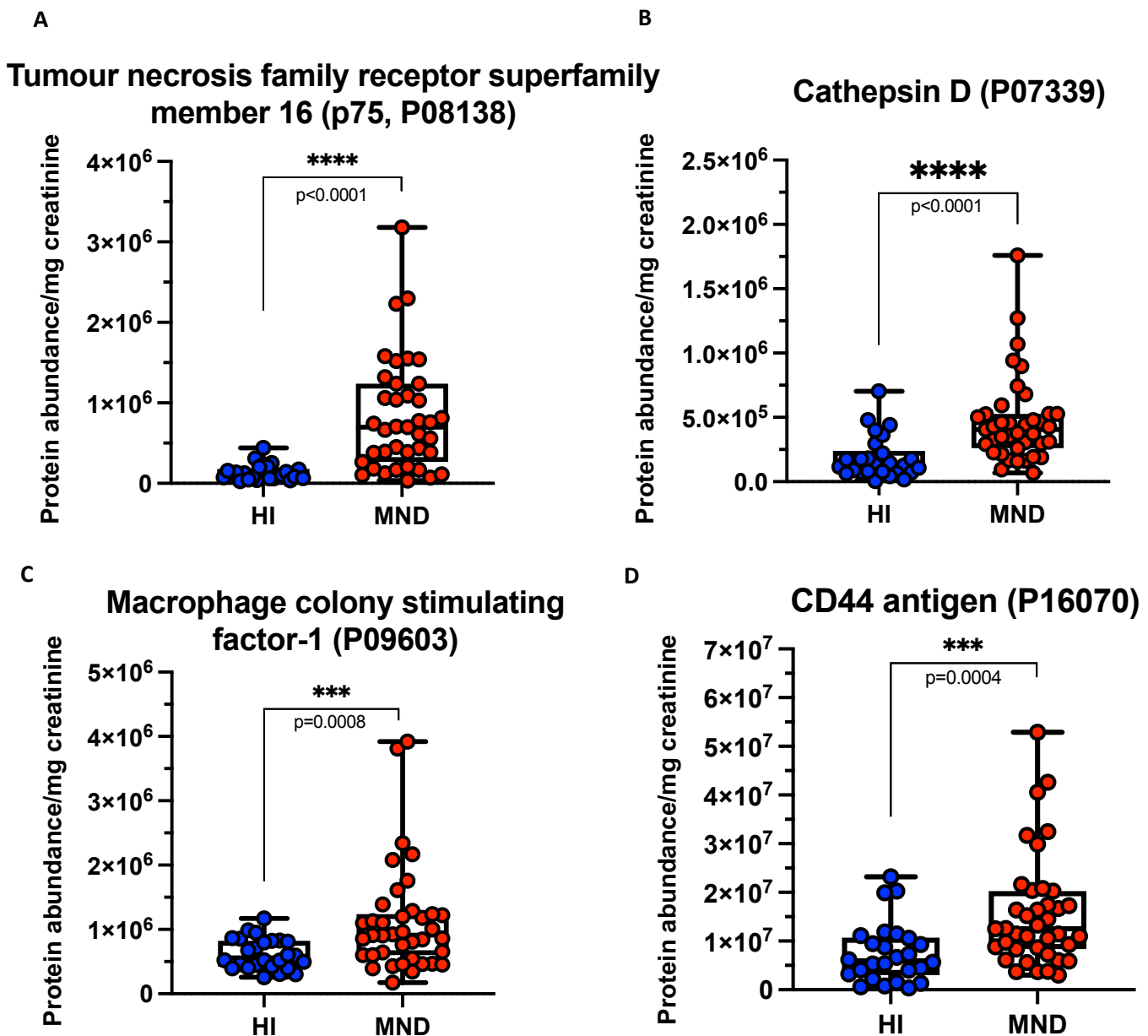


Figure 6.19: Significant urinary protein candidates related to neuronal injury and macrophage activation quantified by PRM at baseline

Significant urinary biomarker levels were compared between 39 people with MND and 26 healthy individuals by 2-way T-test in Prism 10. In **(A)** urinary p75 was significantly higher in MND; $1.33\text{E}^5 \pm 9.55\text{E}^4$, compared to HI; $8.24\text{E}^5 \pm 6.98\text{E}^5$, $p<0.0001$. **(B)** Cathepsin D was higher in MND; $1.86\text{E}^5 \pm 1.67\text{E}^5$, compared to HI; $4.70\text{E}^5 \pm 3.36\text{E}^5$, $p<0.0001$. **(C)** Macrophage colony stimulating factor-1 was higher in MND; $6.20\text{E}^5 \pm 2.40\text{E}^5$, compared to HI; $1.12\text{E}^6 \pm 8.20\text{E}^5$, $p=0.0008$, and in **(D)** CD44 antigen was higher in MND; $7.51\text{E}^6 \pm 6.14\text{E}^6$, compared to HI; $1.57\text{E}^7 \pm 1.14\text{E}^7$, $p=0.0004$. Values presented as mean $\pm$ SD.

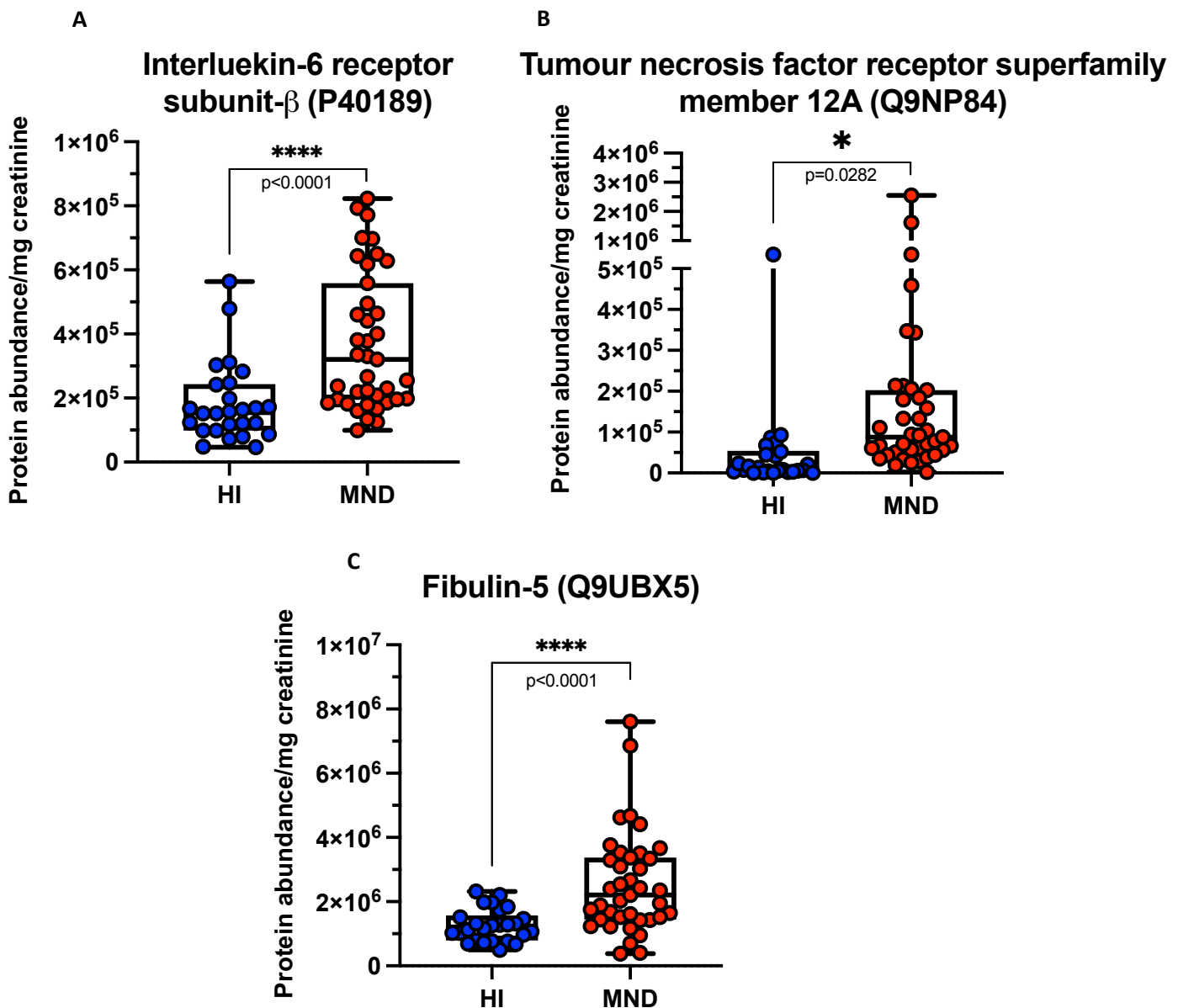


Figure 6.20: Significant urinary protein candidates related to cytokine signalling and extracellular matrix remodelling quantified by PRM at baseline

Significant urinary biomarker levels were compared between 39 people with MND and 26 healthy individuals by 2-way T-test in Prism 10. In **(A)** interleukin-6 receptor subunit beta was significantly higher in MND; $1.84E^5 \pm 1.24E^5$, compared to HI; $3.73E^5 \pm 2.15E^5$, $p < 0.0001$. **(B)** Tumour necrosis factor receptor superfamily member 12A was higher in MND; $5.00E^4 \pm 1.28E^5$, compared to HI; $2.29E^5 \pm 4.66E^5$, $p = 0.0282$, and **(C)** fibulin-5 was higher in MND; $1.26E^6 \pm 4.99E^5$, compared to HI; $2.55E^6 \pm 1.58E^6$, $p < 0.0001$. Values presented as mean $\pm$ SD.

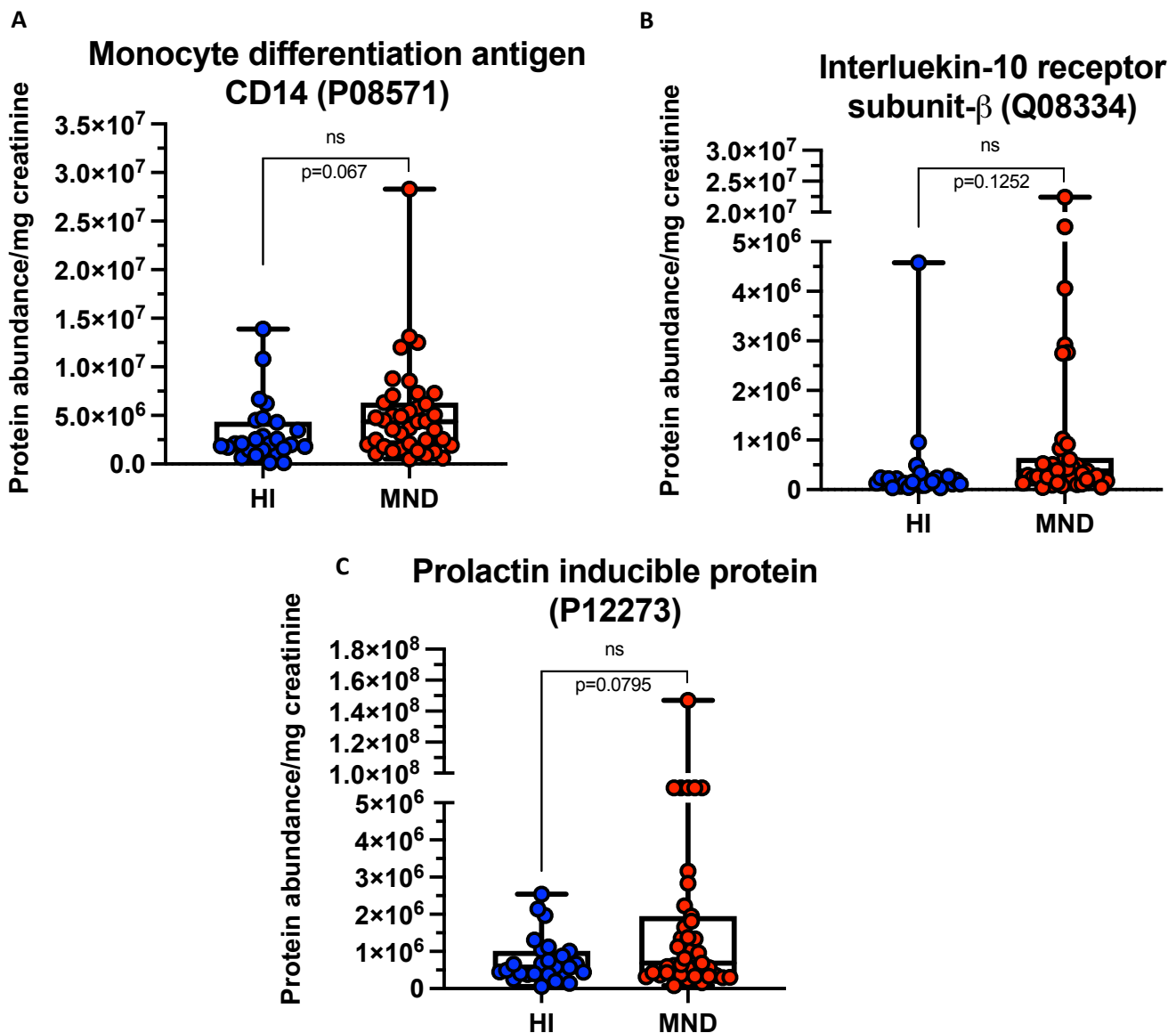


Figure 6.21: Not significant urinary protein candidates quantified by PRM at baseline

Not significant urinary biomarker levels were compared between 39 people with MND and 26 healthy individuals by 2-way T-test in Prism 10. In **(A)** monocyte differentiation antigen CD14 was not significantly different in MND; $3.22\text{E}^6 \pm 3.19\text{E}^6$, compared to HI; $5.11\text{E}^6 \pm 5.00\text{E}^6$, $p=0.067$. **(B)** Prolactin inducible protein was not different in MND; $7.62\text{E}^5 \pm 6.22\text{E}^5$, compared to HI; $8.54\text{E}^6 \pm 2.70\text{E}^7$, $p=0.0795$, and in **(C)** interleukin-10 receptor subunit-beta was not changed in MND; $3.69\text{E}^5 \pm 8.78\text{E}^5$, compared to HI; $1.32\text{E}^6 \pm 3.65\text{E}^6$, $p=0.1252$. Values presented as mean $\pm$ SD.

Table 6.8: Summary of significant urinary proteins by PRM at baseline

Candidate protein groups were quantified by merging the measurement of one or two unique peptides by PRM mass spectrometry (protein abundance/mg creatinine) in 39 people with MND and 26 healthy individuals. Statistical analyses were performed by 2-way T-tests in Prism 10 (*p* value) and fold-change was calculated in Excel. Values presented as mean $\pm$ SD.

Protein name (accession)	Quantified from unique peptides	Healthy individuals (n=26)	People with MND (n=39)	<i>p</i> value	Fold difference in MND/HI
Tumour necrosis factor receptor superfamily member 16 (p75, P08138)	<u>C</u> AYGYYQDETTGR	$1.33\text{E}^5 \pm 9.55\text{E}^4$	$8.24\text{E}^5 \pm 6.98\text{E}^5$	<0.0001	6.18
	WADAE <u>C</u> EEIPGR				
Cathepsin D (P07339)	LSPEDYTLK	$1.86\text{E}^5 \pm 1.67\text{E}^5$	$4.70\text{E}^5 \pm 3.36\text{E}^5$	<0.0001	2.53
Macrophage colony stimulating factor-1 (P09603)	AFLLVQDIMEDTMR	$6.20\text{E}^5 \pm 2.40\text{E}^5$	$1.12\text{E}^6 \pm 8.20\text{E}^5$	0.0008	1.80
	SHSSGSVLPLGELEG R				
CD44 antigen (P16070)	FAGVFHVEK	$7.51\text{E}^6 \pm 6.14\text{E}^6$	$1.57\text{E}^7 \pm 1.14\text{E}^7$	0.0004	2.09
	YGFIEGHVVIPR				
IL-6 receptor subunit beta (P40189)	VTSDHINFDPVYK	$1.84\text{E}^5 \pm 1.24\text{E}^5$	$3.73\text{E}^5 \pm 2.15\text{E}^5$	<0.0001	2.03
	LTWTNPSIK				
Tumour necrosis factor receptor superfamily member 12A (Q9NP84)	GSSWSADLDK <u>C</u> MD <u>C</u> ASC (missed 1)	$5.00\text{E}^4 \pm 1.28\text{E}^5$	$2.29\text{E}^5 \pm 4.66\text{E}^5$	0.0282	4.57
Fibulin-5 (Q9UBX5)	DQPFTILYR	$1.26\text{E}^6 \pm 4.99\text{E}^5$	$2.29\text{E}^5 \pm 4.66\text{E}^5$	<0.0001	2.02
	QSGQ <u>C</u> LDIDE <u>C</u> R				
CD14 (P08571)	LTVGAAQVPAQLLV GALR	$3.22\text{E}^6 \pm 3.19\text{E}^6$	$5.11\text{E}^6 \pm 5.00\text{E}^6$	0.0673	1.59
	VLDL <u>S</u> CNR				
Prolactin inducible protein (P12273)	ELGI <u>C</u> PDDAAVIPIK	$7.62\text{E}^5 \pm 6.22\text{E}^5$	$8.54\text{E}^6 \pm 2.70\text{E}^7$	0.0795	11.19
	YTA <u>C</u> L <u>C</u> DDNPK				
IL-10 receptor subunit beta (Q08334)	NILQWESPAFAK	$3.69\text{E}^5 \pm 8.78\text{E}^5$	$1.32\text{E}^6 \pm 3.65\text{E}^6$	0.1252	3.59
	IENEYETWTMK				

6.3.7 Correlation of unique peptides; variability and consistency

In this study, 18 unique peptides corresponding to 10 protein candidates were quantified by PRM to be analysed for their association with people with MND by statistical analyses (**Table 6.8**). As stated previously, protein quantification was based on two unique peptides with exceptions of two proteins: cathepsin D and tumour necrosis factor receptor superfamily member 12A. For the remaining 8 proteins, two unique peptides were quantified. Interestingly, in three of these cases, the concentration of the two peptides derived from the same protein did not show a significant linear correlation as assessed by Pearson's correlation coefficient in SPSS. The Pearson's correlation coefficient (r) of each peptide pair, where applicable, was reported in **Table 6.9** alongside significance (p , two-tailed), upper, and lower 95% confidence intervals (two-tailed) with an estimation based on Fisher's r -to- z transformation performed in SPSS.

The concentrations of two peptides from tumour necrosis factor receptor superfamily member 16 (p75) did not demonstrate significant linearity ($r=0.311$, $p=0.054$), as suggested by a large 44.01-fold-change for peptide WADAECEEIPGR compared to a difference of 2.18-fold in CAYGYQDETTGR in people with MND (**Table 6.7**). Two other proteins were found with discrepancies in peptide concentrations, including macrophage colony stimulating factor-1 ($r=-0.041$, $p=0.804$), and interleukin-10 receptor subunit beta ($r=0.0269$, $p=0.098$). CD44 antigen ($r=0.571$, $p<0.001$), interleukin-6 receptor subunit beta ($r=0.901$, $p<0.001$), fibulin-5 ($r=0.444$, $p=0.0005$), monocyte differentiation antigen CD14 ($r=0.702$, $p<0.001$), and prolactin inducible protein ($r=0.996$, $p<0.001$), reported strong linearity between peptides from the same protein.

Table 6.9: Correlation of urinary peptides by PRM-MS at baseline

18 Unique peptides were quantified from 10 candidate protein groups. Two unique peptides from the same protein group (where applicable) were analysed for correlation to one another. This analysis compared the Statistical analyses were performed by Pearson's correlation (r) and simple linear regression (p) in Prism 10 with upper and lower confidence intervals (CI: 95%).

Protein name (accession)	Quantified from unique peptides	Pearson Correlation (r)	p value	95% confidence intervals	
				lower	upper
Tumour necrosis factor receptor superfamily member 16 (p75, P08138)	<u>C</u> AYGYYQDETTGR	0.311	0.054	-	-
	WADAE <u>C</u> EEIPGR				
Cathepsin D (P07339)	LSPEDYTLK	NIL	NIL	NIL	NIL
Macrophage colony stimulating factor-1 (P09603)	AFLLVQDIMEDTMR	-0.041	0.804	-	-
	SHSSGSVLPLGELEG R				
CD44 antigen (P16070)	FAGVFHVEK	0.571	<0.001	0.312	0.751
	YGFIEGHVVIPR				
IL-6 receptor subunit beta (P40189)	VTSDHINFDPVYK	0.901	<0.001	0.818	0.947
	LTWTNPSIK				
Tumour necrosis factor receptor superfamily member 12A (Q9NP84)	GSSWSADLDK <u>C</u> MD <u>C</u> ASC (missed 1)	NIL	NIL	NIL	NIL
Fibulin-5 (Q9UBX5)	DQPFTILYR	0.444	0.005	0.150	0.666
	QSGQ <u>C</u> LDIDE <u>C</u> R				
Monocyte differentiation antigen CD14 (P08571)	LTVGAAQVPAQLLV GALR	0.702	<0.001	0.497	0.833
	VLDLS <u>C</u> NR				
Prolactin inducible protein (P12273)	ELGI <u>C</u> PDDAAVPIK	0.996	<0.001	0.993	0.998
	YTA <u>C</u> LCDDNPK				
IL-10 receptor subunit beta (Q08334)	NILQWESPAFAK	0.269	0.098	-	-
	IENEYETWTMK				

6.3.8 Cross sectional analysis of candidate protein groups

While quantification and fold-change differences in peptide concentrations provide insight into protein expression changes in MND urine, they do not indicate how candidate proteins may be related to survival, or clinical outcomes, at baseline, which are key factors in assessing the potential of these proteins as prognostic biomarker candidates.

All 10 candidate proteins were explored for their ability to predict survival at baseline in 39 people with MND. It was shown previously that urinary p75 detected by ELISA was found to share a significant correlation with ALSFRS-R scores at baseline ($r=-0.33$, $p=0.041$, **Figure 5.2B**, *section 5.3.3*). Each protein (sum of all peptides) was tested for correlation to the ALSFRS-R score of 39 people with MND at baseline using a Pearson's correlation coefficient in Prism 10 (**Figure 6.22-6.24**). Of the 7 proteins that reported significant change, two were significantly correlated with the ALSFRS-R score at baseline, including cathepsin D (**Figure 6.22A**, $r=-0.40$, $p=0.011$), and fibulin-5 (**Figure 6.22B**; $r=-0.35$, $p=0.031$). However, the methodology being only one technical replicate means these comparisons need validation with replicate PRM studies in the future. The remaining protein candidates did not demonstrate any significant correlation with the ALSFRS-R score of people with MND. **Table 6.10** summarizes the correlation of each unique peptide, reporting that only one peptide from Fibulin 5, DQPFTILYR ($r=-0.35$, $p=0.027$), and Cathepsin D, LSPEDYTLK ($r=-0.35$, $p=0.027$), were significantly correlated to the ALSFRS-R score in 39 people with MND at baseline. This highlights the need to refine this method using stable isotope standard (SIS) peptides that can co-ionize with each target peptide and generate absolute quantitative data in the future.

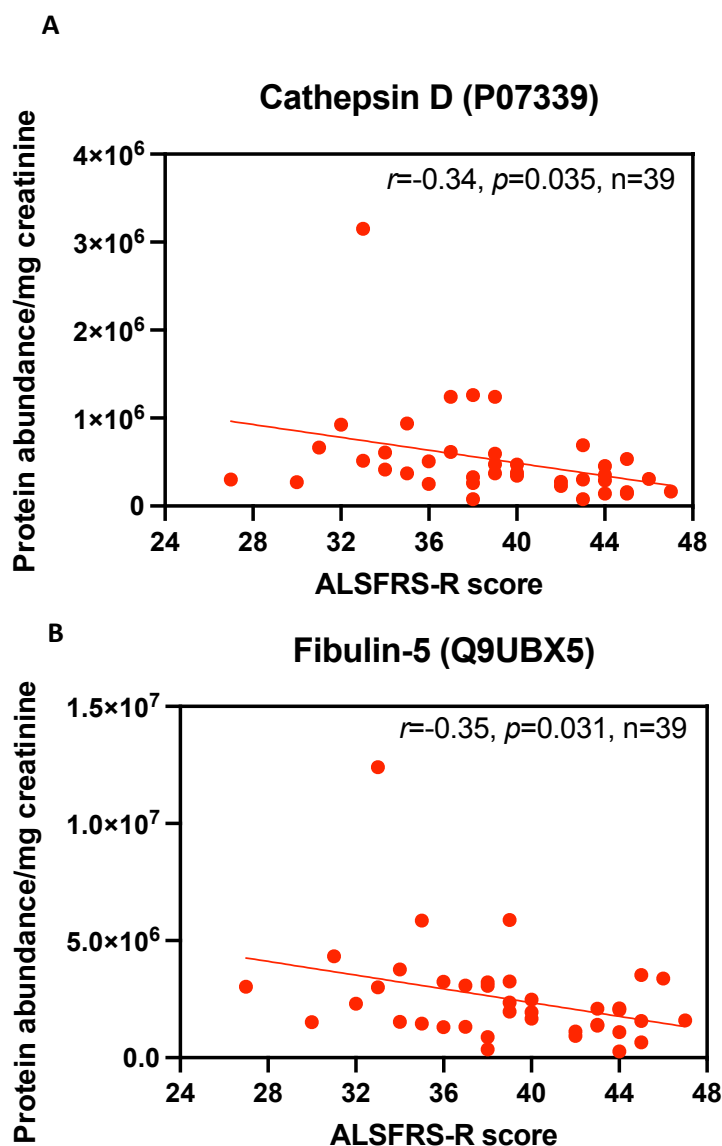


Figure 6.22: Verified urinary proteins significantly correlated with baseline ALSFRS-R scores

Pearson's correlation (r) between protein abundance by PRM mass spectrometry normalised to mg/creatinine (protein abundance, y axis) and the ALSFRS-R score of 39 people with MND at baseline. **(A)**; cathepsin D (●, $r=-0.34$, $p=0.035$) and **(B)**; fibulin-5 (●, $r=-0.35$, $p=0.031$) shared a significant inverse correlation with the ALSFRS-R at baseline. Statistical significance was determined using Pearson's correlation, Prism 10.

Table 6.10: Correlation of unique peptides quantified by PRM and ALSFRS-R scores

18 unique peptides were compared to ALSFRS-R scores in 39 people with MND. Each row represents a peptide identified by accession number and sequence. Statistical analysis for Pearson's correlation (r) and confidence intervals was performed in Prism 10.

Protein name (accession)	Quantified from unique peptides	Pearson Correlation (r)	p value	95% confidence intervals	
				lower	upper
Tumour necrosis factor receptor superfamily member 16 (p75, P08138)	<u>C</u> AYGYYQDETTGR	-0.27	0.099	-	-
	WADAE <u>C</u> EEIPGR	-0.18	0.263	-	-
Cathepsin D (P07339)	LSPEDYTLK	-0.40	0.011	-0.64	-0.10
Macrophage colony stimulating factor-1 (P09603)	AFLLVQDIMEDTMR	0.20	0.230	-	-
	SHSSGSVLPLGELEG R	-0.19	0.238	-	-
CD44 antigen (P16070)	FAGVFHVEK	0.056	0.735	-	-
	YGFIEGHVVIPR	-0.13	0.424	-	-
IL-6 receptor subunit beta (P40189)	VTSDHINFDPVYK	-0.16	0.319	-	-
	LTWTNPSIK	-0.12	0.484	-	-
Tumour necrosis factor receptor superfamily member 12A (Q9NP84)	GSSWSADLDK <u>C</u> MD <u>C</u> ASC (missed 1)	-0.13	0.428	-	-
Fibulin-5 (Q9UBX5)	DQPFTILYR	-0.35	0.027	-0.60	0.04
	QSGQ <u>C</u> LDIDE <u>C</u> R	0.0078	0.962	-	-
Monocyte differentiation antigen CD14 (P08571)	LTVGAAQVPAQLLV GALR	0.069	0.676	-	-
	VLDL <u>S</u> CNR	0.0071	0.966	-	-
Prolactin inducible protein (P12273)	ELGI <u>C</u> PDDAAVPIK	-0.097	0.557	-	-
	YTA <u>C</u> L <u>C</u> DDNPK	-0.082	0.621	-	-
IL-10 receptor subunit beta (Q08334)	NILQWESPAFAK	-0.089	0.589	-	-
	IENEYETWTMK	-0.17	0.295	-	-

6.3.9 Prognostic characteristics of ten biomarker candidates

Although candidate peptides were measured by PRM only once, a preliminary investigation of prognosis was performed. As expected, none of the candidate peptides measured by PRM were prognostic for survival in people with MND. Univariate Cox-Regression survival analysis was performed on 39 people with MND. In this cohort, 38 people with MND had reached end-stage disease or PAV. Univariate Cox-Regression analysis was performed by SPSS, independently assessing prognostic factors to predict survival; site of disease onset (bulbar compared to limb onset), change in ALSFRS-R per month from symptom onset (Δ FRS), gender (male compared to female), age at sample collection, baseline urinary p75^{ECD} quantified by ELISA, and the validated concentration of 18 unique peptides that corresponded to 10 protein candidates by PRM. The univariate Cox-Regression analysis was reported in **Table 6.11**, demonstrating that the Δ FRS (HR: 2.60, 95% CI: 1.49-4.55, $p < 0.001$), and urinary p75^{ECD} detected by ELISA (HR: 1.27, 95% CI: 1.08-1.49, $p = 0.004$) were both independent prognostic factors for survival ($n = 38$). Bulbar onset MND (HR: 1.68, 95% CI: 0.85-3.33, $p = 0.135$), male gender (HR: 1.40, 95% CI: 0.64-3.04, $p = 0.400$), or age at sample collection (HR: 0.99, 95% CI: 0.96-1.03, $p = 0.748$) were not prognostic factors for survival in this cohort. This confirmed that clinical factors like bulbar onset MND, gender, and age at collection were not confounding factors influencing the significance of the 10 candidate proteins analysed for prognosis.

All 18 unique peptides and their 10 candidate proteins reported in **Table 6.11** were tested independently for their ability to predict survival in 38 people with MND. However, no peptide or protein group validated by PRM mass spectrometry was able to provide any prognostic value beyond what could be offered by the Δ FRS and urinary p75^{ECD} quantified by ELISA at baseline. The hazard ratio of all peptides and proteins validated by PRM at baseline was equal to 1.00. This demonstrated no apparent change in the hazard per unit change on the covariate in relationship to survival, or time to death, followed by the 95% confidence interval to reflect this (Tabachnick & Fidell, 2013). This indicated that urinary p75 by PRM, alongside all other validated proteins, did not provide any greater prognostic value compared to the significance of the Δ FRS which was consistent with previous studies (Shepherd et al., 2022).

Table 6.11: Univariate Cox proportional hazards model for proteins and peptides

Candidate protein groups and peptides were compared to clinical prognostic covariates to predict survival outcomes in 39 people with MND. Only the Δ FRS and urinary p75^{ECD} by ELISA were prognostic at baseline, although Cathepsin D was trending towards significance.

Parameter group	Parameter	Univariate Cox-Regression			
		<i>p</i> value	Hazard ratio	95% confidence interval	
				lower	upper
Clinical covariates	Δ FRS	<0.001	2.601	1.488	4.548
	p75 ^{ECD} by ELISA	0.004	1.269	1.081	1.490
	Disease onset	0.135	1.683	0.850	3.331
	Gender	0.400	1.396	0.642	3.035
	Age at collection	0.748	0.990	0.962	1.030
Tumour necrosis factor receptor superfamily member 16 (p75, P08138)	CAYGYYQDETTGR	0.386	1.000	-	-
	WADAECEEIPGR	0.226	1.000	-	-
	<i>p75</i>	0.178	1.000	-	-
Cathepsin D (P07339)	LSPEDYTLK	0.083	1.000	-	-
Macrophage colony stimulating factor-1 (P09603)	AFLLVQDIMEDTMR	0.444	1.000	-	-
	SHSSGSVLPLGELEGR	0.953	1.000	-	-
	<i>MCSF-1</i>	0.507	1.000	-	-
CD44 antigen (P16070)	FAGVFHVEK	0.319	1.000	-	-
	YGFIEGHVVIPR	0.905	1.000	-	-
	<i>CD44</i>	0.395	1.000	-	-
IL-6 receptor subunit beta (P40189)	VTSDHINFDPVYK	0.737	1.000	-	-
	LTWTNPSIK	0.995	1.000	-	-
	<i>IL-6RB</i>	0.880	1.000	-	-
Tumour necrosis factor 12A (Q9NP84)	GSSWSADLDK <u>C</u> MD <u>C</u> ASC	0.773	1.000	-	-
Fibulin-5 (Q9UBX5)	DQPFTILYR	0.420	1.000	-	-
	QSGQ <u>C</u> LDIDE <u>C</u> R	0.578	1.000	-	-
	<i>FIB-5</i>	0.492	1.000	-	-
Monocyte differentiation antigen CD14 (P08571)	LTVGAAQVPAQLLVGALR	0.920	1.000	-	-
	VLDLSC <u>N</u> R	0.627	1.000	-	-
	<i>CD14</i>	0.790	1.000	-	-
Prolactin inducible protein (P12273)	ELGIC <u>P</u> DDAAVPIK	0.215	1.000	-	-
	YTA <u>C</u> L <u>C</u> DDNPK	0.276	1.000	-	-
	PIP	0.302	1.000	-	-
IL-10 receptor subunit beta (Q08334)	NILQWESPAFAK	0.636	1.000	-	-
	IENEYETWTMK	0.562	1.000	-	-
	<i>IL-10RB</i>	0.630	1.000	-	-

6.4 Urinary p75^{ECD} measurement by ELISA, DIA, and PRM

Urinary p75 has been detected and quantified numerous times throughout this study using three different three distinct methods. Urinary p75^{ECD} was firstly quantified by our in-house sandwich ELISA assay (Shepherd et al., 2017) in 39 people with MND at baseline from *Chapter 4*. These measurements were followed up in *Chapter 5*, where p75 was detected and quantified in the same cohort using DIA by orbitrap-mass spectrometry. To improve upon the quantitative accuracy and precision of p75 detection by DIA, the study of *Chapter 6* identified three unique peptides belonging to tumor necrosis factor receptor superfamily member 16, where two unique peptides CAYGYQDETTGR and WADAECEEIPGR belonging to the ECD region of p75 were quantified using PRM to improve the sensitivity and specificity of p75 quantification in 39 people with MND and 26 healthy individuals.

To compare the measurement of each method of p75 quantification, a Pearson's correlation (r) analysis was performed in Prism 10. In 39 people with MND, the measurement of p75 quantification was compared by ELISA to DIA-mass spectrometry (**Figure 6.23A**; $r=0.37$, $p=0.022$), ELISA to PRM mass spectrometry (**Figure 6.23B**; $r=0.084$ $p=0.61$) by combining the abundance of CAYGYQDETTGR and WADAECEEIPGR, and DIA to PRM mass spectrometry (**Figure 6.25C**; $r=0.045$ $p=0.79$). It was reported that p75 values quantified by ELISA and DIA-mass spectrometry shared a significant positive correlation (**Figure 6.23A**, $r=0.37$, $p=0.022$), however, the measurement of p75 by PRM was not statistically correlated. When comparing the mass spectrometry and ELISA data of p75, there were differences in experimental replication. The DIA and PRM methods were based on a single mass spectrometry injection, providing a snapshot of protein abundance in a single measurement. However, the ELISA method sampled p75^{ECD} by 3 technical replicates per plate, across a minimum of 3-5 plates. By averaging across multiple replicates and plates, the ELISA data was more robust to potential errors and inconsistencies that can arise from noise and run-to-run variation, and it was inferred that the statistical reliability and precision of the two measurements are not comparable with up to 18 ELISA measurements of urinary p75^{ECD} for one urine sample.

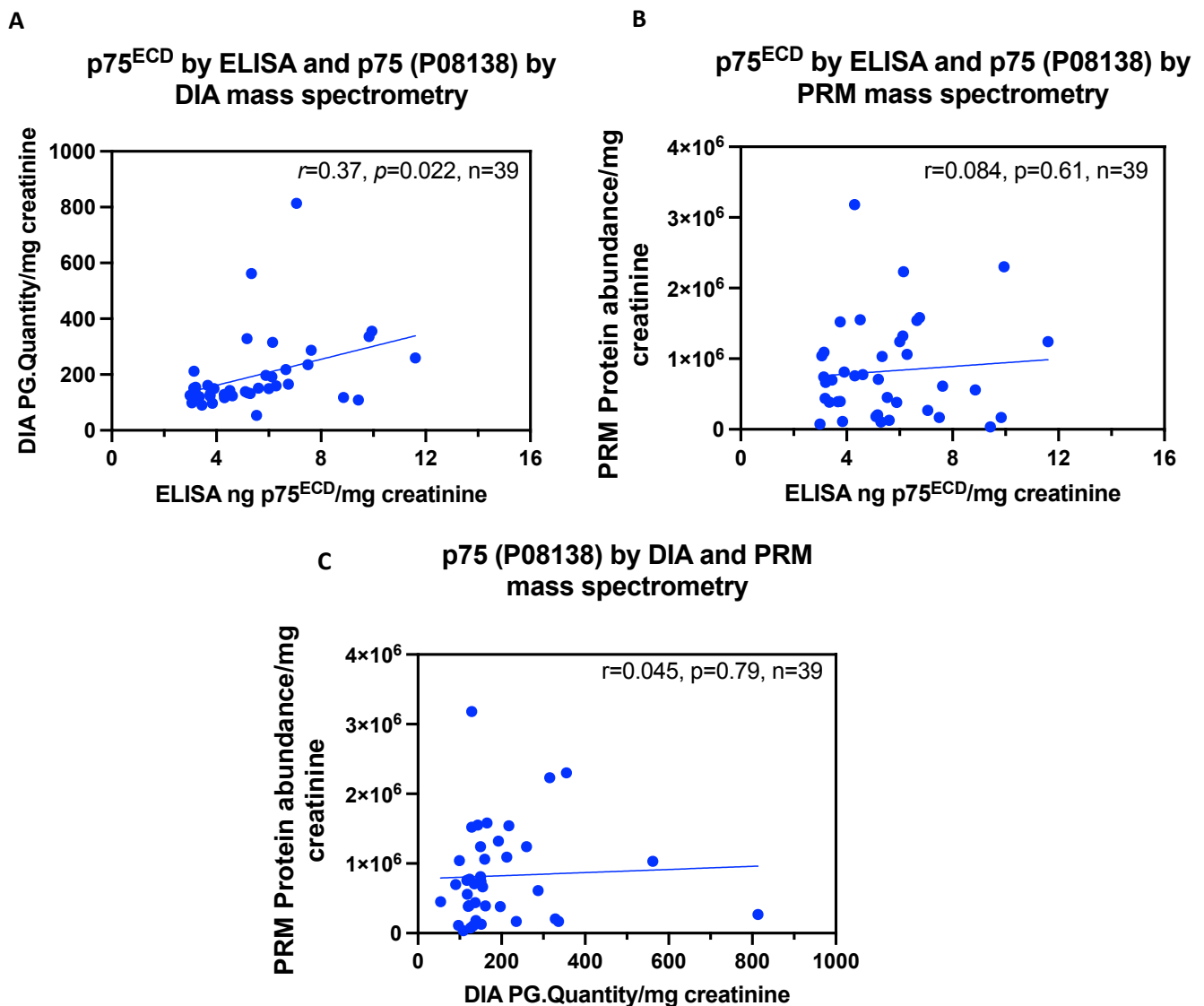


Figure 6.23: Urinary p75 quantification by ELISA compared to DIA and PRM

The quantification of urinary p75^{ECD} by in-house sandwich ELISA was tested for statistical correlation against mass spectrometry techniques in 39 people with MND. (A) Found that p75 measurement by in-house ELISA shared a significant positive correlation to DIA-mass spectrometry in 39 people with MND ($r=0.37$, $p=0.022$), compared to (B) which found that in-house ELISA measurement was not significantly correlated to PRM mass spectrometry ($r=0.084$, $p=0.61$), and (C) that DIA and PRM mass spectrometry quantification techniques were not significantly correlated to one another ($r=0.045$, $p=0.79$). Statistical significance was determined using Pearson's correlation, Prism 10.

6.4.1 Urinary p75 and ALSFRS-R by ELISA, DIA, and PRM

To further critique this analysis, a comparison was performed on the ability of these three p75 quantification techniques; ELISA, DIA-mass spectrometry, and PRM mass spectrometry, to correlate with ALSFRS-R scores at baseline. The results revealed differences in the strength and significance of the correlation between protein concentrations and clinical outcome measures. Using a Pearson's correlation (r) in 39 people with MND, their respective ALSFRS-R scores at baseline were compared to the measurement of urinary p75^{ECD} quantified by ELISA (**Figure 6.24A**; $r=-0.33$, $p=0.041$), and relative abundance analysis of p75 by DIA-mass spectrometry (**Figure 6.24B**; $r=-0.23$, $p=0.15$), and PRM mass spectrometry (**Figure 24C**; $r=-0.28$, $p=0.081$). This reported that p75^{ECD} values quantified by ELISA shared a significant negative correlation with functional disease decline as indicated by ALSFRS-R score at baseline (**Figure 6.24A**; $r=-0.33$, $p=0.041$), and that p75 values relatively quantified by PRM mass spectrometry were trending towards significance. However, there was no statistical correlation between DIA mass spectrometry analysis and ALSFRS-R scores.

These findings highlight the strengths and limitations of each method in measuring p75 in the urine of people with MND and correlating those measurements with clinical outcomes of the ALSFRS-R at baseline. The ELISA demonstrated the strongest correlation due to its robust repeatability, while PRM mass spectrometry reported a trend towards significance but lacked the technical replication to achieve statistical significance. DIA-MS although powerful in its broad proteomic coverage, did not correlate with ALSFRS-R scores at baseline, as expected with its lower sensitivity for detecting changes in specific protein abundances. These results suggest that for assessing protein biomarkers related to MND prognosis and predicting functional decline by pathological mechanisms, methods that provide high precision and targeted measurement, like an ELISA or PRM mass spectrometry, are strong quantitative techniques, however, technical factors like robustness and repeatability are equally important for sample replicability and statistical reliability.

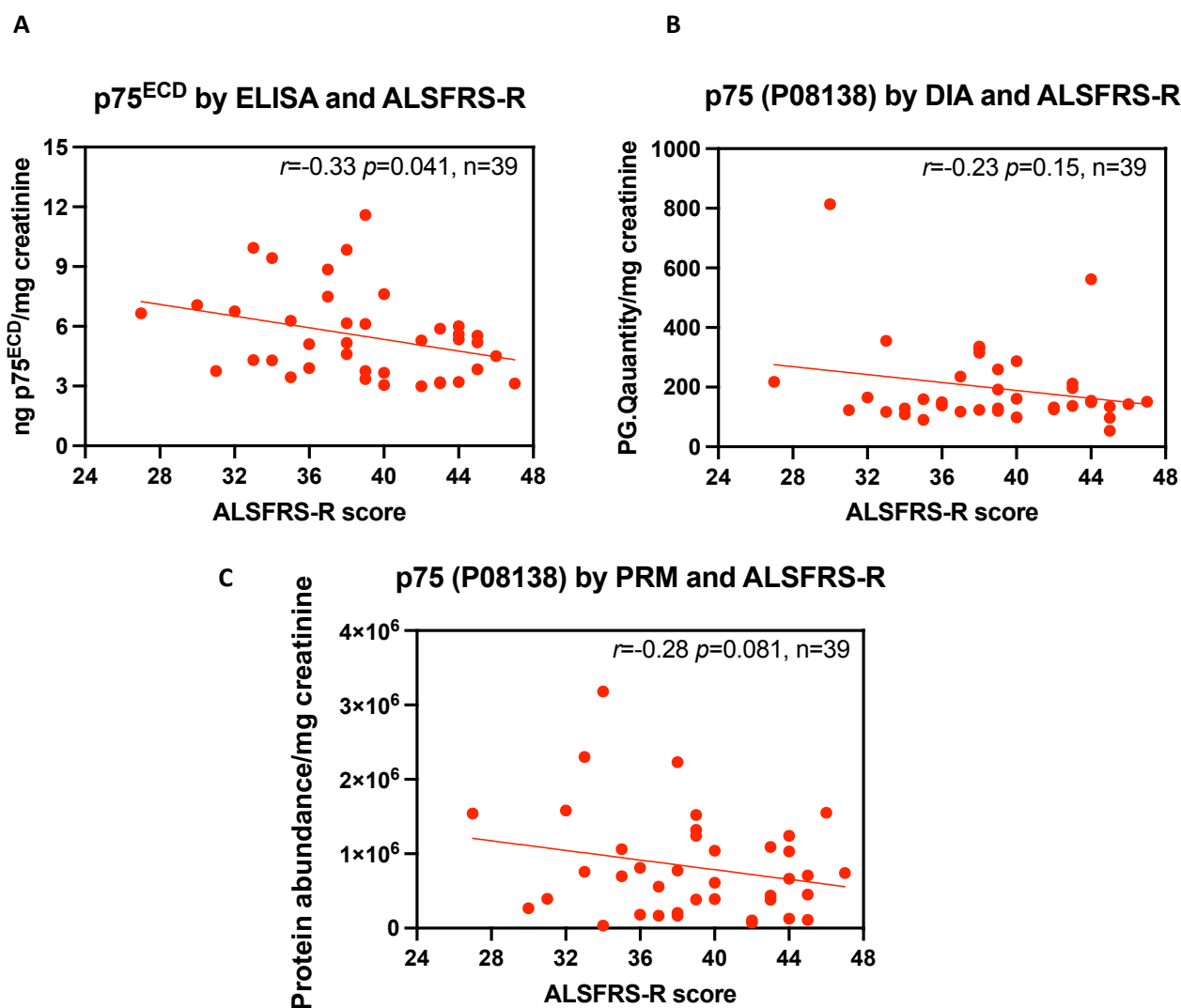


Figure 6.24: Comparison of ALSFRS-R between measurement methods by Pearson's correlation

The quantification of urinary p75^{ECD} by in-house sandwich ELISA and mass spectrometry techniques was tested for statistical correlation against ALSFRS-R scores in 39 people with MND at baseline. **(A)** found that urinary p75^{ECD} measurement by sandwich ELISA was significantly inversely proportional to ALSFRS-R score at baseline ($r=-0.33$, $p=0.041$), compared to **(B)** which found that urinary p75 by DIA mass spectrometry was not significantly correlated to ALSFRS-R score at baseline ($r=-0.23$, $p=0.15$), and in **(C)** that urinary p75 quantified by PRM mass spectrometry was not significantly correlated to ALSFRS-R score at baseline ($r=-0.28$, $p=0.081$). Statistical significance was determined using Pearson's correlation, Prism 10.

6.5 Discussion

The result of this investigation demonstrates the ability to generate a semi-automated PRM mass spectrometry technique that can simultaneously detect 18 unique peptides in the urine of people with MND. The aim of this investigation was to conduct a pilot study that would verify the sensitivity, specificity, and precision of protein-based biomarkers detected and relatively quantified by DIA mass spectrometry in *Chapter 5*. This is the first time that orbitrap mass spectrometry has been used to conduct a parallel reaction monitoring (PRM) analysis in real urine of people with MND to simultaneously detect 10 biomarker candidates. The resulting quantity of peptides and protein groups were compared to the ALSFRS-R score of people with MND as a validated clinical outcome measure, and to urinary p75^{ECD} quantified by in-house ELISA and DIA mass spectrometry, to determine the ability of any candidate proteins to predict MND survival outcomes from baseline.

Of the urinary peptides and proteins targeted by PRM analysis in this study, two peptides from the p75 neurotrophin receptor; CAYGYQDETTGR and WADAECEEIPGR, were investigated as another method of p75 protein quantification. The mass spectrometry data confirmed that peptides from the p75 neurotrophin receptor were present in human urine, and that three peptides could be derived from the extracellular domain (ECD) region between 29- and 250- of the 421 amino acid sequence, agreeing with previous data demonstrating that peptides from the ECD can be found in MND urine (Shepherd et al., 2014). The p75 neurotrophin receptor (TNFRS16, UniProt ID: P08138) is only expressed embryonically, however, during the process of neuronal cell damage the neurotrophin receptor is re-expressed from injury. During this event, pro-apoptotic ligands bind to p75NTR, and the extracellular domain (p75^{ECD}) is cleaved leading to neuronal cell death (Kenchappa et al., 2010). The appearance of the ECD was first described by radioimmunoassay in rat urine (DiStefano and Johnson, 1988). This was followed up by our laboratory, using immunoprecipitation to discover ECD fragments in urine from people with MND with a molecular weight of 35-50 kDa, and confirmed by mass spectrometry (Shepherd et al., 2014). Five peptides located in the ECD (AA: 29-250) were found after immunoprecipitation of p75 from MND urine (Shepherd et al., 2014). In the current study, urinary p75 was detected and quantified by PRM mass spectrometry, confirming the specificity of three unique peptides CAYGYQDETTGR, VCEAGSGLVFSCQDK, and WADAECEEIPGR which were all located in the ECD, confirming our previous findings. In the interest of time, only two unique peptides were targeted by PRM analysis, including CAYGYQDETTGR (AA: 209-121) and WADAECEEIPGR (AA: 183-194) for their higher

sensitivity to ensure robust and repeatable analysis in 39 people with MND and 26 healthy individuals. Interestingly, CD14 has been previously identified as increased in MND urine (Beers et al., 2020), and in the current study trended towards significance. This protein warrants further investigation using SIS peptides for absolute quantitation in MND urine.

To support the aim of this investigation and ensure the specificity of each targeted peptide to their precursor ion (MS1, m/z), three transition (y^+) ions were selected based on their highest sensitivity in Skyline v24.1 for all 18 unique peptides relatively quantified by PRM. The three highest abundance transition (y^+) ions for CAYGYQDETTGR and WADAECEEIPGR were reported in **Figure 6.5**, demonstrating a seamless overlap of their precursor ions with each transition ion to confirm the specificity of the targeted peptide for PRM quantification. **Table 6.6** reported three transition y^+ ions respective to each precursor ion belonging to all 18 unique peptides, highlighting the outcomes of their MS1 intensity and retention times on a C18 chemistry 25 cm analytical column. The number of points per peak were reported, with a minimum of 7 points across the peak belonging to peptide GSSWSADLDKCMDCASC of tumour necrosis factor receptor superfamily member 12A, reporting the lowest sensitivity of $8.01E^6$. Optimal peak width resolution was important for well-defined chromatography allowing for accurate quantification, ensuring the collection of a minimum of 7 data points across the peak for robust peak integration (Zeng and Bateman, 2023). Depending on the charge state (z) of each peptide, parameters including automatic gain control (AGC) percentage and Q1 resolution were optimised to ensure the application of a robust and repeatable method for the scheduled targeting of 18 peptides in human urine within an 80-minute gradient.

The developed PRM method was used to confirm the specificity of 18 unique peptides from 10 protein candidates. Assay robustness was reported across **Figures 6.4-6.7**, demonstrating highly sensitive and robust repeatability of urinary p75 peptides across three technical replicates. However, this study came with the caveat of using only one biological replicate to generate this data. It has been previously reported that the intra-assay variation of p75^{ECD} measurement using the ELISA should fall below 10% for validation (Shepherd et al., 2014). This PRM mass spectrometry method generated an average intra-assay CV of 2.9% for CAYGYQDETTGR and 7.0% for WADAECEEIPGR, producing an average intra-assay CV of 3.6% for p75. This co-aligns with p75^{ECD} measured by ELISA, however, the PRM method may not be robust for at least one peptide (WADAECEEIPGR, 15% median CV, **Figure 6.6**). Interestingly, p75 peptide CAYGYQDETTGR was trending towards a significant correlation with the ALSFRS-R, each requiring further validation.

There were some limitations around the methodology as performed by this investigation that, if rectified, may lead to more robust and accurate peptide quantification. This method was used to perform a relative abundance analysis with higher quantitative precision than previously performed in *Chapter 5*. This was applied to firstly examine the specificity of peptides identified from each protein group, confirming their presence in MND urine, and to further examine the ability of each unique peptide or protein group to add prognostic value to urinary p75 and the ALSFRS-R scores as clinical outcome measures. The major limitations of this investigation were the lack of testing biological replicates and the lack of using stable isotope standard (SIS) peptides for absolute quantitative accuracy. This study was designed to verify the presence of 10 protein groups in the urine of people with MND and healthy individuals and should not be considered conclusive; rather, more detailed analytical validation using methods of absolute quantification over multiple biological replicates are needed to confirm if these urinary peptides are useful prognostic or predictive biomarkers for people with MND. Many scheduled PRM assays have been performed using SIS synthetic peptides to perform absolute quantitative proteomics in human urine from other diseases (Gallien et al., 2015, Rauniyar et al., 2018, Ponce and Zhang, 2023), with (Joshi et al., 2024) expanding on the catalogue of proteins detected in the urine from a variety of quantitative techniques.

To translate this study into an accurate quantitative investigation, SIS peptides that are chemically identical to their endogenous analyte peptide counterparts should be used to co-elute during chromatographic separation on a Vanquish Neo uHPLC system combined with the latest orbitrap high resolution accurate mass (HRAM) mass spectrometer to enhance peptide and protein quantification. The sensitivity of electrospray ionisation is inversely proportional to flow rate (Luo et al., 2006), thereby using nano-flow (i.e., 300 nL/min) to increase the sensitivity of protein detection. The low flow rate of the nano-spray emitter is tuned for improved ion sensitivity, peak shape, and ionisation efficiency by desolvation for increased proteome coverage (Swearingen et al., 2012). However, the ionisation efficiency and total ion current (TIC) signal are decreased with increasing solvent flow rates by capillary-flow liquid chromatography (Tang et al., 2006). When taken together, micro-flow Vanquish Neo uHPLC systems combined with the latest sensitive ion detectors belonging to orbitrap HRAM mass spectrometers can perform crude mass measurements with higher sensitivity, speed, and reproducibility (Bian et al., 2020). For accurate and absolute quantification with lower coefficient of variation (CV%) and increased signal-to-noise ratios, the ratios of peak areas under the monitored fragment ions of endogenous peptides can be

compared to known amounts of SIS peptides to calculate the concentration of candidate proteins with high sensitivity and across a wide dynamic range (Meng and Veenstra, 2011, Kettenbach et al., 2011).

Differential peptide behaviour was observed during the relative quantification of protein groups that were determined by two unique peptides. In **Table 6.9**, the relative abundance of each peptide was reported, with disparity between some peptides derived from the same protein group. Peptides belonging to tumour necrosis factor receptor superfamily member 16 (p75): CAYGYQDETTGR ($p < 0.0001$, fold-change=2.18), WADAECEEIPGR ($p < 0.0001$, fold-change=44.01), Fibulin-5: DQPFTILYR ($p = 0.0741$, fold-change=1.45), QSGQCLDIDECR ($p < 0.0001$, fold-change=2.09), and Interleukin-10 receptor subunit beta: NILQWESPAFAK ($p = 0.1468$, fold-change=3.71), IENEYETWTMK ($p = 0.0004$, fold-change=2.54) reported significant differences in peptide behaviour. Further, **Table 6.11** reported disparity between the linear correlation of peptides from urinary p75 ($p = 0.054$), macrophage colony stimulating factor-1 ($p = 0.804$), and interleukin-10 receptor subunit beta ($p = 0.098$), providing evidence for peptide-specific changes, biological differences, or sample heterogeneity. However, the caveat of only one mass spectrometry injection creates a challenge of sourcing the variation.

In hindsight, we acknowledge that we may not have targeted ideal peptides for PRM-based quantitation due to interferences from other co-eluting peptides in the urinary proteome. The sequence, charge state, and chemical structure of peptides can influence their ionisation efficiency, and co-eluting peptides or background matrices can suppress ionisation (Abdul-Khalek et al., 2023). Evidence of peptides co-eluting was reported in **Table 6.7**, highlighting the retention time of peptides QSGQCLDIDECR, CAYGYQDETTGR, and VLDSLCNR within a scheduled 30–34-minute window. Peptide hydrophobicity and size can cause variation in signal intensity, commonly eluting later in the reversed-phase C18 column (Mant et al., 2007). Peptide DQPFTILYR eluting between 53–59 minutes in the 80-minute gradient, and subsequent peptides up to 75-minutes, are examples of hydrophobic peptides that may have been affected compared to their hydrophilic counterparts, like QSGQCLDIDECR eluting between 30–34 minutes in highly chimeric spectra. Based on this knowledge, this may have significantly changed the relative abundance of peptides to the TIC as applied by Skyline. Post-translational modifications (PTMs) like phosphorylation, oxidation, or deamidation events can alter the detectable signal or divide them into multiple forms (Will et al., 2023), however, carbamidomethylation of cysteine residues (C, +57 Da) was the only fixed modification introduced by iodoacetamide (IAA) during the sample preparation of this

investigation (Kuznetsova et al., 2021) which may have introduced peptide heterogeneity within the population, as only 7 of 18 peptides contained this modification.

Further, protein splicing is a naturally occurring post-translational modification that can alter the structure of protein groups and biological relevance of unique peptides (Aranko et al., 2009). When proteins transition from their cellular origin through the bloodstream and into the urine, they are subject to multiple forms of proteolytic cleavage, post-translational modifications, and compartmental degradation. When comparing the unity of peptides from one protein group, one peptide may be derived from a region affected by alternative splicing, proteolytic processing, or partial degradation by renal proteases, meaning only one peptide reflects biological change (Paulus, 2000). In the instance of p75, proteolytic activity may have influenced the cleaving of the ECD causing some variance in peptide quantification between peptides CAYGYYQDETTGR and WADAECEEIPGR. Notably, the average abundance $\pm$ SD of peptide WADAECEEIPGR was $1.27E^4 \pm 5.37E^3$ in healthy individuals, which was the lowest signal reported in the study. The signal was two orders of magnitude higher in people with MND, $5.61E^5 \pm 6.31E^5$, generating a 44-fold difference and a CV of 7.0%, from which may have derived from the circumstances described above. To avoid this, protease inhibitors like serine and threonine may be applied to the urine upon collection to rule out protein degradation by endogenous proteases (Siddiqui et al., 2015), and ultimately, acquiring SIS peptides for each endogenous peptide with the same sequence, ionization efficiency, hydrophobicity, and fragmentation pattern to improve the experimental design can reduce technical bias and provide a clinically robust, repeatable, and accurate measure of candidate peptides for the first time in people with MND.

To investigate if any urinary proteins were related to mechanisms of disease, the quantity of each peptide and protein were correlated with baseline ALSFRS-R scores, a validated biomarker of MND prognosis (Cedarbaum et al., 1999). Only two of the significant proteins quantified within this pilot study, lysosomal autophagy protein cathepsin D (P07339) and elastic fibre protein fibulin-5 (Q9UBX5), were found to be significantly correlated with the ALSFRS-R score, or rate of disease decline, at baseline. However, this study is limited without biological replication or SIS peptides to accurately compare to neurological characteristics and further validation is needed. The univariate Cox proportional hazards model was used to determine if any peptides were prognostic for survival at baseline. Lysosomal autophagy protein cathepsin D (P07339), significantly correlated with the ALSFRS-R scores, was the only protein that trended towards significance ($p=0.083$). This is not to say that none of the proteins detected by DIA and PRM mass spectrometry are

related to disease survival, rather than that this is a pilot study confirming the presence and specificity of unique peptides belonging to protein groups involved in people with MND detected by DIA mass spectrometry, and that further analytical validation and improvement in study design is required to uncover potential changes in protein activity in people with MND.

Peptide and protein-based biomarkers for people with MND can offer sensitive, specific, and reliable tools for reducing disease heterogeneity, determining prognosis, and predicting disease pathogenesis and treatment outcomes to improve clinical trial design. Peptides and proteins detected and quantified by PRM mass spectrometry in people with MND may reflect changes in disease resulting from neuroinflammation, muscle degeneration, and lysosomal dysfunction compared to healthy physiological and pathophysiological processes that can serve as clinically useful biomarkers. This study has demonstrated that DIA-mass spectrometry can be used to perform an early-discovery phase investigation of differentially changed proteins in people with MND, and that a PRM technique can be designed and applied to analytically validate the specificity of unique peptides belonging to those protein groups. There are many possible biomarkers that may be related to MND pathology that have been identified in the urine, and this study builds upon the aim of generating a panel of biomarkers to add to the value of serum NfL, urinary p75^{ECD}, neopterin, and other clinical markers like ALSFRS-R scores and the Δ FRS that have been discussed to represent different pathophysiological processes to better stratify individuals and determine therapeutic outcomes beyond phase 2 clinical trials.

CHAPTER 7.

DISCUSSION, FUTURE DIRECTIONS, AND CONCLUDING REMARKS

7.1 Overview

Motor neuron disease is a progressive neurodegenerative disease with no cure. The only approved treatments for sporadic MND by the TGA are riluzole (Bensimon et al., 1994) and edaravone (O'Neill et al., 2024b), and tofersen is approved by the FDA to people who carry mutations in the SOD1 gene (Miller et al., 2022). MND is a multi-system disease with a highly heterogeneous clinical presentation, and validated biofluid biomarkers are our best hope of bridging the gap between characterising disease pathology and implementing treatments specific to a disease phenotype in clinical trials. The ALSFRS-R is the principal measure of clinical outcomes for people with MND (Kimura et al., 2006), and recently serum NfL has been validated as a prognostic and pharmacodynamic biofluid marker for use with the ALSFRS-R and ENCALIS predictor scores for ongoing and future clinical trials (Benatar et al., 2024b, Benatar et al., 2025). Importantly, reductions in NfL following Tofersen treatment were a key biomarker outcome supporting its conditional FDA approval (Miller et al., 2022). This milestone demonstrates a growing confidence in biomarker based endpoints for evaluating drug efficacy, although full regulatory approval by the TGA will require concordant improvements in clinical measures such as the ALSFRS-R. Outside of these validated clinical measures, objective biomarker candidates that can predict survival outcomes, characterise disease processes, and improve clinical trial design is at the core of this research. While the number of clinical trials failing in phase 2 continues to rise, the most obvious solution is to identify and validate candidate biomarkers that can stratify individuals by their disease trajectory or a predicted response to experimental therapeutics to improve clinical trial outcomes.

Recent drug trials have incorporated exploratory biomarkers to monitor pharmacodynamic effects and treatment responses in MND clinical trials. These include experimental therapeutics like IC14, a monoclonal antibody against CD14, in phase 2 clinical trials using NfL and p75^{ECD} as exploratory markers (Henderson et al., 2021); Tecfidera in phase 2 and 3 clinical trials assessing increases in T-regulatory cells using p75^{ECD} (Vucic et al., 2020); and Triumeq, an anti-retroviral investigated in phase 2a and 3 trials using NfL and p75^{ECD} (Gold et al., 2019). Others using NfL and p75^{ECD} include Rescue-ALS using gold nanoparticles {Vucic, 2023 #1080}, and Rock-ALS with a Rho-kinase inhibitor (Koch et al., 2024), highlighting the growing use of biofluid biomarkers to complement the ALSFRS-R in determining clinical endpoints and providing insight into drug mechanisms. Alongside validated measures like the ALSFRS-R and NfL, such biomarkers will strengthen the ability

of future trials to determine meaningful treatment effects and support regulatory drug approval.

It is well understood that neuroinflammation and the associated pathways play a key and distinct role in MND pathology, in particular affecting mechanisms that accelerate neurodegeneration, muscle degeneration, and axon degeneration. Every system is involved with one another, and therefore biomarkers reflective of the immune response, alongside others reflecting important disease mechanisms are required in a panel to push past the current barrier of futility in phase 2 clinical trials. The high cost and lengthy development times, combined with limited multiplexing capability, prevents techniques like the ELISA from verifying thousands of newly discovered biomarker protein candidates. Novel HPLC-MS assays using DDA, DIA, MRM, and PRM can be rapidly developed at low costs that provide a high level of multiplexed protein quantification that is ideal for biomarker discovery, quantification, and verification.

Due to the complexity and heterogeneity of MND, a single protein or molecular biomarker is not likely to have adequate sensitivity for determining prognosis or predicting disease pathology to improve stratification in clinical trials, and a panel of biomarkers are desirable (Rogers et al., 2023, Benatar et al., 2024b). Since traditional clinical trial outcome measures such as the ALSFRS-R are underpowered in phase 2 (Benatar et al., 2024b) and only a fraction of clinical trials have progressed to phase 3 since the approval of Riluzole almost 30-years ago (Pupillo et al., 2024), the central aim of this investigation was to determine if urinary neopterin could be candidate predictive biomarker of pro-inflammation in people with MND, and to determine if other protein candidates related to pro-inflammation, or other parts of MND pathology, could be discovered as significantly changed in MND urine compared to healthy individuals. The underlying technique for biomarker detection, quantification, and verification in this investigation was the use of HPLC-MS for biomarker detection to perform protein-based biomarker screening for the first time in MND urine, as has been achieved in the CSF (Dellar et al., 2024, Trautwig et al., 2025). We have identified a number of future candidates that require further analytical and clinical validation steps as done with serum NfL (Benatar et al., 2020) to see their implementation into clinical trials.

For an objective biological marker to be useful, it needs to be indicative of an underlying pathological process of MND. The major subtypes of biomarker candidates for people with MND have been categorized as proteins reflecting microglial activation and neuroinflammatory proteins, mitochondrial dysfunction and oxidative stress proteins, and

dense TDP-43 and associated transposable element de-silencing proteins (Muntel et al., 2015, Swensen et al., 2021). This investigation identified candidate proteins that may characterize microglial activation and neuroinflammation in people with MND. Although MND is a disease that specifically affects the motor neurons in the brain and spinal cord, the innate and adaptive immune system are involved in MND pathology (Murdock et al., 2015, Lyon et al., 2019, Béland et al., 2020). The workflow of this investigation and the processes discovered are summarized in **Figure 7.1**.

In response to ongoing cellular stress and motor neuron damage occupied during disease, microglia, astrocytes, macrophages, and other resident immune cells have shown to increase or become activated at baseline, supporting evidence for a microglial response (Appel et al., 2011, Wang et al., 2022) and interferon response (Yu et al., 2020, Decout et al., 2021) and their involvement in disease. Outside of the immune response, it is evident that other processes are elevated at baseline resulting in programmed cell death and neurodegeneration (Shepherd et al., 2017), and the degeneration of elastic fibers and collagen binding resulting in muscle atrophy (Shefner et al., 2023).

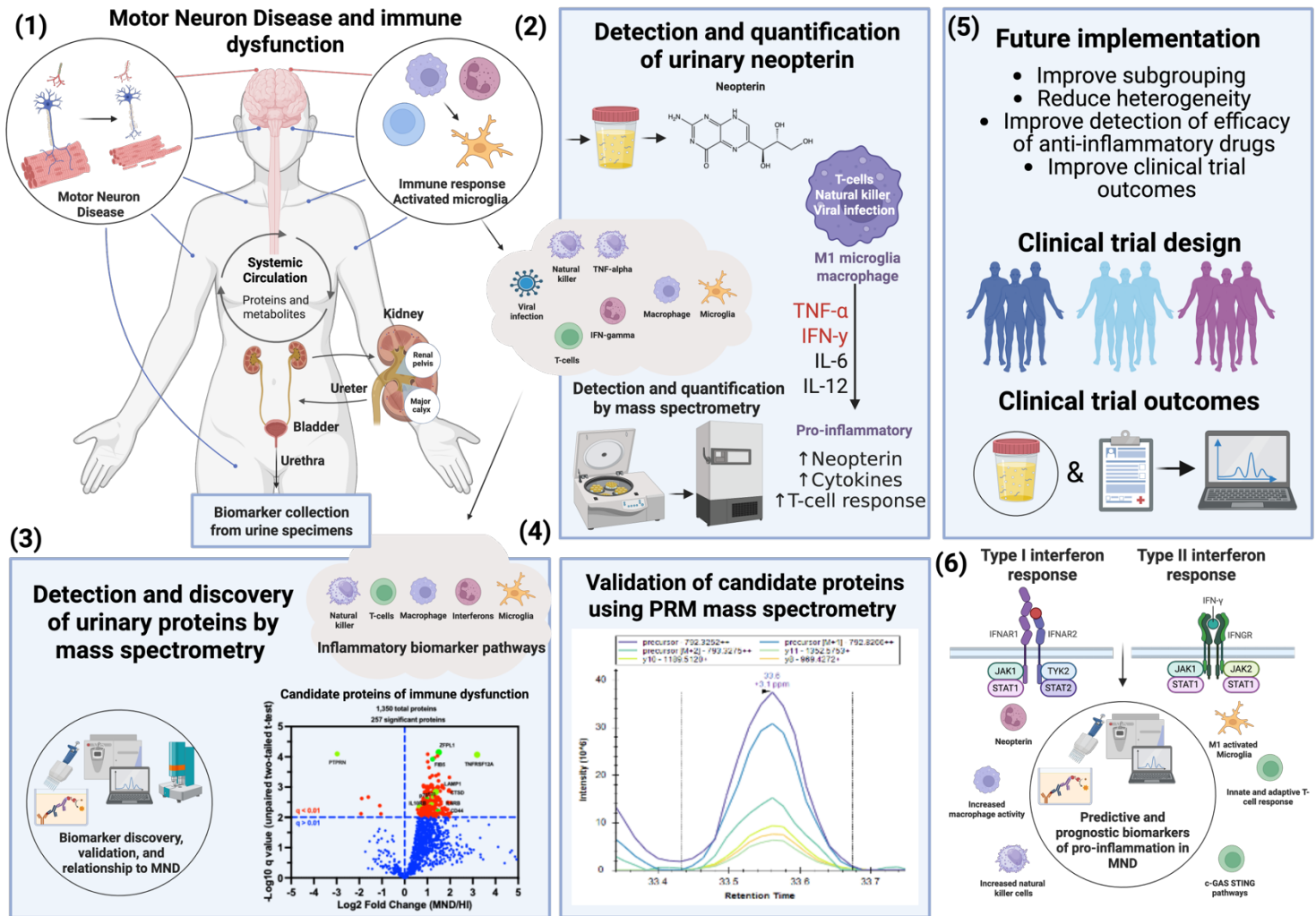


Figure 7.1: Motor Neuron Disease and immune dysfunction: a summary of this investigation.

(1) MND is a non-cell autonomous and heterogenous disease, bringing a challenge to characterize disease processes using biomarkers. Urine is a resourceful biofluid and was investigated for urinary neopterin in a targeted analysis (2). Urinary neopterin was identified as a candidate predictive biomarker of pro-inflammation, characterized by an increase in the interferon and activated microglial response. Other urinary proteins were explored in an untargeted analysis using mass spectrometry (3), identifying <257 differentially changed candidates, from which 10 proteins relating to neuroinflammation, programmed cell death, and muscle degeneration by GO analysis were validated by PRM (4). (5) The aim of this investigation was to identify biomarkers that can help improve clinical trial outcomes by characterizing disease processes and relating them to neurological and prognostic factors of disease to improve sub-grouping in clinical trials and the detection of efficacy for therapeutic drug candidates. (6) This investigation identified biomarker candidates that are involved with the interferon and activated microglial response in MND, proposing candidate pathways and proteins for future studies to validate biomarkers for use in clinical trials. *Made in Biorender.*

7.2 Urinary neopterin detection and quantification

Urine samples from people with MND were first tested by an IBL competition ELISA to determine if neopterin was detectable in people with MND. As described in *Chapter 3*, the IBL competition ELISA resulted in the detection and quantification of neopterin in the urine of people with MND at baseline, and longitudinally over disease progression. This study was published in (Shepherd et al., 2022) and was able to infer that neopterin was a novel biomarker candidate of systemic pro-inflammation and MND progression. This was not without its caveat of a small sample size and a non-linear calibration curve. *Chapter 3* went on to describe the adaption of a novel liquid chromatography and mass spectrometry technique, adapted from (Burton et al., 2016) for urinary neopterin quantification in MND urine for the first time using triple quadrupole mass spectrometry. The optimized LCMS method for urinary neopterin used a *D*-neopterin calibration standard for linearity in neopterin detection, and *D*-neopterin-¹³C₅ internal standard for absolute neopterin quantification in MND urine. Using one calibration curve, the optimized LCMS assay could detect and quantify urinary neopterin in people with MND and healthy individuals with high sensitivity, specificity, and repeatability. This method was first applied to the same cohort of individuals from (Shepherd et al., 2022), verifying and refining the results of the IBL competition ELISA with a strong linear correlation of urinary neopterin quantification between two independent techniques.

The utility of urinary neopterin as a biomarker candidate of pro-inflammation in people with MND was investigated in *Chapter 4*, addressing the caveats of the previous study with a larger sample size. Urinary neopterin levels were significantly increased in people with MND in comparison to healthy individuals at baseline. Urinary neopterin exhibited a significant correlation with people over the age of 78 years, reflecting age-related inflammatory processes in people with MND and healthy individuals that was observed by ELISA (Shepherd et al., 2022). When the cohort was restricted to people between the ages of 42 and 78 years of age, neopterin was correlated with ALSFRS-R scores at baseline and was an independent predictor of survival by univariate Cox proportional hazards models when detected by LCMS or ELISA in 65 people with MND. While inflammatory biomarkers like neopterin can be elevated in disease and associated with survival outcomes, they are not always reliable prognostic indicators on their own (Hu et al., 2017). The prognostic value of neopterin is limited when compared other clinical characteristics (i.e., Δ FRS) and biomarkers related to disease processes (i.e., urine p75^{ECD}, serum NfL) (Benatar et al., 2024b). However, the predictive capability and dynamic changes in inflammatory markers over

disease progression, or with treatment intervention may help to stratify people with MND most likely to respond to anti-inflammatory treatments and monitor the effects of those treatments over time in clinical trials.

Urinary neopterin as a predictive biomarker candidate of pro-inflammation was significantly correlated to urinary p75^{ECD} reflecting programmed cell death and neurodegeneration in people with MND. With future analytical and clinical validation studies, neopterin and p75^{ECD} together may have potential utility and predictive and pharmacodynamic biomarkers in phase 2 clinical trials of experimental therapeutics for people with MND. Despite this study not having serum-matched data for neurofilament light (NfL) levels as a now-validated biomarker of axonal and neurodegeneration (Benatar et al., 2020, Benatar et al., 2024b), generating this data to compare all three biomarkers in the future would contribute towards the overarching idea of generating a biomarker panel for use in phase 2 clinical trials and beyond to better stratify individuals by predicted therapeutic response, and understand the safety and tolerability of experimental therapeutics.

7.3 Detection, discovery, and validation of differentially expressed proteins in MND

Urinary protein discovery was the first phase in uncovering novel biomarker candidates in people with MND. *Chapter 5* describes the application of an un-targeted biomarker discovery workflow using liquid chromatography and mass spectrometry in data dependent acquisition (DDA) and data independent acquisition (DIA) modes. This generated the structure for an early-discovery phase of protein-based biomarker candidates that could characterize MND pathology, with a focus on systemic inflammation. This study aimed to discover urinary proteins that were differentially changed at baseline, and to determine how urinary proteins may be related to disease mechanisms by exploring gene ontology pathways. Human urine from people with MND advantageous to explore, having a simple proteome that contained a low complexity matrix compared to serum, and a large dynamic range of up to 30% of proteins that can reflect systemic processes (Rogers et al., 2023). Using DDA mass spectrometry, a spectral library of 1,970 unique proteins was generated that identified 1,350 common protein groups in greater than 50% of samples in a relative abundance using a DIA analysis between 39 people with MND and 26 healthy individuals. Of those proteins, >257 candidates were significantly changed belonging to gene ontology pathways representing multiple inflammatory pathways of pro-inflammatory cytokines, neuroimmune glial cells, and lysosomal proteins, amongst apoptotic pathways involving death receptor and tumour necrosis factor activity, and muscle degeneration pathways from elastic fibers, collagen

fibers, and basement membrane proteins which have been previously established in people with MND (Kiernan et al., 2021, Rogers et al., 2023).

To evaluate the relevance of our findings, we compared the differentially expressed urinary proteins identified in this DIA study with those reported in previous studies (Rogers et al., 2023). Three proteins related to the immune response were identified, including cystatin C (P01034), transthyretin (P02766), and CD14 (P08571). Cystatin C has been previously detected in the CSF of people with MND by ELISA and has been correlated with survival as a candidate prognostic biomarker (Pasinetti et al., 2006, Tsuji-Akimoto et al., 2009, Wilson et al., 2010), and transthyretin has been identified by mass spectrometry in CSF and was shown not to change with disease progression (Ryberg et al., 2010); however, neither of these proteins were significantly changed in this study. CD14 (P08571) has been previously described in serum, urine, and CSF as a candidate prognostic biomarker of MND (Süssmuth et al., 2010, Beers et al., 2020). CD14 was identified and trended towards significance in the current study, warranting further investigation using SIS peptides. Urinary p75 (P08138) related to neurodegeneration as a prognostic, progression, and pharmacodynamic biomarker (Shepherd et al., 2017) was identified, confirming previous findings from (Shepherd et al., 2014) that used immuno-precipitation and mass spectrometry to identify five unique peptides from the extracellular domain region, and titin (Q8WZ42) related to muscle degeneration was identified as a candidate prognostic biomarker for lower limb MND (Yamada et al., 2021), but in this study was not significantly changed. Many other proteins were identified, including multiple collagen chain proteins between collagen alpha-1 (P39059, P02452) and alpha-3 chains (P12111) that may be correlated to MND duration (Ono et al., 1999), and disease specific proteins including *SOD1* (P00441) previously identified in plasma (Sproviero et al., 2018), and TDP-43 (Q13148) that has been linked to secretion in human biofluids by extracellular vesicles as a key pathway for the clearance of pathological TDP-43 (Dellar et al., 2025). The relationship of these proteins to disease was summarized in (Rogers et al., 2023), and future studies are required in the urine to identify and validate protein candidates with improved sensitivity.

Lastly, some important proteins were not discoverable in MND urine, namely NfL and pNfH that have previously been identified in serum and CSF by ELISA and SIMOA techniques (Benatar et al., 2024d). Both NfL and pNfH are quantified in low abundance in serum and CSF, measuring between 0-500 pg/mL for NfL and 0-2,000 pg/mL for pNfH (Benatar et al., 2020). NfL has been identified by mass spectrometry in CSF (Trautwig et al., 2025), but only when TMTs were used to improve the sensitivity of detection. Hence, neurofilaments in

biofluids are difficult to detect using untargeted mass spectrometry techniques, but when using a highly sensitive and targeted SIMOA assay they are more easily detected. This suggests that using both targeted and un-targeted techniques to identify and validate biomarkers for people with MND is an important consideration.

Ten protein candidates were verified in *Chapter 6* describing the development of a novel parallel reaction monitoring (PRM) technique for the targeted quantification of 18 unique peptides belonging to 10 protein candidates using orbitrap-mass spectrometry. These proteins were selected on a rationale described in *Chapter 5*, validating proteins that may characterize the immune response or that were related to other mechanisms of disease. After performing verification using parallel reaction monitoring, these proteins were identified in MND urine with greater precision and sensitivity. The relative quantification of each protein using PRM allowed us to investigate pilot measurements of target peptides to clinical characteristics including the ALSFRS-R to identify if any proteins were related to prognosis or disease outcomes in people with MND. Lysosomal protein cathepsin D (P07339), and elastic fibre protein fibulin-5 (Q9UBX5) were related to ALSFRS-R scores in people with MND, however, this data was not considered conclusive. Future validation studies using SIS peptides across multiple biological replicates may determine if they can be useful as prognostic and possible predictive biomarkers in clinical trials for MND. Predictive biomarkers will be useful tools for classify people with MND into response and non-responder groups based on their clinical phenotype (i.e., pro-inflammatory state, high rate of neurodegeneration, axonal loss, etc.) which is critical for drug design and administration in phase 2 and 3 clinical trials (Amiri-Dashatan et al., 2018).

7.4 Study limitations

This study presented with the challenge of enrolling age-matched healthy individuals for biomarker assessment. It may be assumed that family members, partners, or caregivers of similar age to their counterparts living with MND would be willing to donate urine to research at the same time, however, this was not the case. Most of the time, confounding conditions like diabetes or infection met our exclusion criteria and could not be included. Although urine is a convenient biofluid to obtain and explore for people with MND, compared to an intrusive lumbar puncture to obtain CSF, in some cases it was found that people with MND produced small volumes of urine. In these cases, this was a limiting factor to perform testing for urinary p75^{ECD}, neopterin, creatinine, osmolality, and diafiltration that required up to 5 mL of debris-free urine. Additional to all limitations with obtaining urine from MND and age-matched controls at the clinic, our criteria for participation of people under the age of 78 years to prevent reflecting age-related inflammatory processes reduced our available cohort size. Hence, the number of people that reached statistical analysis was far less than what was available from our biobank source.

Neopterin was previously measured in people with MND at baseline and longitudinally using the IBL competition ELISA (Shepherd et al., 2022), however, mass spectrometry measurement was limited to baseline samples only. Neopterin was not cross-validated as a candidate biomarker of disease progression in longitudinal follow-up samples using mass spectrometry. This limitation was at the caveat of reliable instrument time that could be offered from the Thermo Scientific Altis triple quadrupole mass spectrometer. Briefly, this instrument suffered from a technical issue after neopterin was measured at baseline in people with MND and other conditions and could not be used for further testing longitudinally. The instrument was replaced under warranty immediately after this investigation was completed. Due to this variation, investigation into the sources of variability coming from the sample preparation technique was limited, and this study needs to be repeated on a newer instrument with further investigation into sample preparation variances. Further, neopterin production increases with various diseases and infections associated with increased oxidative stress (Widner et al., 2002b), or when T-cells, macrophages, or microglia are activated in a neuroinflammatory response (Melichar et al., 2006). Neopterin was not compared to disease mimics of MND as done in previous studies (Shepherd et al., 2017). These mimics might include people with Multiple Sclerosis or Parkinson's disease (Turner and Talbot, 2013).

Although urinary neopterin may characterise inflammation at earlier stages of disease, further studies need to be completed to validate this conclusion. Understanding the relationship between differentially changed proteins and disease in a relative abundance analysis proved difficult when compared to prognostic markers for survival, and the challenge of performing mass spectrometry without stable isotope standard (SIS) peptides needs to be overcome in the future.

7.5 Future directions

This study demonstrated by mass spectrometry that urinary neopterin is an objective biomarker candidate of MND that can characterize pro-inflammation. However, further studies are required to test the feasibility of using urinary neopterin to determine prognosis and predict people most likely to respond to anti-inflammatory treatments, and to monitor treatment outcomes in clinical trials from people with sporadic MND, familial MND, and pre-familial MND over their course of disease. Future studies should investigate urinary neopterin levels in pre-clinical MND models to work towards differentiating when neuroinflammation becomes a characterizing feature of disease (Béland et al., 2020). Exploring neopterin in pre-clinical models of MND may uncover if neuroinflammation is a cause of motor neuron cell death, or if the inflammatory signature is a result of disease. It is understood that inflammation is not the primary cause of disease, since SOD1-^{G93A} mice deficient in B cells still develop MND-like symptoms (Naor et al., 2009), and ablating microglia made no effect on motor neuron degeneration (Gowing et al., 2008). Neopterin was detectable in SCID mice injected with human PBMCs almost 20 years ago (Amadori et al., 2001), and this may be a technique that is transferable to the SOD1-^{G93A} mouse model to identify when urinary neopterin levels start to change, and if they can be influenced with the addition of a disease modifying treatment before advancement to the clinic. Ultimately, defining immune activation at the time of diagnosis may characterize people with MND at higher risk of disease progression, and improving clinical trial design and therapeutic outcomes (Femiano et al., 2024).

Significant progress has been made in understanding the pre-symptomatic, or prodromal phase of MND (Benatar et al., 2022). Although biomarkers like neopterin that can characterize the immune response may not offer the ability to diagnose MND, they may have some value in predicting the likely timing of clinically manifest disease based on the extent of neuro- or pro-inflammation occurring at an earlier time point than baseline. Future studies exploring the interference of inflammation and age in pre-fMND, that was observed this study at baseline, may uncover age-related inflammation as a contributor to disease by the multistep hypothesis proposed by (Al-Chalabi et al., 2014) over a decade ago. This should be applied not only to targeted analysis, observing changes in urinary neopterin across the prodromal spectrum of disease, but also through un-targeted biomarker discovery studies that may identify differential changes in protein and gene ontology pathways that may be contributing towards disease manifestation. Although urine is a feasible biological fluid for non-invasive collection, optimizing mass spectrometry workflows

to complete studies in the future that can identify neopterin simultaneously in time-point matched urine, serum, and CSF samples will be advantageous to characterize changes in the immune response both centrally (i.e., CSF) and peripherally (i.e., serum and urine) on both sides of BBB. This will allow future studies to compare biomarker candidates to matched serum NfL levels as a validated prognostic biomarker involved in drug development (Benatar et al., 2023). It is understood that a single biomarker cannot capture the complex biology of MND (Turner and Benatar, 2015), and this research builds a foundation for generating a panel of biomarkers that can objectively define disease mechanisms, including neuroinflammation. There is no one 'ideal biomarker for MND' (Otto et al., 2012), but ideal biomarker panels can exist, as done in other diseases to create an algorithm of disease activity in Multiple Sclerosis (Paul et al., 2019, Gonyou et al., 2025) and Alzheimer's disease (Krishna et al., 2024).

This study is the first to investigate urine as a source of novel biomarkers by differential protein changes using DIA mass spectrometry. Similar studies have been performed in the CSF of people with both sporadic and fMND (Dellar et al., 2024, Trautwig et al., 2025). The urinary proteome should be explored with sufficient power in people with pre-fMND, fMND, MND at baseline, and longitudinally. The results of the current study at baseline, and future longitudinal studies, are promising; however, these proteins need to be validated in future PRM studies in larger sample sizes and using SIS peptides for absolute quantification before they can be used in clinical trials as possible prognostic, progression, or pharmacodynamic biomarkers. Exploring the proteome of people with known genetic mutations will be of interest in the future to determine which biomarkers change in pre-familial disease before symptom onset. This will not only consider candidate diagnostic biomarkers but will also provide information regarding pathological changes that may occur at disease onset. In addition, a number of genes have been associated with neuroinflammation, including *TBK1* involved in antiviral innate immunity (Ahmad et al., 2016), and *C9orf72* that is characterized by immune phenotypes and dysregulation of autophagy-lysosome pathways in MND-FTD (Beckers et al., 2021). This is interesting because a number of lysosomal pathways were up-regulated in the gene ontology analysis performed in this study, and cathepsin D (P07339) was correlated to the ALSFRS-R at baseline when measured by PRM. Cathepsin D is an important protein involved in lysosomal recycling and degradation that has been studied in other neurodegenerative diseases Parkinson's and Alzheimer's disease (Bunk et al., 2021) that should be studied in the future in familial and sporadic forms of MND. All of

these directions will improve the effectiveness of biomarker translation to the clinic to identify treatment outcome measures in a highly heterogeneous disease.

To confirm these pathways and their relationship to disease, improvements can be made to the mass spectrometry methods used to discover and validate candidate proteins in the future. Quantification has been performed in these studies using label-free or relative abundance quantification, without spiking in any labeled reference peptides for DIA or PRM mass spectrometry methods. Isobaric labeling strategies including tandem mass tag (TMT) (Thompson et al., 2003) and isobaric tags for relative quantification (iTRAQ) (Wiese et al., 2007) should be explored in future study designs to perform a label-based relative abundance analysis of novel proteins using shotgun proteomics. This comes with the advantage of increased precision and accuracy of lower abundance peptides using reporter ion and fragment ion peaks to infer the relative intensity of unknown peptides (Rauniyar and Yates, 2014), and has been used to explore metabolism and inflammatory-related proteins in mice that could be adapted to suit this work (Yu et al., 2020). However, targeted approaches such as PRM can offer sensitive, reproducible, accurate, and absolute quantification of target peptides (Ronsein et al., 2015) using SIS peptides as internal standards to validate candidate peptides (Carr et al., 2014). Monitoring both heavy and light peptides is a technique revision that can be implemented into this method to quantify the abundance of endogenous peptides relative to the SIS spike-in, which has been used to perform validation on 340 target peptides using the same instrumentation (Stopfer et al., 2021).

Significant advances have been made in understanding the inflammatory architecture and pathology of MND. The best strategy moving forward is to improve the accuracy of stratification and sub-classification of people with MND entering clinical trials using objective biomarkers to identify the people most at risk (i.e., prognostic biomarkers) and most likely to respond to a treatment (i.e., predictive biomarkers). To improve the outcomes of those clinical trials, novel progression and pharmacodynamic biomarkers alongside the ALSFRS-R and serum NfL (Benatar et al., 2025) are needed to track disease changes with the administration of candidate treatments to monitor disease outcomes. These can be used in a panel to interpret data from phase 2 clinical trials and offer better translation to phase 3. This will inherently enable the validation of several disease pathways (i.e., neuroinflammation, neurodegeneration, programmed cell death, elastic fibres and muscle degeneration) seen within this investigation, and provide a rationale for pharmaceutical companies to explore therapeutic interventions tailored to those mechanisms.

Reflecting on data published from 2023, the most represented target category for candidate therapeutics was neuroinflammation, with 16 therapeutic agents being applied in clinical trials ranging from mechanisms that attempt to increase the levels of endogenous T-regulatory cells, antibodies targeting CD4⁺ T- and B-cells, and using recombinant human interleukin-2 as a candidate treatment to reduce pro-inflammation in MND. Enhanced microglial activation has been found in multiple areas of the brain in people with MND (Zürcher et al., 2015) and represents a potential way to monitor treatment interventions targeting neuroinflammation. Outside of these invasive mechanisms, urinary neopterin, or other biomarkers that can characterise this pathology in disease, will be critical for advancing these clinical trials from phase 2 to phase 3 to better determine their effectiveness in improving disease outcomes for people living with MND. Since the discovery of riluzole 30 years ago (Bensimon et al., 1994), over 179 experimental therapeutics have been tested in phase 2 clinical trials for sporadic MND. Only 26 made it to phase 3 clinical trials, highlighting the need to reduce MND heterogeneity in phase 2 and 3 clinical trials. The discovery, characterisation, and validation of prognostic, pharmacodynamic, progression, and predictive biomarkers is of utmost importance for future studies (Goyal et al., 2020).

7.6 Concluding remarks

MND is a highly heterogeneous and fatal neurodegenerative disease with no cure. Despite the efforts of many of phase 2 clinical trials, progress towards effective treatments has been limited by vastly heterogeneous phenotypes and underpowered measures of treatment outcomes. This highlights the importance of advancing traditional biomarker detection techniques using mass spectrometry, and the need to discover, validate, and implement novel biofluid biomarkers to improve clinical trial outcomes. By incorporating biomarkers into rigorous testing of experimental therapeutics, the design and outcomes of clinical trials will be improved and there is great potential for developing improved treatments for MND in the near future. The aim of this study was to investigate urinary neopterin as a candidate predictive biomarker of pro-inflammation, and to identify differentially changed urinary proteins at baseline and verify their relationship to MND. This investigation will be the first to explore urinary neopterin and identify novel biomarkers in the urinary proteome of people with MND using mass spectrometry, generating the infrastructure for a panel of biofluid biomarkers that may allow clinicians to stratify people according to their disease phenotype, or predict a response to an experimental therapeutic. The focus was on biomarkers of immune dysfunction to compete with the increasing interest of treatment candidates targeting neuroinflammation as the best represented therapeutic target mechanism. The results of this investigation are promising; however, these biomarker candidates need to be validated in the clinical space in larger sample sizes and across multiple disease phenotypes to understand their utility as candidate prognostic and predictive biomarkers.

These findings highlight the pressing need for robust biomarkers that can characterise disease heterogeneity to accurately stratify people with MND into clinical trials by their predicted response to treatment outcomes. While further validation in larger and more diverse cohorts is important, this work represented a critical step towards bridging the gap between disease characterisation and therapeutic intervention. The question is starting to change from 'what' is going on to 'how' can this be resolved, marking a shift from understanding MND pathogenesis to translating actionable clinical strategies that can improve the quality of life of people living with MND.

Appendices

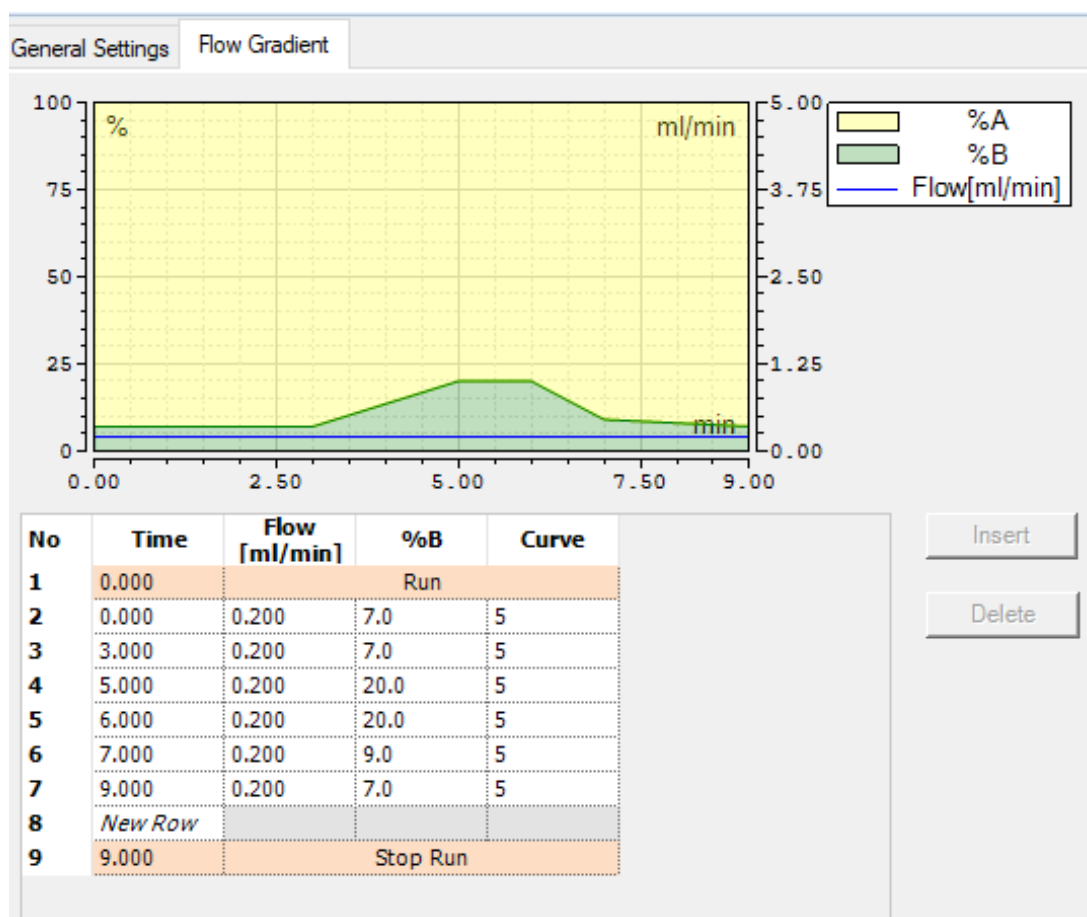
Appendix 8.1

TSQ Altis Multiple Reaction Monitoring (MRM) Method

Liquid Chromatography

Instrument: Vanquish Flex uHPLC (Thermo Fisher Scientific)
 Mobile Phases: Buffer A: 1% Acetonitrile, 0.1% Formic Acid
 Buffer B: 99.9% Methanol, 0.1% Formic Acid
 Guard Column: Phenomenex SecurityGuard™ Guard Cartridge Kit (KJ0-4282)
 SecurityGuard™ Cartridge (AJ0-4350, 4x2.0mm ID)
 Analytical Column: Luna^(R) 3 μ m Phenyl-Hexyl 100Å LC column 150 x 3.0 mm (00F-4256-Y0)

uHPLC Gradient: 9 minutes



Mass Spectrometry

Instrument: TSQ Altis™ Mass Spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
 MS Source: H-ESI Ion Source (ES071, Thermo Fisher Scientific)
 Column Oven: Vanquish™ uHPLC Column Oven (Thermo Fisher Scientific)
 Oven Temp: 40°C

Method Summary

Method Settings

Method Duration (min): **9**

Global Parameters

Ion Source

Ion Source Type: **H-ESI**
 Spray Voltage: **Static**
 Positive Ion (V): **3500**
 Negative Ion (V): **2500**
 Current LC Flow (µL/min): **200**
 Sheath Gas (Arb): **35**
 Aux Gas (Arb): **7**
 Sweep Gas (Arb): **0**
 Ion Transfer Tube Temp (°C): **325**
 Vaporizer Temp (°C): **275**
 APPI Lamp: **Not in Use**
 FAIMS Mode: **Not Installed**

MS Global Settings

Default Charge State: **1**
 Internal Mass Calibration: **Off**
 Current Lock Mass: **Current**

Experiment#1 [SRM]

Start Time (min): **0**
 End Time (min): **9**

Master Scan:

MRM

Polarity: **Positive**
 Chromatographic Peak Width (sec): **12.0**
 Use Chromatographic Filter: **True**
 Use Cycle Time: **True**
 Cycle Time (sec): **0.8**
 Points Per Peak: **15.0**
 Use Dwell Time Factor: **True**
 Minimum Dwell Time (ms): **90.0**
 Use Calibrated RF Lens: **False**
 Q1 Resolution (FWHM): **0.7**
 Q3 Resolution (FWHM): **0.7**
 CID Gas (mTorr): **1.5**
 Source Fragmentation: **0**
 Use Retention Time Reference: **False**
 Display Retention Time: **False**

Use Quan Ion: **False**Show Visualization: **False**

MRM Table

SRM Table							
Compound	Start Time (min)	End Time (min)	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)	Min Dwell Time (ms)
D+Neopterin	0	9	254	190.125	20.92	69	65.344
D+Neopterin	0	9	254	194.125	19.28	69	65.344
D+Neopterin	0	9	254	206.113	18.4	69	65.344
D+Neopterin	0	9	254	236.125	15.19	69	65.344
13C5 Neopterin	0	9	259	194.125	21.01	73	65.344
13C5 Neopterin	0	9	259	197.125	19.32	73	65.344
13C5 Neopterin	0	9	259	210.125	18.31	73	65.344
13C5 Neopterin	0	9	259	241.125	15.28	73	65.344

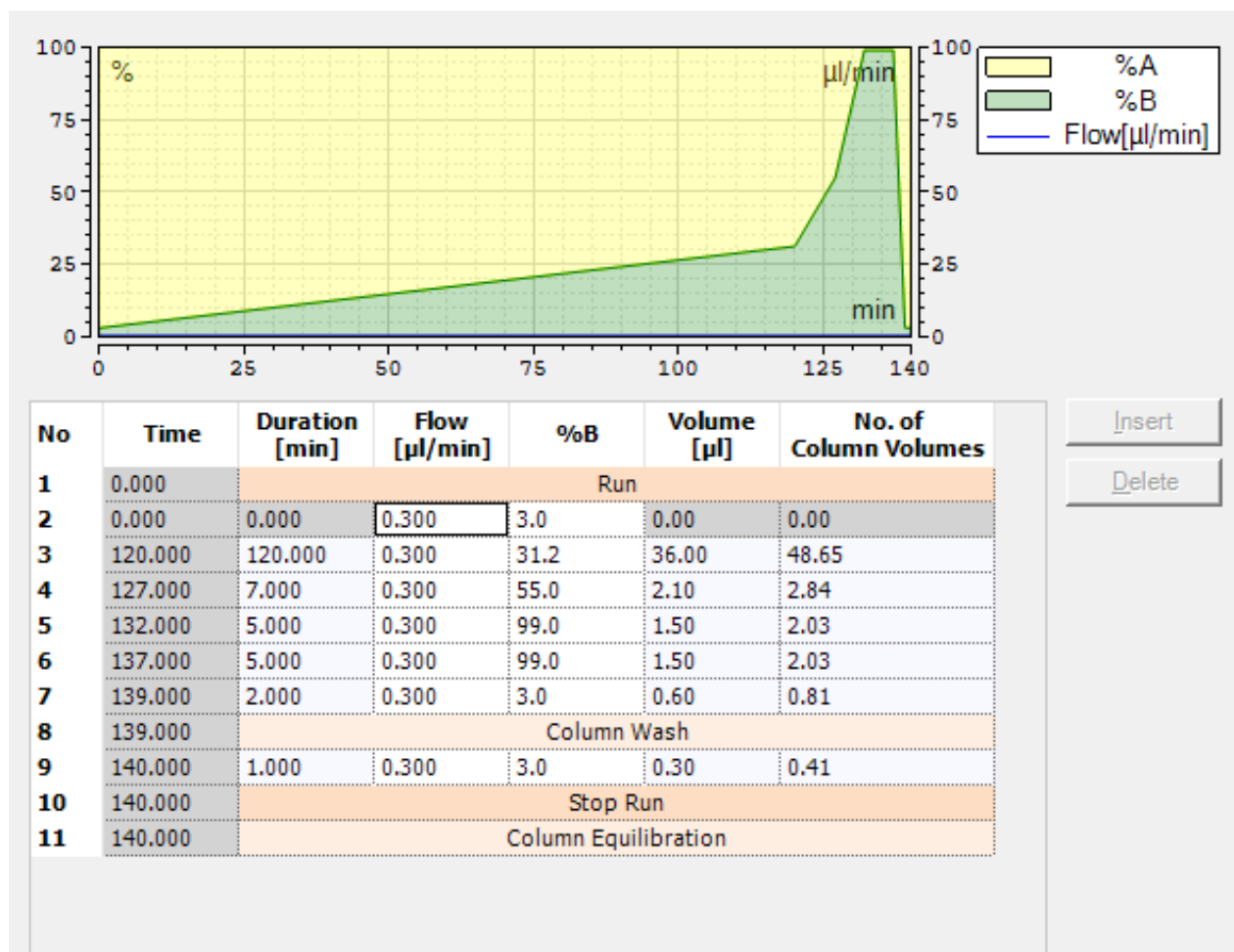
Appendix 8.2

Exploris 480 Data Dependent Acquisition (DDA) Method

Liquid Chromatography

Instrument: Dionex Ultimate 3000 UPLC (Thermo Fisher Scientific)
 Mobile Phases: Buffer A: Water, 0.1% Formic Acid
 Buffer B: 79.9% Acetonitrile, 20% Water, 0.1% Formic Acid
 Trap Column: PepMap™ 100 trap cartridge (0.3 x 5 mm, 5 µm C18, Thermo Fischer)
 Analytical Column: Inhouse pulled column created from 75 µm inner diameter fused silica capillary packed with 3 µm ReproSil-Pur C18 beads (Dr. Maisch, Ammerbuch, Germany) to 25cm

uHPLC Gradient: 140 minutes



Mass Spectrometry

Instrument: Orbitrap Exploris™ 480 Mass Spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
 MS Source: Nanospray Flex™ Ion Source (ES071, Thermo Fisher Scientific)
 Column Oven: Nanospray Flex™ Column Oven (Sonation)
 Oven Temp: 60°C
 Spray Voltage: 2400 V
 Ion Transfer Tube Temp: 275°C

Method Settings

Application Mode: **Peptide**

Method Duration (min): **150**

Global Parameters**Ion Source**

Use Ion Source Settings from Tune: **True**

FAIMS Mode: **Not Installed**

MS Global Settings

Infusion Mode: **Liquid Chromatography**

Expected LC Peak Width (s): **30**

Advanced Peak Determination: **True**

Default Charge State: **2**

Enable Xcalibur AcquireX Ab method modifications: **False**

Lock Mass Correction: **User-defined Lock Mass**

Mode: **Scan-to-Scan**

Mass Tolerance (ppm): **15**

Current Lock Mass: **Current**

Experiment #1

Start Time (min): **5**

End Time (min): **150**

Master Scan:**Full Scan**

Orbitrap Resolution: **120000**

Scan Range (m/z): **350-1400**

RF Lens (%): **40**

AGC Target: **Custom**

Normalized AGC Target (%): **200**

Absolute AGC Value: **2.000e6**

Maximum Injection Time Mode: **Auto**

Microscans: **1**

Data Type: **Profile**

Polarity: **Positive**

Source Fragmentation: **Disabled**

Lock Mass Injection: **False**

Scan Description:

Monoisotopic peak determination: **Peptide**

Relax restrictions when too few precursors are found: **True**

Filter Type: **Intensity Threshold**

Intensity Threshold: **5.0e4**

Include charge state(s): **2-6**

Include undetermined charge states: **False**

Dynamic Exclusion

Share dynamic exclusion list with other selected dynamic exclusion filters: **False**
Dynamic Exclusion Mode: **Custom**
Exclude after n times: **1**
Exclusion duration (s): **45**
Mass Tolerance: **ppm**
Low: **10**
High: **10**
Exclude isotopes: **True**
Perform dependent scan on single charge state per precursor only: **False**
Data Dependent
Data Dependent Mode: **Number of Scans**
Number of Dependent Scans: **20**
Scan Event Type 1:
ddMS²
Multiplex Ions: **False**
Isolation Window (m/z): **1.4**
Isolation Offset: **Off**
Collision Energy Type: **Normalized**
HCD Collision Energy (%): **32**
Orbitrap Resolution: **15000**
TurboTMT: **Off**
Scan Range Mode: **Define First Mass**
First Mass (m/z): **120**
AGC Target: **Standard**
Maximum Injection Time Mode: **Auto**
Microscans: **1**
Data Type: **Centroid**
Lock Mass Injection: **False**

Appendix 8.3

Table 8.1: Sequential windows used for data-independent acquisition (DIA)

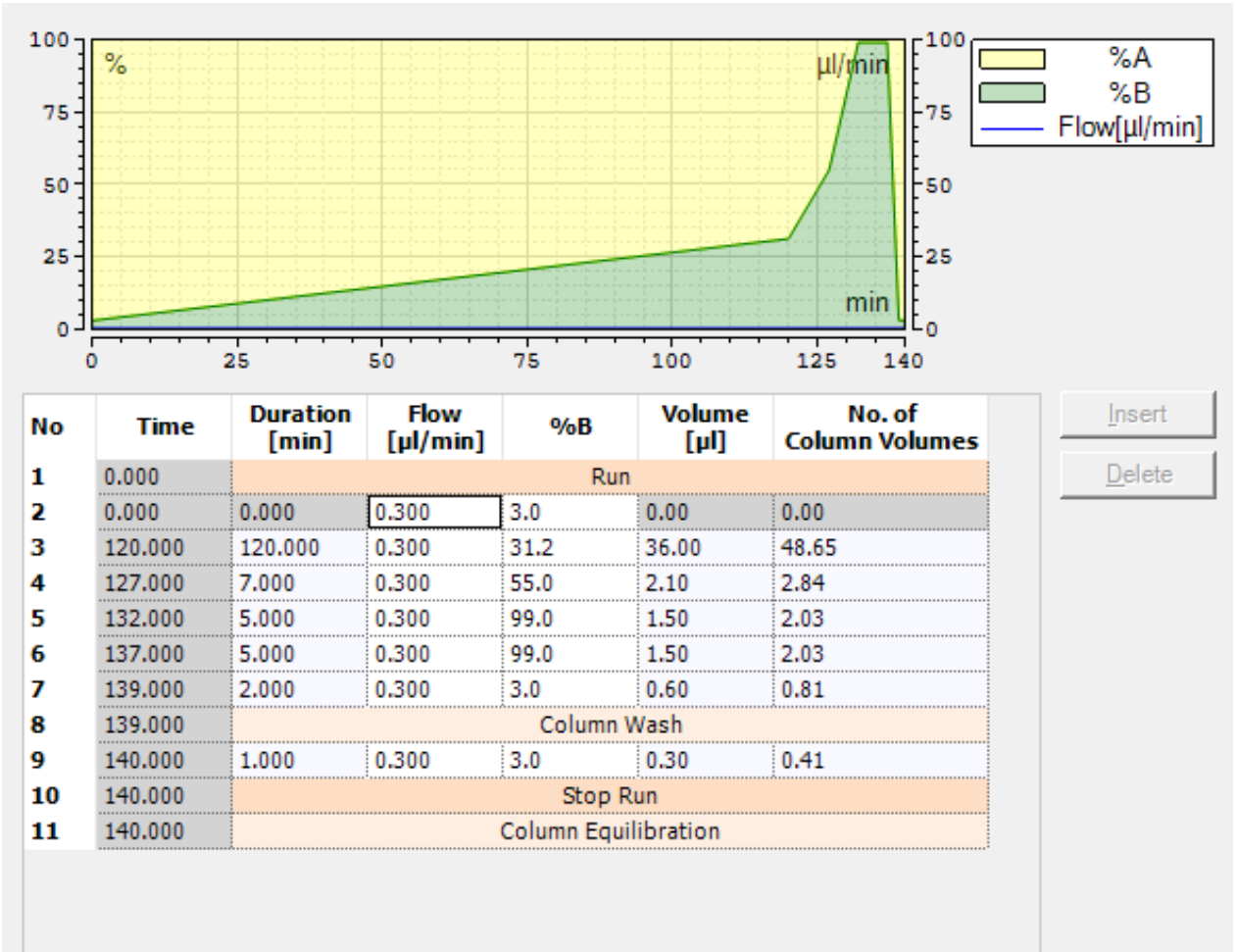
DIA Experiment #	Window range (m/z)	window size (m/z)	Window #
1	350.5-409	60	1
2	409.5-432	23	1
3	432.5-462	15	2
4	462.5-482	10	2
5	482.5-500	9	2
6	500.5-510	10	1
7	510.5-517	7	1
8	517.5-527	10	1
9	527.5-536	9	1
10	536.5-546	10	1
11	546.5-564	9	2
12	564.5-576	6	2
13	576.5-587	11	1
14	587.5-597	10	1
15	597.5-606	9	1
16	606.5-616	10	1
17	616.5-628	12	1
18	628.5-637	9	1
19	637.5-643	6	1
20	643.5-655	12	1
21	655.5-664	9	1
22	664.5-688	12	2
23	688.5-704	16	1
24	704.5-721	17	1
25	721.5-736	15	1
26	736.5-750	14	1
27	750.5-767	17	1
28	767.5-788	21	1
29	788.5-808	20	1
30	808.5-821	13	1
31	821.5-837	16	1
32	837.5-856	19	1
33	856.5-878	22	1
34	878.5-907	29	1
35	907.5-945	38	1
36	945.5-972	27	1
37	972.5-1015	43	1
38	1015.5-1083	68	1
39	1083.5-1199	116	1
			45

Appendix 8.4

Exploris 480 Data Independent Acquisition (DIA) Method

Liquid Chromatography

Instrument: Dionex Ultimate 3000 UPLC (Thermo Fisher Scientific)
Mobile Phases: Buffer A: Water, 0.1% Formic Acid
Buffer B: 79.9% Acetonitrile, 20% Water, 0.1% Formic Acid
Trap Column: PepMap™ 100 trap cartridge (0.3 x 5 mm, 5 µm C18, Thermo Fischer)
Analytical Column: Inhouse pulled column created from 75 µm inner diameter fused silica capillary packed with 3 µm ReproSil-Pur C18 beads (Dr. Maisch, Ammerbuch, Germany) to 25cm
uHPLC Gradient: 140 minutes



Mass Spectrometry

Instrument: Orbitrap Exploris™ 480 Mass Spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
 MS Source: Nanospray Flex™ Ion Source (ES071, Thermo Fisher Scientific)
 Column Oven: Nanospray Flex™ Column Oven (Sonation)
 Oven Temp: 60°C
 Spray Voltage: 2400 V
 Ion Transfer Tube Temp: 275°C

Method Summary

Method Settings

Application Mode: **Peptide**

Method Duration (min): **140**

Global Parameters

Ion Source

Use Ion Source Settings from Tune: **True**

FAIMS Mode: **Not Installed**

MS Global Settings

Infusion Mode: **Liquid Chromatography**

Expected LC Peak Width (s): **30**

Advanced Peak Determination: **True**

Default Charge State: **3**

Enable Xcalibur AcquireX Ab method modifications: **False**

Lock Mass Correction: **User-defined Lock Mass**

Mode: **Scan-to-Scan**

Mass Tolerance (ppm): **15**

Current Lock Mass: **Current**

m/z	Polarity
445.12003	Positive

Experiment #1 [MS]

Start Time (min): **5**

End Time (min): **140**

Master Scan:

Full Scan

Orbitrap Resolution: **120000**

Scan Range (m/z): **350-1200**

RF Lens (%): **50**

AGC Target: **Custom**

Normalized AGC Target (%): **200**

Absolute AGC Value: **2.000e6**

Maximum Injection Time Mode: **Auto**

Microscans: **1**

Data Type: **Profile**

Polarity: **Positive**

Source Fragmentation: **Disabled**

Lock Mass Injection: **False**

Scan Description:

Experiment #2 [DIA]

Start Time (min): **5**

End Time (min): **140**

Master Scan:
 DIA
 Precursor Mass Range (m/z): **350.5-409**
 DIA Window Type: **Auto**
 Multiplex Ions: **False**
 Isolation Window (m/z): **60**
 Window Overlap (m/z): **1**
 Window Placement Optimization: **On**
 Number Of Scan Events: **1**
 DIA Window Mode: **m/z Range**
 Collision Energy Type: **Normalized**
 HCD Collision Energy (%): **30**
 Orbitrap Resolution: **30000**
 Scan Range Mode: **Define First Mass**
 First Mass (m/z): **110**
 RF Lens (%): **50**
 AGC Target: **Custom**
 Normalized AGC Target (%): **3000**
 Absolute AGC Value: **3.000e6**
 Maximum Injection Time Mode: **Dynamic**
 Desired minimum points across the peak: **9**
 Microscans: **1**
 Data Type: **Centroid**
 Polarity: **Positive**
 Source Fragmentation: **Disabled**
 Lock Mass Injection: **False**
 Loop Control: **All**
 Scan Description:
 DIA m/z window

DIA m/z window
m/z range
350.4091625-411.43690225

Repeated [Experiment #] for all windows in **Appendix 8.1** between 350.5 – 1199.0 m/z

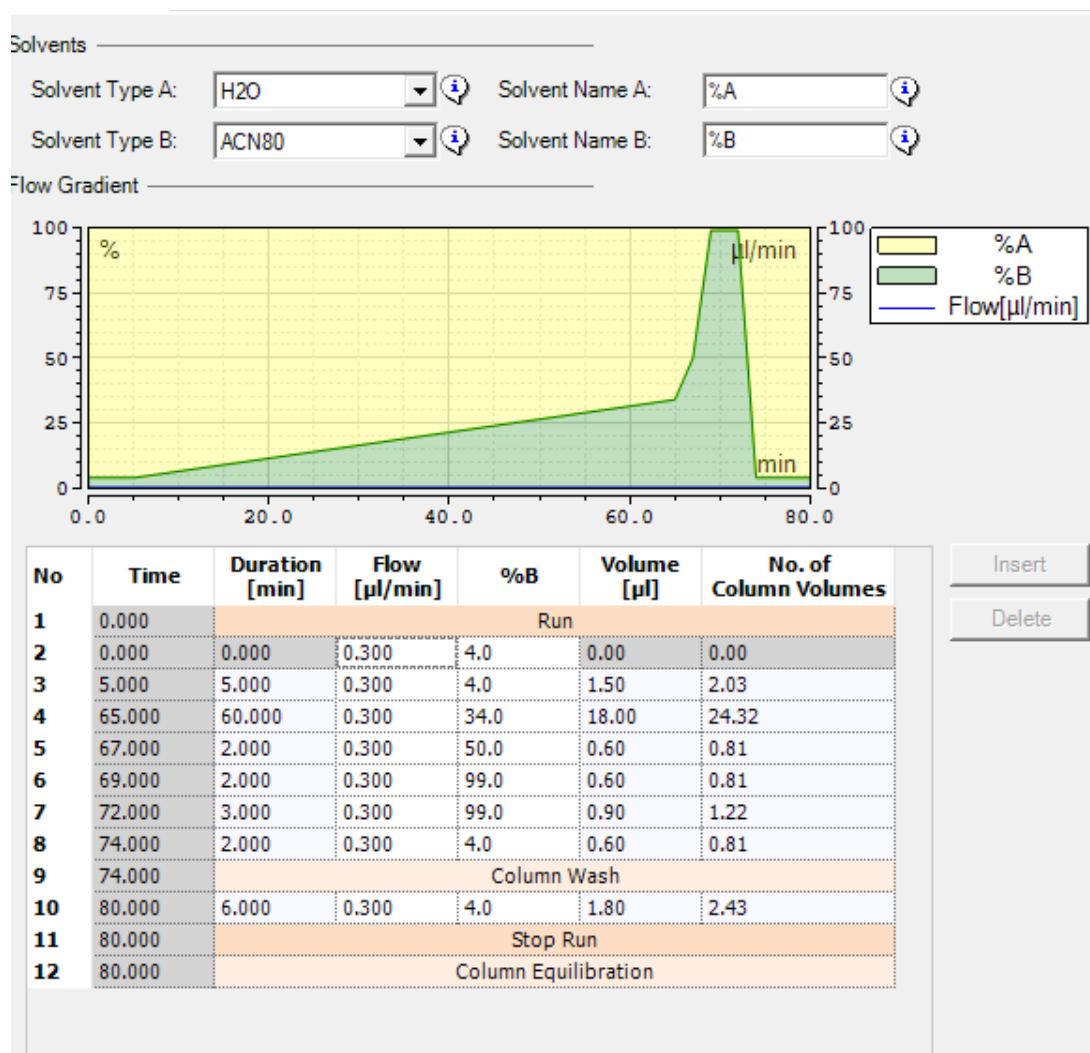
Appendix 8.5

Exploris 480 Parallel Reaction Monitoring (PRM) Method

Liquid Chromatography

Instrument: Vanquish NEO 3000 UPLC (Thermo Fisher Scientific)
 Mobile Phases: Buffer A: Water, 0.1% Formic Acid
 Buffer B: 79.9% Acetonitrile, 20% Water, 0.1% Formic Acid
 Trap Column: None. Direct injection
 Analytical Column: Inhouse pulled column created from 75 μ m inner diameter fused silica capillary packed with 3 μ m ReproSil-Pur C18 beads (Dr. Maisch, Ammerbuch, Germany) to 25cm

uHPLC Gradient: 80 minutes



Mass Spectrometry

Instrument: Orbitrap Exploris™ 480 Mass Spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
 MS Source: Nanospray Flex™ Ion Source (ES071, Thermo Fisher Scientific)
 Column Oven: Nanospray Flex™ Column Oven (Sonation)
 Oven Temp: 60°C
 Spray Voltage: 2400 V
 Ion Transfer Tube Temp: 275°C

Method Summary

Method Settings

Application Mode: **Peptide**

Method Duration (min): **80**

Global Parameters

Ion Source

Use Ion Source Settings from Tune: **True**

FAIMS Mode: **Not Installed**

MS Global Settings

Infusion Mode: **Liquid Chromatography**

Expected LC Peak Width (s): **30**

Advanced Peak Determination: **True**

Default Charge State: **2**

Enable Xcalibur AcquireX Ab method modifications: **False**

Lock Mass Correction: **User-defined Lock Mass**

Mode: **Scan-to-Scan**

Mass Tolerance (ppm): **20**

Current Lock Mass: **Current**

m/z	Polarity
445.12003	Positive

Experiment #1 [Full Scan]

Start Time (min): **0**

End Time (min): **80**

Master Scan:

Full Scan

RF Lens (%): **50**

AGC Target: **Custom**

Normalized AGC Target (%): **600**

Absolute AGC Value: **6.000e6**

Maximum Injection Time Mode: **Dynamic**

Desired minimum points across the peak: **9**

Microscans: **1**

Data Type: **Profile**

Polarity: **Positive**

Source Fragmentation: **Disabled**

Lock Mass Injection: **False**

Scan Description:

Full Scan

Full Scan	
Scan Range (m/z)	Orbitrap Resolution
350-1400	60000

Experiment #2 [Product Ion Scan]

Start Time (min): **26**End Time (min): **32**

Master Scan:

Product Ion

Multiplex Ions: **False**Q1 Resolution (m/z): **0.7**Q1 Offset: **Off**Collision Energy Type: **Normalized**HCD Collision Energy (%): **27**Orbitrap Resolution: **60000**TurboTMT: **Off**Scan Range Mode: **Define First Mass**First Mass (m/z): **120**RF Lens (%): **50**AGC Target: **Custom**Normalized AGC Target (%): **600**Absolute AGC Value: **6.000e5**Maximum Injection Time Mode: **Custom**Maximum Injection Time (ms): **150**Microscans: **1**Data Type: **Profile**Polarity: **Positive**Source Fragmentation: **Disabled**Lock Mass Injection: **False**Loop Control: **All**Dynamic RT: **Off**

Scan Description:

Time Mode: **Start/End Time**

Product Ion Scan

Product Ion Scan						
Compound	Formula	Adduct	Precursor (m/z)	Precursor Charge (z)	t start (min)	t stop (min)
YTACLCDDN PK (PIP)			678.7816	2	26	32

Peptide schedules were repeated as per **Table 6.5**

Appendix 8.6

Normalisation of the urinary proteome

Due to the nature of the sample preparation, neat urine specimens were concentrated roughly ~20x. To effectively normalise to the concentration of creatinine, that was tested in neat urine to avoid exceeding the concentration of the standard curve, the concentration of urine used to achieve a 10 µg digest of peptides for DDA and DIA analysis must be calculated back to its neat **starting volume** and total **injection volume**. Urinary biomarkers derived from spot urine samples were normalised to urinary creatinine as a reflection of their neat concentration.

Values equal for all urine specimens:

Tryptic digest volume (µL) = 210 µL

LCMS injection volume (µL) = 3 µL

Values different for all urine specimens

Diafiltration starting and final volume (µL)

Creatinine (mg/mL)

Starting volume (µL) for 10 µg peptide digest calculation

$$\frac{\text{Diafiltration starting volume (}\mu\text{L)}}{\text{Diafiltration final Volume (}\mu\text{L)}} \times 10 \mu\text{g peptide digest volume} \\ = \text{Hypothetical neat starting volume}$$

Mass spectrometer injection volume (µL) calculation

$$\frac{\text{Hypothetical neat starting volume}}{\text{Tryptic Digest Volume (}\mu\text{L)}} \times \text{LCMS injection volume (}\mu\text{L)} \\ = \text{hypothetical injection volume (E. }\mu\text{L loaded)}$$

Creatinine normalisation calculation

$$\left(\frac{\text{PG. Quantity}}{\text{E. }\mu\text{L loaded}} / 1000 \right) / \text{creatinine (mg/mL)}$$

Appendix 8.7

Significantly abundant urinary proteins in MND normalised to Creatinine

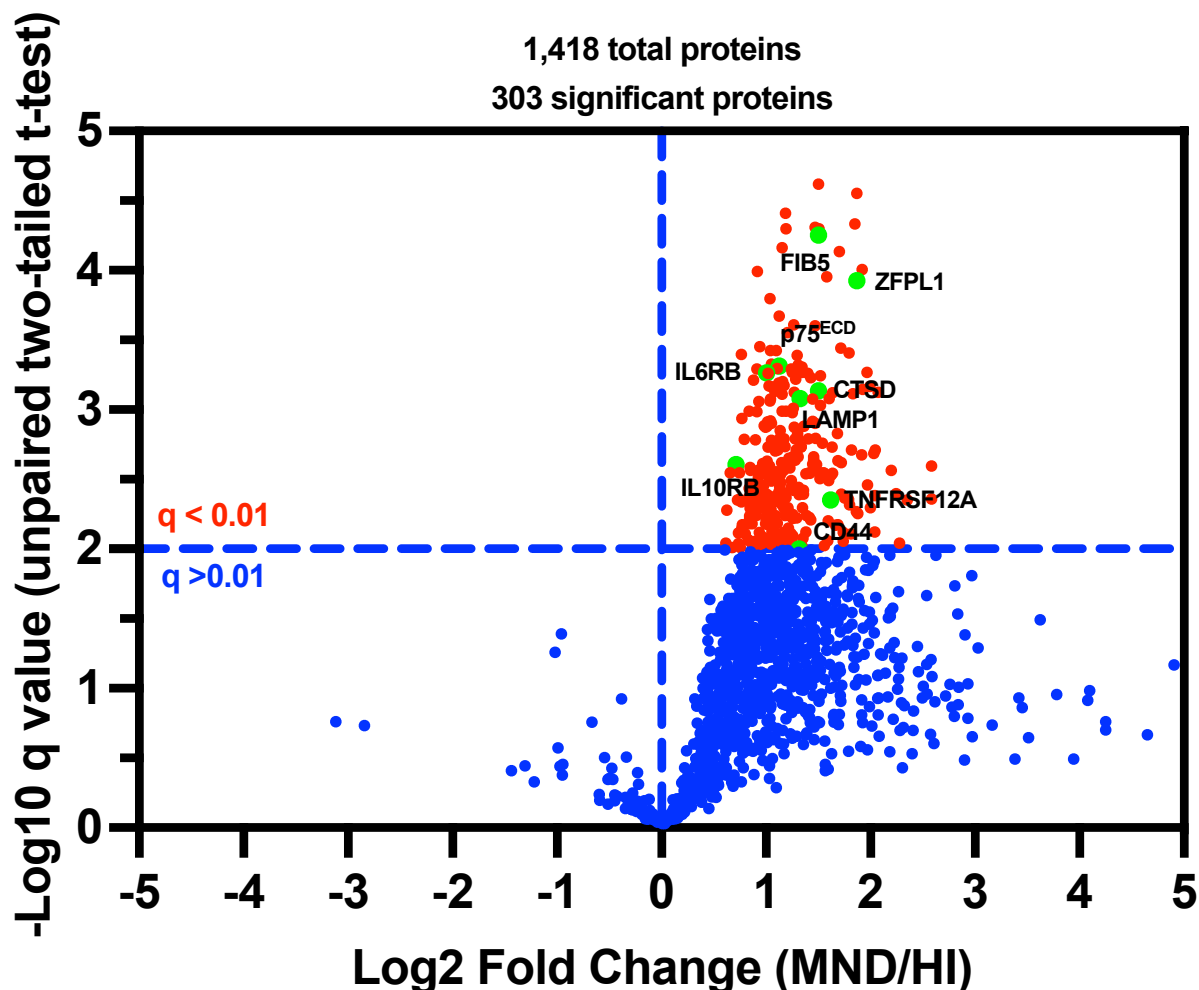


Figure 8.1: Differentially expressed protein groups from study 2 in people with MND compared to healthy individuals normalised to creatinine

Volcano plot of urinary proteins from 39 people with MND and 26 healthy individuals (HI) by DIA mode in *study 2*, processed by Spectronaut, and normalised to creatinine by MatLab. 1,418 total protein groups were identified. 1,115 proteins shown in blue (●) were not significantly changed, and 303 protein groups were significantly changed in people with MND (two-tailed unpaired T-test, multiple comparisons corrected by FDR and BH, ●, $q < 0.01$), when considering protein groups are in at least 50% of all study participants. Protein groups were organised by their $-\text{Log}_{10}$ q value (threshold, $q < 0.01$), compared to their Log_2 fold change in people with MND compared to HI. 303 protein groups were significantly up-regulated, and none were significantly down-regulated. Proteins of interest that overlapped with *study 1* are highlighted in green (●) for further discussion and validation.

Appendix 8.8

Table 8.2: Significantly changed urinary proteins unique to study 2

From study 2, 156 significantly changed urinary proteins were identified that were not common with the protein groups identified in study 1. These unique protein groups were investigated in the same group of 39 people with MND and 26 healthy individuals at baseline. MatLab was used to perform a two-tailed T-test on protein concentrations normalised to creatinine at baseline, revealing 11 significantly changed proteins in green (●, $q < 0.01$) that were otherwise undiscovered in people with MND.

PROTEIN ACCESSIONS	PROTEIN DESCRIPTIONS	<i>q</i> VALUE
Q8WX77	Insulin-like growth factor-binding protein-like 1	0.000716672
P51693	Amyloid beta precursor like protein 1	0.001048327
P08253	72 kDa type IV collagenase	0.002061919
Q9Y639	Neuroplastin	0.002541876
P04233	HLA class II histocompatibility antigen gamma chain	0.003216351
Q08708	CMRF35-like molecule 6	0.004301404
Q96J42	Thioredoxin domain-containing protein 15	0.004811818
P98161	Polycystin-1	0.00525129
Q86YD5	Low-density lipoprotein receptor class A domain-containing protein 3	0.005383535
Q8N423	Leukocyte immunoglobulin-like receptor subfamily B member 2	0.006702905
Q15084	Protein disulfide-isomerase A6	0.008701685
P10721	Mast/stem cell growth factor receptor Kit	0.011462646
P22607	Fibroblast growth factor receptor 3	0.011915429
Q9UQV4	Lysosome-associated membrane glycoprotein 3	0.01228767
Q13751	Laminin subunit beta-3	0.01306783
P23468	Receptor-type tyrosine-protein phosphatase delta	0.014173763
P07225	Vitamin K-dependent protein S	0.014183774
Q9H0E2	Toll-interacting protein	0.014188639
P35475	Alpha-L-iduronidase	0.015999344
Q14703	Membrane-bound transcription factor site-1 protease	0.016749396

P49257	Protein ERGIC-53	0.017693066
O00253	Agouti-related protein	0.019619798
Q8WZA1	Protein O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase 1	0.021369399
Q9UK41	Vacuolar protein sorting-associated protein 28 homolog	0.023166881
Q86XR5	Proline-rich membrane anchor 1	0.023214725
Q7Z692	Carcinoembryonic antigen-related cell adhesion molecule 19	0.028352321
Q9Y274	Type 2 lactosamine alpha-2,3-sialyltransferase	0.028353063
P14784	Interleukin-2 receptor subunit beta	0.029356288
P01705	Immunoglobulin lambda variable 2-23	0.032135427
Q9Y6Z7	Collectin-10	0.032414833
P01275	Pro-glucagon	0.033257498
P05814	Beta-casein	0.034675867
Q9NZ08	Endoplasmic reticulum aminopeptidase 1	0.034681
P61018	Ras-related protein Rab-4B	0.038756979
P08581	Hepatocyte growth factor receptor	0.038946939
P17931	Galectin-3	0.040749928
O75051	Plexin-A2	0.040791063
A6NGN9	IgLON family member 5	0.041949771
P35558	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]	0.044386249
O95971	CD160 antigen	0.045083024
Q695T7	Sodium-dependent neutral amino acid transporter B(0)AT1	0.045831729
O15273	Telethonin	0.047580663
Q96NZ9	Proline-rich acidic protein 1	0.04890764
O14672	Disintegrin and metalloproteinase domain-containing protein 10	0.051353942
Q9BVK6	Transmembrane emp24 domain-containing protein 9	0.051419185
O43157	Plexin-B1	0.052855802
Q9NS93	Transmembrane 7 superfamily member 3	0.054745117
Q16706	Alpha-mannosidase 2	0.055725071
P98155	Very low-density lipoprotein receptor	0.056211863

P17405	Sphingomyelin phosphodiesterase	0.056483591
Q5T749	Keratinocyte proline-rich protein	0.0589489
Q7Z7M8	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8	0.058961998
O14818	Proteasome subunit alpha type-7	0.061647532
P61204;P84077	ADP-ribosylation factor 3;ADP-ribosylation factor 1	0.063894368
Q14697	Neutral alpha-glucosidase AB	0.063943691
P17927	Complement receptor type 1	0.066197318
Q9HDC9	Adipocyte plasma membrane-associated protein	0.066606968
Q86UN2	Reticulon-4 receptor-like 1	0.070621524
P04259	Keratin, type II cytoskeletal 6B	0.076254513
Q99795	Cell surface A33 antigen	0.078831166
P00533	Epidermal growth factor receptor	0.079196284
P61160	Actin-related protein 2	0.08186151
A0A075B6I9	Immunoglobulin lambda variable 7-46	0.083822394
P05981	Serine protease hepsin	0.084567752
Q8NI32	Ly6/PLAUR domain-containing protein 6B	0.085470161
Q14667	Bridge-like lipid transfer protein family member 2	0.087104349
P15428	15-hydroxyprostaglandin dehydrogenase [NAD(+)]	0.091833535
O95154	Aflatoxin B1 aldehyde reductase member 3	0.095140974
P22392	Nucleoside diphosphate kinase B	0.096566219
Q9NZU1	Leucine-rich repeat transmembrane protein FLRT1	0.101371931
Q9NZN1	Interleukin-1 receptor accessory protein-like 1	0.109072943
Q16531	DNA damage-binding protein 1	0.110316524
Q8N4F0	BPI fold-containing family B member 2	0.110454467
Q8N474	Secreted frizzled-related protein 1	0.111577177
A0A087WSZ0	Immunoglobulin kappa variable 1D-8	0.114981624
P62826	GTP-binding nuclear protein Ran	0.121252345
Q09328	Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase A	0.124099775
P12318	Low affinity immunoglobulin gamma Fc region receptor II-a	0.125148797

O75131	Copine-3	0.126439262
O95633	Follistatin-related protein 3	0.128957124
P27918	Properdin	0.132712444
Q9NPR2	Semaphorin-4B	0.136819914
Q9H741	SREBP regulating gene protein	0.146452201
P01031	Complement C5	0.149520771
Q9H3R2	Mucin-13	0.151151811
Q16663	C-C motif chemokine 15	0.15294599
Q9P2B2	Prostaglandin F2 receptor negative regulator	0.153130767
P80108	Phosphatidylinositol-glycan-specific phospholipase D	0.157795952
Q9NY33	Dipeptidyl peptidase 3	0.159176969
P14778	Interleukin-1 receptor type 1	0.163347968
P08246	Neutrophil elastase	0.164995458
P20073	Annexin A7	0.166364097
P0DOX3	Immunoglobulin delta heavy chain	0.173663356
Q06418	Tyrosine-protein kinase receptor TYRO3	0.174941231
P47755	F-actin-capping protein subunit alpha-2	0.176527002
Q8N4L2	Type 2 phosphatidylinositol 4,5-bisphosphate 4-phosphatase	0.178875076
P02747	Complement C1q subcomponent subunit C	0.182180992
O60241	Adhesion G protein-coupled receptor B2	0.209119693
Q9UPR5	Sodium/calcium exchanger 2	0.211932903
Q9BZR6	Reticulon-4 receptor	0.2128676
Q9H0X4	Protein FAM234A	0.21899967
P40855	Peroxisomal biogenesis factor 19	0.222571922
Q9UN37	Vacuolar protein sorting-associated protein 4A	0.226859767
Q9UEF7	Klotho	0.233886725
P43631	Killer cell immunoglobulin-like receptor 2DS2	0.23834657
O00748	Cocaine esterase	0.252643631
Q7Z4F1	Low-density lipoprotein receptor-related protein 10	0.259166713

P16066	Atrial natriuretic peptide receptor 1	0.267781961
Q8NDX1	PH and SEC7 domain-containing protein 4	0.269784785
P0DME0;Q01105	Protein SETSIP;Protein SET	0.277734392
P30740	Leukocyte elastase inhibitor	0.285268076
Q9Y644	Beta-1,3-N-acetylglucosaminyltransferase radical fringe	0.318946557
P01135	Protransforming growth factor alpha	0.3215959
P01111;P01112;P01116	GTPase NRas;GTPase HRas;GTPase KRas	0.322000756
Q9Y5K6	CD2-associated protein	0.322361095
Q8WUT4	Leucine-rich repeat neuronal protein 4	0.350909606
O00182	Galectin-9	0.366095287
P61019	Ras-related protein Rab-2A	0.366664211
P47929	Galectin-7	0.370480981
P02792	Ferritin light chain	0.370647163
P09417	Dihydropteridine reductase	0.371643032
Q9BY43	Charged multivesicular body protein 4a	0.380681692
Q6XQN6	Nicotinate phosphoribosyltransferase	0.393000153
P21281	V-type proton ATPase subunit B, brain isoform	0.415911023
Q13740	CD166 antigen	0.445452459
Q9UGB7	Inositol oxygenase	0.449657337
Q96Q06	Perilipin-4	0.458962723
Q03468	DNA excision repair protein ERCC-6	0.473863799
P52823	Stanniocalcin-1	0.476185374
P20916	Myelin-associated glycoprotein	0.493422752
Q9UKR3	Kallikrein-13	0.503286553
Q5PT55	Sodium/bile acid cotransporter 5	0.514076606
P01225	Follitropin subunit beta	0.522212415
P32754	4-hydroxyphenylpyruvate dioxygenase	0.565720819
P18084	Integrin beta-5	0.569191834
P80511	Protein S100-A12	0.575517177

P20138	Myeloid cell surface antigen CD33	0.608855912
P15924	Desmoplakin	0.62381666
Q96AM1	Mas-related G-protein coupled receptor member F	0.674032927
Q99650	Oncostatin-M-specific receptor subunit beta	0.72970281
P06753	Tropomyosin alpha-3 chain	0.729825933
O75264	Small integral membrane protein 24	0.731645309
Q15691	Microtubule-associated protein RP/EB family member 1	0.739641081
P05387	60S acidic ribosomal protein P2	0.742762213
A8MUH7;Q5T2W1	Putative PDZ domain-containing protein PDZK1P1;Na(+)/H(+) exchange regulatory cofactor NHE-RF3	0.749961098
O15394	Neural cell adhesion molecule 2	0.753479117
Q9H063	Repressor of RNA polymerase III transcription MAF1 homolog	0.76890428
P06703	Protein S100-A6	0.775868169
Q70SY1	Cyclic AMP-responsive element-binding protein 3-like protein 2	0.782635384
Q02083	N-acyl ethanolamine-hydrolyzing acid amidase	0.783279891
P20810	Calpastatin	0.806397285
Q14002	Carcinoembryonic antigen-related cell adhesion molecule 7	0.864595856
P26447	Protein S100-A4	0.869624624
O14618	Copper chaperone for superoxide dismutase	0.871005035
P01877	Immunoglobulin heavy constant alpha 2	0.872769776
P48594	Serpin B4	0.893816689

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