

Optimisation of a Targeted Mass Spectrometry Assay to Investigate Nuclear Lamina Dynamics

GRAYSON, Amy Kathryn

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Optimisation of a Targeted Mass Spectrometry Assay to Investigate Nuclear Lamina Dynamics

Amy Kathryn Grayson

A thesis submitted in partial fulfilment of the requirements of
Sheffield Hallam University for the degree of Doctor of
Philosophy

October 2025

Candidate Declaration

I hereby declare that:

1. I have not been enrolled for another award of the University, or other academic or professional organisation, whilst undertaking my research degree.
2. None of the material contained in the thesis has been used in any other submission for an academic award.
3. I certify that this thesis is my own work. The use of all published or other sources of material consulted have been properly and fully acknowledged.
4. The work undertaken towards the thesis has been conducted in accordance with the SHU Principles of Integrity in Research and the SHU Research Ethics Policy, and ethics approval has been granted for all research studies in the thesis.
5. The word count of the thesis is 74,845.

Name	Amy Kathryn Grayson
Date	October 2025
Award	PhD
Research Institute	Health and Wellbeing
Director(s) of Studies	Professor David Smith

Abstract

Nuclear lamins are intermediate filament proteins within the nuclear membrane that are involved in a number of cellular processes such as providing structural support to the nucleus, transcriptional regulation and mechanosensing. They are also considered biomarkers in a number of different cancers. Here, a targeted multiple-reaction monitoring-based liquid chromatography-tandem mass spectrometry (MRM-LC-MS/MS) assay was developed and optimised to examine the protein expression of the four main mammalian nuclear lamin isoforms in biological samples of human and mouse origin. As part of this assay development, instrument optimisation was performed and the results suggested that optimisation of the collision energy (CE), fragmentor voltage (FV), and several electrospray ionisation source parameters can lead to profound improvements in assay sensitivity. This optimised MS method was then used to provide semi-quantitative analysis of the protein expression of nuclear lamin isoforms in cell lines and mouse models. Analysis of secondary cell lines was conducted to investigate the expression of nuclear lamins in cells representative of different tissue stiffnesses, in cell lines representative of different B-cell malignancies, including Burkitt lymphoma and multiple myeloma, and in a Burkitt lymphoma cell line devoid of activation-induced cytidine deaminase (AID), a key regulating enzyme involved in B-cell activation. Additionally, mouse models were also used to examine nuclear lamin expression in the lamin B1-overexpressing neurodegenerative disease autosomal dominant leukodystrophy (ADLD) and in knock-down mouse models of *LMNB1* and *ATG7*. Use of the optimised LC-MRM-MS/MS method allowed for the limited detection and semi-quantitation of different nuclear lamin isoforms in these samples. The targeted nature of the assay allows for future work to focus on the seamless translation of this method into a fully quantitative assay to be implemented in the measurement of various nuclear lamin isoforms in clinical samples.

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COVID-19 Impact Statement

During my PhD studies, a nationwide lockdown commenced after I had completed my first 17 months of lab work which resulted in the complete closure of the laboratories within the Biomolecular Sciences Research Centre. Due to my long-term health condition and treatment, the UK Government guidelines assigned me as a clinically extremely vulnerable individual, meaning I was to shield from all in-person interactions for a minimum of 12 weeks. In addition, my clinically extremely vulnerable status also meant I had to be absent from the laboratory for longer than most other member of society as the government warned of another wave of COVID-19 infection upon opening of some public spaces and lifting the lockdown restrictions. This meant I had to wait for the subsequent wave of infections to subside before I could return to the lab safely, which further extended my absence from the university.

Upon returning to the lab, the way in which we were expected to work for a further several months included restrictions on the number of days and hours each lab user could be present in the labs, and restrictions to the number of people in each lab at any given time due to social distancing requirements. As a result, conducting lab work around these restrictions was difficult for much of the following year. Given that my project required the use of cultured cell lines, the lack of access to the lab facilities significantly limited the number of cell lines I could maintain and experiments I could perform during this time. Although cell pellets of biological replicates of all cell lines in Chapter 4 were eventually acquired, no extra lab time was possible, resulting in less comprehensive experimental analysis of these samples.

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Abbreviations

2D-PAGE – 2-dimensional polyacrylamide gel electrophoresis

ABC – ammonium bicarbonate

ACN – acetonitrile

ADLD – autosomal dominant leukodystrophy

AID – activation-induced cytidine deaminase

AFM – atomic force microscopy

AJS – Agilent Jet Stream

ALL- acute lymphoblastic leukemia

AML – acute myeloid leukemia

AUC – area under the curve

BCA - bicinchoninic acid

BCR – B-cell receptor

BL – Burkitt lymphoma

BM – bone marrow

CE – collision energy

CEM – chain ejection model

CID – collision-induced dissociation

CLL – chronic lymphocytic leukemia

CNS – central nervous system

CRM – charged residue model

CSR – class switch recombination

CV – coefficient of variation

DC – direct current

DLBCL – diffuse large B cell lymphoma

DMEM - Dulbecco's Modified Eagle's Medium

DoE – design of experiments

DSB – double-strand break

DTT - 1,4-dithiotritol

EBV – Epstein-Bar virus

EDTA - Ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

ESC – embryonic stem cell

ESI – electrospray ionisation

FA – formic acid

FBS – foetal bovine serum

FDC – follicular dendritic cell

FL – follicular lymphoma

FDR – false discovery rate

FV – fragmentor voltage

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

GC – germinal centre

HL – Hodgkin lymphoma

HLB – hypotonic lysis buffer

IAA – iodoacetamide

IEM – ion evaporation model

IF – intermediate filament

Ig – immunoglobulin

IP – intraperitoneal

LAD – lamina-associated domain

LC – liquid chromatography

LCTQ – liquid chromatography coupled triple quadrupole mass spectrometer

LDS – lithium dodecyl sulphate

LINC – linker of nucleoskeleton and cytoskeleton

LIT – linear ion trap

LMNB1 – lamin B1

LOD – limit of detection

LOQ – limit of quantitation

LPS – lipopolysaccharide

MACS – magnetic activation cell sorting

MEF – mouse embryonic fibroblasts

MM – multiple myeloma

MS – mass spectrometry

MS/MS – tandem mass spectrometry

MS^e – tandem mass spectrometry

MRI – magnetic resonance imaging

MRM – multiple reaction monitoring

m/z – mass-to-charge ratio – the unit of measurement of ions

nHL – non-Hodgkin lymphoma

NLS – nuclear localisation signal

NP-CCG - 4-hydroxy-3-nitrophenyl)acetyl-conjugated chicken gamma globulin

OVA – ovalbumin

OVAT – one variable at a time

PAGE - polyacrylamide gel electrophoresis

PBMC – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PCL – plasma cell leukemia

PIC – protease inhibitor cocktail

PS – penicillin and streptomycin

PTM – post-translational modification

PVDF - polyvinylidene difluoride

QQQ – triple quadrupole – a type of mass analyser

QToF – quadrupole time of flight – a type of mass analyser

RF – radio frequency

RH – relative humidity

SD – standard deviation

SDS – sodium dodecyl sulphate

SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM – standard error of the mean

SHM – somatic hypermutation

S:N – signal-to-noise ratio

SNP – single nucleotide polymorphism

sPCL – secondary plasma cell leukemia

SPE – solid phase extraction

TBS – Tris-buffered saline

TBS-T - TBS-Tween

TEAB – triethylammonium bicarbonate

TFA – trifluoroacetic acid

TOF – time of flight

VIF – vimentin intermediate filament

WB – Western Blot

1. Introduction

1.1. Introduction to Nuclear Lamins

All cells require structural integrity for survival. This structural integrity is provided by intermediate filament (IF) proteins, which span throughout the entirety of the cell, from the nucleus through the cytoplasm and to the cell membrane, to provide structural support and mechanical strength to the organelles and the cell, and thus to the tissues in which they reside. There are several different types of IFs, ranging from type I to VI (Eriksson et al., 2009). Within the nucleus, type V IF proteins known as nuclear lamins play a major role in providing structural support to the nuclear membrane, where they polymerise to form a complex network of fibres that create a thin membrane known as the nuclear lamina. This membrane is located on the innermost surface of the inner nuclear membrane (INM) (Höger et al., 1991). Figure 1-1 shows the location of the nuclear lamina within the nucleus.

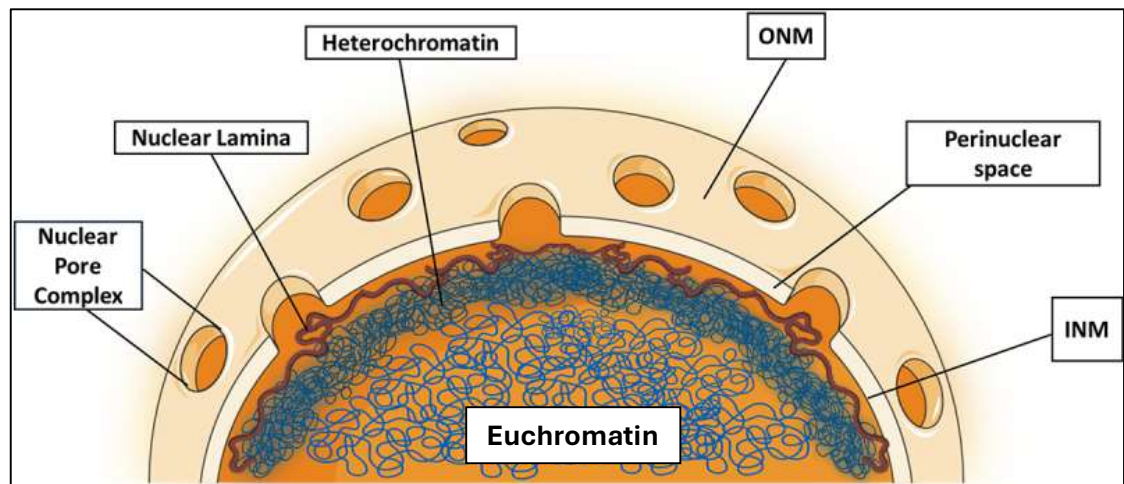


Figure 1-1: Structure of the nucleus indicating the location of the nuclear lamina. The nuclear membrane is composed of the outer nuclear membrane (ONM), the inner nuclear membrane (INM), the perinuclear space between them and the nuclear pore complexes that traverse them. The nuclear lamina sits below the INM and is closely associated with the heterochromatin, while the euchromatin is located towards the centre of the nucleus. Adapted from (Srivastava & Ehrlicher, 2024).

Nuclear lamins are found within most somatic cell nuclei (Odell & Lammerding, 2023; Turgay et al., 2017) with two main groups found in vertebrates: A-type and B-type lamins. A-type lamins are alternative splice variants of the *LMNA* gene located in humans on chromosome 1q22 and include lamin A, lamin A Δ 10, lamin C and lamin C2 while B-type lamins include lamin B1, B2 and B3. Lamin B1 and lamin B2 are transcribed from two separate genes (*LMNB1* and *LMNB2* respectively), while lamin B3 is an alternative splice variant of the *LMNB2* gene. *LMNB1* is located on human chromosome 5q23 while *LMNB2* is located on chromosome 19p13.3. Lamin C2 and lamin B3 are lesser-known isoforms due to their germline-specific expression (Alzheimer & Benavente, 1996; Furukawa & Hotta, 1993; Furukawa et al., 1994; Smith & Benavente, 1992; Vester et al., 1993) while lamin A Δ 10 is a minor *LMNA* isoform resulting from the absence of exon 10 in the mRNA sequence and the creation of a truncated form or lamin A which is linked to progeroid diseases (Machiels et al., 1996). This thesis will focus on the four main nuclear lamin isoforms found in mammalian somatic cells: lamin A, lamin B1, lamin B2, and lamin C.

Nuclear lamins are involved in a wide variety of cellular processes, including chromatin organisation, gene regulation, cell migration, and modulation of the nuclear size, shape and mechanics (Vahabikashi et al., 2022a), however they are often confused with laminins, which are a family of glycoproteins that form the basal lamina – an extracellular matrix layer that lines the basal side of epithelial and endothelial cells (Khalilgharibi & Mao, 2021). While both lamins and laminins play major roles in contributing to the formation of structural membranes, they are fundamentally different in terms of biochemistry, physiology, and structure.

All nuclear lamin genes are evolutionarily conserved (Odell & Lammerding, 2023) and their expression results in proteins with a similar overall structure, consisting of a central coiled-coil domain of four α -helical subdomains joined by unstructured linker regions. The central rod domain is flanked by an N-terminal head domain and C-terminal tail domain. The C-terminal domain contains lamin-specific motifs, including a nuclear localisation signal (NLS), an immunoglobulin (Ig) domain and a CaaX motif (Vahabikashi, et al., 2022a). The CaaX motif is absent in lamin C but present in lamin A, B1 and B2 (Gruenbaum & Foisner, 2015). Figure 1-2 below shows the general structure of a nuclear lamin monomer.

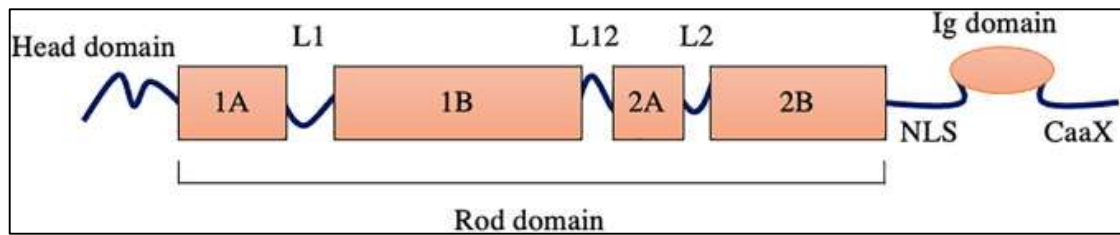


Figure 1-2: The general structure of a nuclear lamin monomer. Nuclear lamin monomers consist of a N-terminal head domain; a central rod domain containing four α -helical sub-domains (coils 1A, 1B, 2A, and 2B) which are joined by three flexible linker regions (L1, L12, and L2); and the C-terminal tail domain that includes the nuclear localization signal (NLS), the globular immunoglobulin (Ig) fold, and a CaaX motif (Vahabikashi et al., 2022a).

To efficiently create the nuclear lamina network, individual lamin monomers dimerise by forming a left-handed superhelical coiled-coil shape through interactions between the central rod domains. Two dimers then associated in a head-to-tail manner and form antiparallel interactions, creating ≈ 3.5 nm thick tetrameric protofilaments. Four protofilaments then interact to form filaments of around 10 nm in thickness, which continue to assemble into well-ordered paracrystalline arrays, resulting in the final formation of the nuclear lamina (Stuurman, et al., 1998; Turgay et al., 2017). Several microscopy techniques have been used to show that the four main nuclear lamin isoforms form individual but interacting meshworks (Shimi et al., 2015) and each isoform meshwork has a specific spatial distribution within the nucleus, with lamin B1 meshwork located closer to the inner nuclear membrane and the lamin A/C network located closer to the nucleoplasm (Nmezi et al., 2020). The thickness and density of the nuclear lamina varies between cell types, ranging from 10 – 30 nm and creates regions of sparsely and densely packed membrane (Höger et al., 1991; Kittisopikul et al., 2021). Figure 1-3 shows the assembly of nuclear lamins from monomers to filament structures.

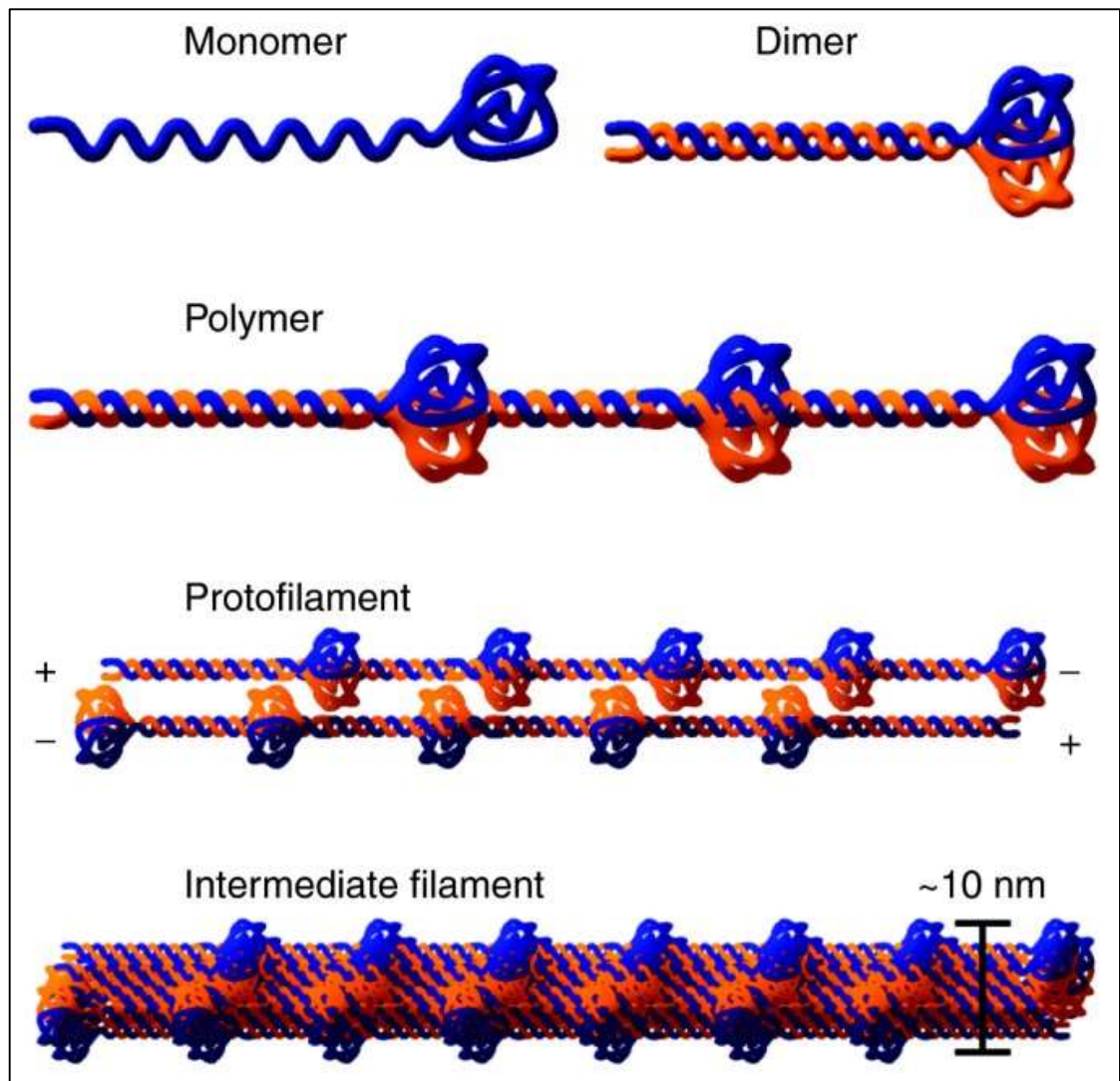


Figure 1-3: Structural assembly of nuclear lamins from monomers to intermediate filaments. Nuclear lamin monomers initially dimerise in a parallel, head-to-head manner through the parallel joining of two α -helical rod domains into a left-handed superhelical coiled-coil dimer. Polymers of lamin dimers then polymerise into tetramer protofilaments by staggered head-to-tail binding. Protofilaments are finally produced through anti-parallel association of two lamin tetramers, and four protofilaments associate laterally to form an intermediate filament around 10 nm thickness. Diagram from (Dittmer & Misteli, 2011).

Post-translational modification (PTM) of nuclear lamins at the C-terminal domain is important for the maturation and function of the different nuclear lamin isoforms. The *LMNA* gene transcribes prelamin A, which is 664 amino acids in length before being

post-translationally modified into mature lamin A. PTM of prelamin A begins by farnesylation of the cysteine residue of the CaaX motif by a farnesyltransferase followed by the removal of the last three amino acids by an aaX endopeptidase and carboxymethylation via a carboxymethyl transferase. Finally, the farnesyl group and the 15 carboxy-terminal amino acids are removed by the Zmpste24 protease, resulting in mature lamin A (Dechat et al., 2008). Conversely, lamin C is produced in its mature form from the *LMNA* gene and does not contain a CaaX motif, thus does not undergo C-terminal PTMs like lamins A, B1 and B2 (Fisher et al., 1986). Lamin B1 and lamin B2 undergo farnesylation and removal of the aaX amino acids similarly to lamin A, however these two B-type lamin isoforms remain farnesylated. This farnesyl group aids in anchoring these isoforms to the nuclear envelope (Dechat et al., 2008).

The human and murine sequences of the four main nuclear lamins are very similar when comparing the same isoform between the two organisms, with between 83-97% sequence homology. The human (*Homo sapiens*) sequences of prelamin A and mature lamin A (Uniprot accession number: P02545 and P02545-1) are only one amino acid shorter than the murine (*Mus musculus*) sequences (P48678 and P48678-1), while lamin C and lamin B1 are two amino acids longer in the mouse sequence (P48678-2 and P14733 respectively) than the human sequence (P02545-2 and P20700 respectively). Conversely, the lamin B2 amino acid sequence is 24 amino acids shorter in mice compared to humans and thus the sequence homology is the lowest in this isoform (mouse: P21619, human: Q03252). Lamins A and C are identical for the first 568 amino acids in mice and for the first 566 amino acids in humans but then differ at the C-terminal domain. Lamin A contains 98 isoform-specific amino acids at the C-terminus, which include the CaaX motif. Murine prelamin A undergoes the same PTM steps as the human protein to produce mature lamin A (Carninci et al., 2005; Fisher et al., 1986; Furukawa et al., 1994; Gerhard et al., 2004; McKeon et al., 1986; Riedel & Werner, 1989). Meanwhile, lamin C contains six isoform-specific amino acids at the C-terminal and lacks a CaaX motif. This sequence is slightly different between the human and mouse sequences. Appendix II Figure 8-3 to Figure 8-6 show the sequences homologies of all four nuclear lamin isoforms in *Homo sapiens* and *Mus musculus*.

In addition to the PTMs required for protein maturation and localisation, nuclear lamins undergo a myriad of other modifications including phosphorylation, ubiquitination, SUMOylation, acetylation and O-linked sugar modifications (Zheng et al., 2022) which contribute to their stability and function. These modifications are key to their involvement in a variety of cellular processes including but not limited to mechanosensing, B-cell activation, gene transcriptional regulation and malignant transformation (Irianto et al., 2016; Klymenko et al., 2018; Nazer, 2022; Vahabikashi et al., 2022a), which will be explored in this thesis. Interestingly, there is no data to suggest that nuclear lamins are N-glycosylated. This is theorised to be due to the absence of N-glycosylation sites, particularly in lamin A/C, as predicted by bioinformatic tools (Şener Uslupehlivan et al, 2022).

1.2. Nuclear Lamins in Mechanosensing

As part of the family of cytoskeletal proteins, one of the main roles of the nuclear lamina is to provide structural and mechanical support to the nucleus. As part of this, lamins play a significant role in the cell's ability to perceive the stiffness of the microenvironment and respond to mechanical forces exerted upon the cell. This is known as mechanosensing and is vital when performing various cellular functions such as responding to environmental forces, adhesion, growth, migration and mitosis (Bao & Suresh, 2003; Sala et al., 2024). To modulate a cell's mechanical properties and morphology, the cell nuclear and cytoskeletal components must interact with one another. This involves interactions between the nuclear lamina and the linker of nucleoskeleton and cytoskeleton (LINC) complexes, which physically couples the nucleus to the cytoskeleton (Méjat & Misteli, 2010). All four nuclear lamin isoforms are involved in interactions with the LINC complexes, however each isoform contributes in different ways to the nuclear and whole-cell mechanics (Stephens et al., 2018; Vahabikashi et al., 2022b). A-type lamins interact with both F-actin and vimentin intermediate filaments (VIFs) through LINC complexes, while B-type lamins mainly interact with VIFs through LINC complexes (Vahabikashi et al., 2022b).

There is a complex interplay between different nuclear lamin isoforms to control global cellular and nuclear stiffness (which allows the cell and nucleus to maintain their structure under applied forces) and cellular elasticity (which influences the cell's ability to restore cellular shape after deformation) (Dahl et al., 2005; González-Cruz et al., 2018a; Osmanagic-Myers et al., 2015; Swift et al., 2013; Vortmeyer-Krause et al., 2020; Wintner et al., 2020). Lamin A is thought to be the dominant isoform in regulating the mechanics of nuclear and cellular mechanotransduction (Lammerding et al., 2006; Swift et al., 2013), however both A- and B-type lamins are somewhat involved in modulating cellular stiffness, as shown by multiple studies where deletions of A-type or B-type lamins disrupt the organisation of the perinuclear cytoskeleton and cause significant softening of the cell (Broers et al., 2004; Lammerding et al., 2004; Lee et al., 2007; Vahabikashi et al., 2022b).

Depletion of lamin B1 does not cause changes in cellular stiffness, however, does result in nuclear blebbing and rupture (Lammerding et al., 2006), implying that A-type lamins have a more dominant role in regulating cellular stiffness while lamin B1 is necessary for retaining the nuclear morphology. In addition, experiments using mouse embryonic fibroblasts (MEFs) showed that both lamin A and lamin B1 are involved in regulating cortical stiffness (stiffness of the cell cortex membrane located just beneath the cell membrane) and nuclear stiffness, however lamin B2 is not (Vahabikashi et al., 2022b). This leads to theories that lamin B2 has a unique role within the cell compared to other nuclear lamin isoforms. It was also found that the loss of A-type lamins increases nuclear and cellular volume, while the loss of B-type lamins results in the opposite effect, with the nuclear and cellular volumes decreasing (Vahabikashi et al., 2022b). Lamin B2 and lamin C have been recently shown to correlate with cellular elasticity (Vortmeyer-Krause et al., 2020).

With respect to different A-type lamins, lamin C knock-down MEFs had softer cytoplasm than lamin A knock-down MEFs, and knock-down of lamin A significantly softened the nucleus, however lamin C knock-down had a marginal effect on nuclear stiffening, further supporting the role of lamin A as the main isoform involved in nuclear stiffness (Vahabikashi et al., 2022b). Furthermore, lamin B1, lamin B2 and lamin C knock-down MEFs migrated at significantly higher rates than wild-type MEFs, however lamin A knock down MEFs migrated slower (Vahabikash et al., 2022b).

Furthermore, there is evidence that lamin A/C and lamin B2 work together, and their structures are dependent on levels of lamin B1. Knockdown of lamin B1 results in a significant increase in the mesh size of lamin A/C and lamin B2 membranes and leads to the formation of nuclear blebs which are enriched in A-type lamins and deficient in B-type lamins (Shimi et al., 2008). In addition, studies in fibroblast-like cells derived from embryonic stem cells (ESC) that were deficient in different combinations of A- and B-type lamins showed that lamin A/C and B2 structures are dependent on lamin B1 for localisation and distribution within the nuclear lamina (Guo et al., 2014). A recent study showed that nuclear mechanics during migration are controlled by A-type lamins, however modulation of lamin A/C and lamin B2, but not lamin B1, controls

nuclear deformation and viscoelasticity, and lamin B2 generally followed lamin A/C-mediated effects (Vortmeyer-Krause et al., 2020).

Overall, different nuclear lamin isoforms play different roles in mechanosensing and the control of cellular and nuclear morphology, and the literature suggest that modulations in the expression of one isoform may affect the expression and localisation of others. It is thought that lamin A regulates cortical and nuclear stiffness and migration, while lamin B1 regulates nuclear morphology and stiffness, and lamins B2 and C are involved in modulating nuclear elasticity and deformation. There is also evidence that the expression of different isoforms is interconnected, where it is thought that lamin B1 expression affects the expression and localisation of lamin A/C and lamin B2, and lamin A/C and lamin B2 may follow a similar pattern of expression within cells.

As a result of the critical role of nuclear lamins in mechanosensing, researchers have explored the expression of nuclear lamins in different tissue types and cells with different stiffnesses (Lammerding et al., 2004; Lee et al., 2007; Swift et al., 2013). One of the most influential research groups studying this area of biology is that of Dennis Discher, who has published extensive primary literature and review articles documenting the importance of nuclear lamins in mechanosensing, and also their involvement in cancer metastasis (Dahl et al., 2005; Irianto et al., 2016; Pajerowski et al., 2007; Pfeifer et al., 2019; Shin et al., 2013; Shin & Discher, 2015; Swift et al., 2013). It has been reported that A-type lamins are highly expressed in stiff tissues (e.g. bone, cartilage and muscle) whereas the lamin A:lamin B ratio is low in soft tissues (such as brain or bone marrow), with B-type lamins being implicated in conveying the plasticity necessary for cells to pass through micron-scale constrictions (Denais et al., 2016; Swift et al., 2013). In addition, lamin A, more than lamin B1 and B2, varies across cells within different tissue types and its expression scales with tissue microelasticity. Lamin A is also involved in modulating the nuclear stiffness in relation to the extracellular stiffness (Swift et al., 2013). Haematopoietic progenitors have been shown to have low lamin A/C levels (Swift et al., 2013), suggesting a reliance on lamin B1 for nuclear integrity in cells of this lineage. Several studies have investigated the ratio of nuclear lamin

isoforms with respect to mechanosensing across different cell types and found that the strongest correlation to cellular stiffness is the ratio between lamin A:lamin B isoforms (González-Cruz et al., 2018a; Shin et al., 2013; Swift et al., 2013).

In consideration of this, one of the aims of this thesis is to investigate the protein expression of different nuclear lamin isoforms across cell lines with various cellular stiffnesses, to explore the hypothesis that lamin A/C expression increases with cellular stiffness while the expression of B-type lamins show a weak correlation with cell stiffness.

1.3. Nuclear Lamins and the Immune System

To understand the role of nuclear lamins in the adaptive immune response, it is important to understand the process of B-cell differentiation and maturation.

1.3.1. B-cell Differentiation and Maturation

B-cells, also known as B-lymphocytes, are a population of white blood cells whose main function is to produce a diverse range of antibodies that recognize and bind with high affinity to foreign antigens within the body. Mammalian B-cells develop from haematopoietic stem cells within the primary lymphoid tissues (human fetal liver, and fetal and adult bone marrow). Upon differentiation from immature B-cells, mature B-cells express a transmembrane protein known as B-cell receptor (BCR) on their cell surface and migrate to the secondary lymphoid tissues such as the lymph nodes, spleen, and tonsils, where B-cell activation and functional maturity occurs.

During B-cell activation, mature inactivated B-cells, known as naïve or follicular B-cells, are activated by the presentation of an antigen by T-helper cells and other antigen-presenting cells such as follicular dendritic cells (FDCs) within the secondary lymphoid tissues. Activation of B-cells by antigen exposure leads to a cascade of cellular processes with the primary aim of developing large quantities of highly specific antibodies targeting a specific antigen. This process begins with the formation of germinal centres (GCs) within the secondary lymphoid organs. GCs are specialised microanatomical structures that are transiently formed within the B-cell follicles of secondary lymphoid organs. GCs are composed of dark zones and light zones and B-cells traverse these zones at different stages of maturation and during antibody diversification processes which allow for highly specific antibodies to be produced.

Figure 1-4 shows a diagram of the GC microenvironment and includes details on the cells within the microenvironment, the differentiation stages of activated B-cells and

the cellular processes that occur at each stage, and the location of each cell type within the GC microenvironment.

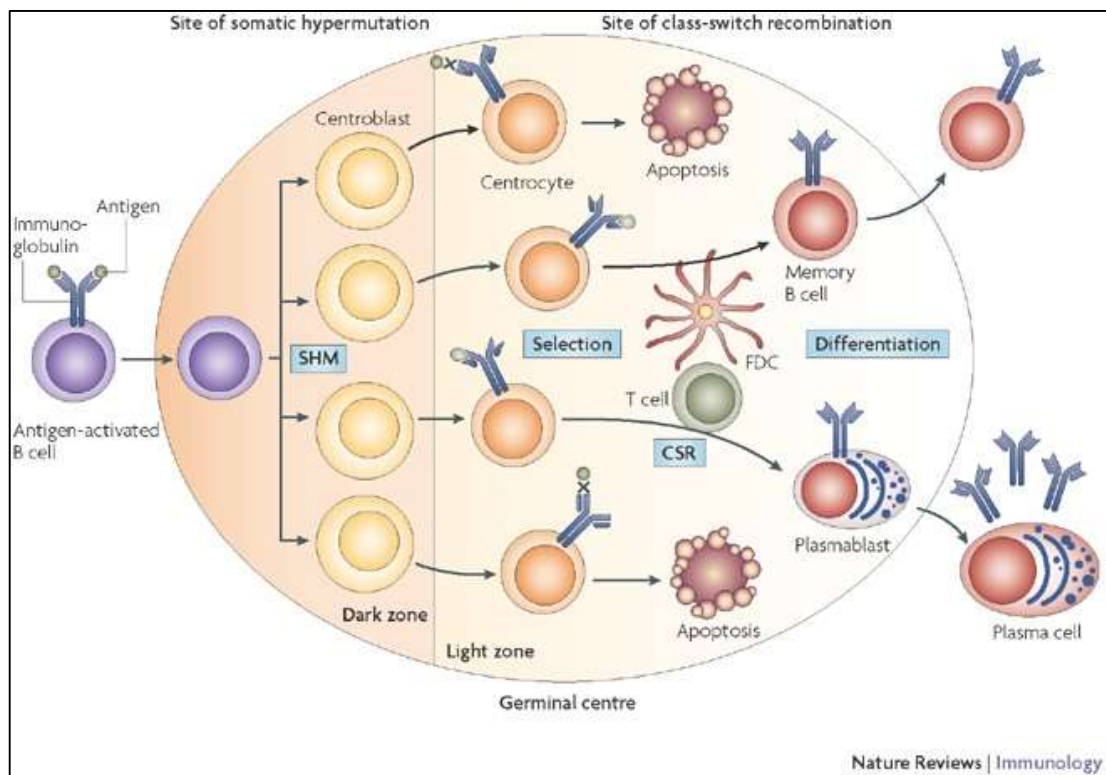


Figure 1-4: The germinal centre (GC) microenvironment. Mature inactivated B-cells are activated by the presentation of an antigen, which leads to the transient formation of germinal centres (GCs) within the secondary lymphoid organs. GCs are composed of dark zones and light zones and B-cells traverse these zones at different stages of maturation and during antibody diversification processes such as somatic hypermutation (SHM) which occurs in the dark zones, and class switch recombination, which occurs in the light zones. After successful SHM and CSR and the production of highly specific antibodies, mature B-cells exit the GCs and differentiate to plasma cells or memory B-cells. Diagram from (Klein & Dalla-Favera, 2008).

To produce a wide range of different antibody types of various specificities, B-cells undergo several antibody diversification mechanisms which allows the immune system to target a wide variety of antigens (Alt et al., 2013; Yeap & Meng, 2019). There are

three main mechanisms to antibody diversification in B-cells: V(D)J recombination, somatic hypermutation (SHM) and class-switch recombination (CSR). V(D)J recombination occurs during the early stages of B-cell development in the bone marrow, where variable (V), diversity (D) and joining (J) immunoglobulin gene segments are rearranged to generate the variable region of the antibody (Schatz & Swanson, 2011). This process uses two endonuclease enzymes, RAG1 and RAG2, to introduce double strand breaks at specific points between the V, D and J gene segments that can be repaired through the non-homologous end joining mechanism to add or remove nucleotides. This creates a diverse range of antibody gene sequences that are capable of generating a wide variety of antibodies with unique variable regions (Jung et al., 2006).

Once the primary antibody repertoire has been established, SHM and CSR events occur in activated B-cells located within the dark zones of the GCs (Hwang et al., 2015; Methot & Di Noia, 2017; Vitoria & Nussenzweig, 2022). SHM involves the introduction of point mutations within the variable regions of the Ig genes (Muramatsu et al., 2000). These mutations fine-tune the antibody-antigen binding affinity. CSR provides antibodies with a different constant region by generating deletion mutations within the switch (S) portion of the Ig heavy region of the antibody genes, resulting in the replacement of the constant region of an antibody, to produce IgG, IgA, IgE, IgD and IgM class antibodies (Hwang et al., 2015; Methot & Di Noia, 2017). Each class of antibody has a different function and contribute to the adaptive immune response in distinct ways. These functions include neutralisation, opsonisation, or the activation of the complement system (Hao et al., 2024).

Both SHM and CSR heavily involve the activity of an essential enzyme known as activation-induced cytidine deaminase (AID) which is transcribed from the *AICDA* gene (Muramatsu et al., 2000). During SHM, AID works by binding to single-stranded DNA during the gene transcription process and hydrolysing cytosine residues into uracil residues that are then recognised as thymine residues by gene transcription factors

(Chaudhuri et al., 2003; Pham et al., 2003). These C-T mutations aid the affinity maturation of B-cells through slight modifications to the variable region of antibodies, which lead to a gradual increase in antibody-antigen binding affinity over subsequent rounds of SHM. In CSR, AID induces double strand breaks within the S portion of the Ig heavy genes, which are repaired by the cell's double strand break (DSB) repair mechanism by joining two broken S regions, leading to deletion of all other Ig heavy chain sequences (Chaudhuri et al., 2003, 2007).

Cyclic re-entry of the B-cells which produce low affinity antibodies to the target antigen from the light zone to the dark zone allows for more mutations to be gained and an increased affinity for the antigen to be developed. After several rounds of SHM and CSR, the B-cells express their modified B-cell receptors on the cell surface and the receptor's affinity for the antigen is challenged by the presentation of the antigen by the FDCs and T-cells within the light zone. B-cells which produce antibodies with a higher affinity for the antigen are selected for survival and proliferation, while those producing low affinity antibodies or autoantibodies often undergo apoptosis (Victora & Nussenzweig, 2022). These surviving B-cells undergo a final stage of differentiation before leaving the GC as either memory B-cells, which remain quiescent within the secondary lymphoid organs and are activated upon re-exposure to the same target antigen or leave the GC as plasmablasts which can quickly proliferate and secrete small amounts of antibodies before terminal differentiation into non-proliferative plasma cells occurs. Plasma cells continually synthesise and secrete large amounts of antibodies with high specificity to the target antigen and can migrate to the bone marrow, become long-lived plasma cells, with the extended lifespan of plasma cells being dependent on bone marrow microenvironment signals (Klein & Dalla-Favera, 2008; Liew, 2012; Victora & Nussenzweig, 2022).

1.3.2. Nuclear Lamins during B-cell Activation

With respect to nuclear lamins in immune cells, studies have shown that the expression of different isoforms varies during haematopoiesis and immune cell

activation (Saez et al., 2020; Shin & Discher, 2015). Considering the expression of lamin A/C, cells found within the GCs such as T-cells, FDCs, activated B-cells, memory B-cells and plasmablasts have been shown to undergo rapid changes in lamin A/C protein levels during differentiation, activation and migration (Contu et al., 2018; González-Granado et al., 2014; Rocha-Perugini & González-Granado, 2014; Saez et al., 2020; Yabuki et al., 1999). Several studies have shown that inactivated T-cells express barely detectable amounts of lamin A/C (González-Granado et al., 2014; Guilly et al., 1990; Röber et al., 1990; Saez et al., 2020) while activated T-cells have an increased expression of lamin A/C (González-Granado et al., 2014; Jansen et al., 1997; Rocha-Perugini & González-Granado, 2014). Regarding B-cells, one study showed that the expression of lamin A/C was undetectable in resting human peripheral B-cells but increased upon treatment with LPS (Contu et al., 2018). Furthermore, in mice, lamin A/C expression in B-cells was undetectable by Western Blot analysis (Thiam et al., 2016). Additionally, a recent paper suggests that lamin A/C expression increased in B-cells activated by CD40 treatment (Caruso et al., 2022). An increased expression of lamin A/C in activated B-cells may be due to lamin A/C being known to increase during differentiation in several cell types (Dubik & Mai, 2020). It has also been speculated that lamin A/C plays an important role in the development of T- and B-cells, as *LMNA*^{-/-} mice display notable defects in T- and B-cell development, however, this observation has been suggested to be a knock-on effect of the lack of lamin A/C in non-immune cells (Hale et al., 2010). Considering other immune cells within the GCs, FDCs were shown to express high levels of lamin A/C (Gieseler et al., 1991, 1993; Prechtel et al., 2006; Thiam et al., 2016), however the expression of lamin A/C in memory B-cells and plasmablasts has not been investigated.

Regarding B-type lamins, their expression in haematopoietic cells is highly variable (Shin et al., 2013; Shin & Discher, 2015) however, in B-cells, it has been previously shown that lamin B1 is downregulated upon activation (Klymenko et al., 2018). In Klymenko and colleagues' study, activation of GC B-cells was associated with a decrease in lamin B1 expression and initiation of SHM. In addition, the authors suggested that the expression of lamin B1 is restored in memory B-cells (Klymenko et

al., 2018). The question of whether the nuclear lamin composition affects other aspects of B-cell maturation besides SHM was posed in Walling and Murphy's review article published in Trends in Immunology in 2021 (Walling & Murphy, 2021). They also questioned whether memory B-cell or plasma cell formation was impacted by lamin expression changes, indicating that this research topic is being questioned by scientists globally and is a topic of interest. This thesis is not the first time these questions have been probed, but this shows that scientists worldwide are intrigued by the role of nuclear lamins in B-cell development, activation, maturation, differentiation and malignant transformation. Additionally, it was recently shown that in Huntington's disease (a neurodegenerative disease in which lamin B1 has been shown to be increased in neuronal cells), analysis of patient-derived peripheral blood mononuclear cells (PBMCs) showed that only B-lymphocytes displayed an increased expression of lamin B1, and no significant change in lamin B1 was observed in monocytes or T-lymphocytes when comparing cells isolated from Huntington's disease patients to control individuals (Garcia-Forn et al., 2023). This suggests that lamin B1 may have a specific role in B-cells compared to other haematopoietic cells.

Finally, when exploring the expression of lamin B2 in immune cells, no studies have specifically investigated the expression of lamin B2 in haematopoietic cells. Considering this, the expression of lamin B2 in hepatocellular carcinoma did correlate with the amount of immune cell infiltration in one 2020 study. It was shown that an increased lamin B2 expression in hepatocellular carcinoma tumours affected the amount of immune cell infiltration, including the infiltration of B-cells, T-cells, macrophages, neutrophils and dendritic cells (Kong et al., 2020). The lack of literature exploring the expression of lamin B2 in immune cells may be due to its low levels of expression. Previous proteomic analysis has shown that lamin B2 is expressed at much lower levels than lamin B1 in haematopoietic cells (Shin et al., 2013). Difficulties in reliably quantifying this isoform in this cell type may lead to a reduced effort in its investigation. In addition, studies suggest that lamin B2 is less relevant to the structure of the nuclear envelope (Shimi et al., 2015) and very few diseases are linked to mutations in lamin B2 (Evangelisti et al., 2022). Currently, the expression level of lamin

B2 in normal tissue has not been systematically studied, however its role in disease has been recently reviewed (Li et al., 2023).

Considering this, the expression of different nuclear lamin isoforms has not been fully elucidated. There is a lack of literature surrounding the expression of lamin B2 upon B-cell activation, which may be crucial to understanding the cellular dynamics involved in B-cell activation and maturation. This thesis seeks to advance current knowledge on the changes in the protein expression of nuclear lamins during B-cell activation. This will be performed by investigating the expression of nuclear lamins in activated B-cells and in other GC cells within immunised mouse models.

1.3.3. Nuclear Lamin Expression and AID

One of the many roles of nuclear lamins is the regulation of gene transcription (Nazer, 2022), which is particularly important during B-cell activation and antibody production. Within the nucleus, chromosomes are organised into non-random territories and specific chromosomal domains are located in close proximity to the nuclear periphery and associate with the nuclear lamina (Briand & Collas, 2020). Studies have shown that lamin B1 binds to the chromatin in regions known as lamin-associated domains (LADs) and this binding has been shown to regulate gene expression (Guelen et al., 2008). These LADs are enriched with histones related to transcriptional repression such as di- and tri-methylated histone H3 lysine 9 (H3K9me2 and H3K9me3) and trimethylated histone H3 lysine 27 (H3K27me3), and genes within these domains are transcriptionally repressed (Guelen et al., 2008). In addition, cell differentiation has been correlated with repositioning of genes away from the nuclear periphery and subsequent gene expression (Noguchi et al., 2021; Peric-Hupkes & van Steensel, 2010). There are different subtypes of LADs which vary in their lamin occupancy and gene density, suggesting LADs may be heterogeneous across different cell types and stages of differentiation (Shah et al., 2023). In addition, the association of LADs with the nuclear lamina has been shown to be dynamic (Alagna et al., 2023).

Within B-cells, LADs have been shown to include Ig variable genes which are crucial during SHM (Klymenko et al., 2018). In addition, it has been previously shown that lamin B1 expression decreases upon B-cell activation. This was demonstrated through a decrease in perinuclear lamin B1 observed in GC B-cells isolated from mice spleens challenged with lipopolysaccharide and ovalbumin to illicit an immune response, and in the BL-2 Burkitt lymphoma cell line with induced SHM (Klymenko et al., 2018). This suggests that during B-cell activation, lamin B1 levels decrease and LADs containing Ig variable genes are released from binding to lamin B1, allowing these previously silenced genes to be transcribed (Klymenko et al., 2018). Furthermore, shRNA knockdown of lamin B1 in the BL-2 cell line resulted in spontaneous SHM and aberrant Ig kappa light chain gene expression on the cell surface (Klymenko et al., 2018). This suggests that expression of lamin B1 may regulate SHM and maturation of B-cells (Walling & Murphy, 2021).

The mechanism by which lamin B1 regulates SHM has been theorised to be, in part, associated with the binding of lamin B1 to the genome however, the relationship between the key regulating enzyme AID and nuclear lamins, particularly lamin B1, is of interest. Klymenko and colleagues investigated the potential role of lamin B1 as a mediator of SHM and AID expression in the Burkitt lymphoma cell line BL-2. They initially showed that BL-2 cells expressed detectible levels of AID via WB and qRT-PCR analysis whereas the BL-2 AID^{-/-} cell line had no detectible levels of AID. In addition, AID expression did not significantly change after LMNB1 RNAi in BL-2 cells, therefore the downregulation of lamin B1 was not thought to affect AID upregulation during SHM. Since lamin B1 expression does not seem to affect AID expression, it was theorised that the expression of AID during SHM may cause the downregulation of lamin B1, leading to the re-localisation of genes involved in SHM away from the nuclear periphery and subsequent expression of genes involved in SHM.

One of the aims of this thesis is to investigate if AID expression during B-cell activation is the cause of the decreased expression of lamin B1. This will be explored using the BL-2 cell line with and without AID expression.

1.4. Nuclear Lamins in Cancer

Since nuclear lamins play a significant role in maintaining nuclear shape and regulating gene expression (Prokocimer et al., 2009), their expression in cancers has been a topic of interest for many years. The mechanisms by which the aberrant expression of nuclear lamins promotes cancer development and progression include changes in gene expression and chromatin organisation through interactions with LADs, and nuclear deformability and cell motility leading to metastasis and extravasation (Denais & Lammerding, 2014). Aberrant expression of both A- and B-type lamins have been noted in many solid tumours and haematopoietic cancers. Here, the expression of nuclear lamins in cancers will be discussed.

1.4.1. Nuclear Lamins in Solid Tumours

1.4.1.1. Lamin A/C

The expression of lamin A/C has been shown to be downregulated in cervical cancer (Capo-chichi et al., 2016; Moss et al., 1999), uterine cancer (Moss et al., 1999; Wang et al., 2019a), gastric cancer (Moss et al., 1999; Wu et al., 2009), osteosarcoma (Evangelisti et al., 2020; Urciuoli et al., 2020), adrenal tumours (Wang et al., 2019a) and some skin cancers (Oguchi et al., 2002). Decreases in lamin A/C are associated with metastasis in skin cancer (Fracchia et al., 2020), breast cancer (Alhudiri et al., 2019; Galal et al., 2021a; Ishii et al., 2020), colon cancer (Belt et al., 2011), endometrial carcinoma (Cicchillitti et al., 2016), lung cancer (Broers et al., 1993; Kaspi et al., 2017) and prostate cancer (Saarinen et al., 2015; Zuo et al., 2018). On the other hand, lamin A/C has been shown to be upregulated in malignant mesothelioma (Giusti et al., 2014).

Conflicting results for the expression of lamin A/C in cancers have been obtained for bladder cancer (Setti Boubaker et al., 2021; Wang et al., 2019a), kidney cancer (Fracchia et al., 2020; Wang et al., 2019a), oesophageal cancer (Moss et al., 1999; Zhao et al., 2007), hepatocellular carcinomas (Fracchia et al., 2020; Hudson et al., 2007; Liu et al., 2020), ovarian cancer (Bengtsson et al., 2007; Capo-chichi et al., 2011a; Gong et al., 2015; Hudson et al., 2007; Wang et al., 2009, 2019a), colorectal cancer (Belt et al.,

2011; Moss et al., 1999; Roth et al., 2010; Willis et al., 2008) and glioblastoma (Gatti et al., 2021; Gupta et al., 2024). In some cancers, the specific loss of lamin A but not lamin C correlated with malignancy (Aljada et al., 2016; Gong et al., 2015).

The expression of lamin A/C in malignancy is not always simple, as in some cancers, the expression of lamin A/C has been shown to change throughout the course of the disease progression. In prostate cancer, lamin A/C has been shown to be downregulated compared to non-malignant cells (Fracchia et al., 2020; Khan et al., 2018; Kong et al., 2012; Reis-Sobreiro et al., 2018; Skvortsov et al., 2011; Zuo et al., 2018). However, increased lamin A/C expression was correlated with tumour stage and grade (Helfand et al., 2012; Kong et al., 2012; Skvortsov et al., 2011; Zuo et al., 2018), suggesting an initial decrease in lamin A/C upon malignant transformation but an increase in expression of lamin A/C upon progression of the disease.

Additionally, the expression of lamin A/C has been shown to differ between tumour subtypes, as has been observed in testicular germ cell tumours (Machiels et al., 1997; Wang et al., 2019a), breast cancer, (Antmen et al., 2019; Capo-chichi et al., 2011b; Ishii et al., 2020; Matsumoto et al., 2015; Roncato et al., 2021; Setijono et al., 2018; Wazir et al., 2013) lung cancer (Broers et al., 1993; Giusti et al., 2014; Hudson et al., 2007; Jia et al., 2019; Kaspi et al., 2017; Kaufmann et al., 1991; Moss et al., 1999; Wang et al., 2019b) and skin cancer (Fracchia et al., 2020; Oguchi et al., 2002; Roncato et al., 2021; Tilli et al., 2003).

Metastasis is one of the leading hallmarks of cancer (Hanahan, 2022), and since nuclear lamins are involved in cellular and nuclear stiffness (see Section 1.2), the influence of A-type lamins on metastasis has been heavily debated. Some studies report the lack of lamin A/C positively influencing the cell's invasive potential (Friedl, et al., 2011) while other studies reported that live metastatic cancer cells were more than 70% softer than benign cells (Cross et al., 2007), with the stiffness of cancerous cells being inversely proportional to their migration and invasive potential (Swaminathan et al., 2011). On the other hand, it has also been reported that cancer cells deficient in A-type lamins experience increased apoptosis due to shear stress and are not as capable

at surviving within the circulatory system during metastasis (Mitchell et al., 2015). Furthermore, depletion of A-type lamins has been shown to hinder cellular migration due to disruptions within the nucleocytoskeletal pathway and inhibition of the formation of focal adhesions that promote cellular adhesion and migration (Chancellor et al., 2010). This suggests the expression of lamin A/C in malignant cells is a double-edged sword, with overexpression of A-type lamins leading to reduced cellular plasticity and invasive potential, while reductions in A-type lamins potentially limit cellular survival within the circulation (Harada et al., 2014).

1.4.1.2. Lamin B1

Analysis of the current literature gives mixed results pertaining to the expression of lamin B1 in solid tumours. A consensus of downregulation of lamin B1 leading to malignancy is apparent in some cancer types. In colorectal cancer, it is generally accepted that lamin B1 mRNA and protein expression is downregulated (Alfonso et al., 2008; Izdebska et al., 2018; Marshall et al., 2010). In gastric cancers, downregulation of lamin B1 is common and associated with tumour differentiation, stage and poor prognosis (Moss et al., 1999; Tan et al., 2016; Yu et al., 2020) while in uterine cancer, decreases in lamin B1 were associated with malignant transformation (Moss et al., 1999).

In contrast, there is overwhelmingly strong evidence that upregulation of lamin B1 is found in hepatocellular carcinoma and several studies have shown this leads to increased tumour size and more advanced tumour stage (Abdelghany et al., 2018; Fracchia et al., 2020; Gu et al., 2020; Idriss et al., 2019; Lim et al., 2002; Sun et al., 2010). In addition, lamin B1 is transcriptionally upregulated in kidney cancer and is associated with poor prognosis (Fracchia et al., 2020; Radspieler et al., 2019). Upregulation of lamin B1 has also been observed in multiple studies in ovarian cancer (Bengtsson et al., 2007; Capo-chichi et al., 2011a; Sengupta et al., 2023), retinoblastoma (Danda et al., 2018; Naru et al., 2016), pancreatic cancer (Li et al., 2013; Moss et al., 1999), glioblastoma (Ruggieri et al., 2019), osteosarcoma (Urciuoli et al., 2020) and thyroid cancer (Wang et al., 2015).

Similarly to the observations of lamin A/C expression, there are controversies within the literature pertaining to the effects of lamin B1 expression in malignant transformation and disease progression within the same tissue type. In breast cancer, lamin B1 upregulation has been documented at a transcriptional and translational level and has been associated with increased tumour grade and cell migration (Capo-chichi et al., 2011b; Fracchia et al., 2020; Saleh et al., 2021; Setijono et al., 2018). Conversely, the expression of lamin B1 was shown to be reduced in several studies and, in some cases, was linked to poor prognosis and metastasis (Kurpińska et al., 2019; Moss et al., 1999; Wazir et al., 2013). To complicate matters further, the expression of lamin B1 was found to be a distinguishable factor in different subtypes of breast cancer in one study, with a lower expression of lamin B1 found in luminal A and HER2-positive breast cancers while a higher expression of lamin B1 was found in luminal B and triple negative breast cancer subtypes (Cotarelo et al., 2016).

Furthermore, studies in small cell lung cancer and Lewis lung carcinoma suggest that lamin B1 is downregulated (Jia et al., 2019), while examination of non-small cell lung cancer samples have given mixed results (Jia et al., 2019; Yi et al., 2020). With respect to prostate cancer, a majority of publications have identified the overexpression of lamin B1 in cancer (Coradeghini et al., 2006; Fracchia et al., 2020; Luo et al., 2021; Saarinen et al., 2015) however, several studies have noted the opposite result, with downregulation of lamin B1 observed in prostate malignancies (Helfand et al., 2012; Khan et al., 2018). Limited studies on skin cancer (Fracchia et al., 2020; Oguchi et al., 2002; Tilli et al., 2003) and cervical cancer (Moss et al., 1999; Yang et al., 2019) have also been published and so far, no consensus on the expression of lamin B1 in these cancer types have been reached.

1.4.1.3. Lamin B2

There is a trend that most literature associates the upregulation of lamin B2 with malignant transformation and progression, particularly in hepatocellular carcinoma (Gu et al., 2020; Kong et al., 2020; Mu et al., 2021; Zheng & Yu, 2021), colorectal cancer (Dong et al., 2021; Kuga et al., 2014), malignant mesothelioma (Giusti et al., 2014), lung

cancer (Broers et al., 1993; Ma et al., 2017; Misono et al., 2018; Zhang et al., 2020a), ovarian cancer (Bengtsson et al., 2007), bladder cancer (Ji et al., 2022), oesophageal cancer (Su et al., 2020) and skin cancer (Oguchi et al., 2002). In prostate cancer, a study by Saarinen and colleagues found a statistically significant increase in the protein expression of lamin B2 in cancerous tissue compared to normal prostate tissue however, low lamin B2 expression was significantly associated with lymph node metastasis (Saarinen et al., 2015). This may suggest that downregulation of lamin B2 may lead to less mechanical stiffness around the nucleus, allowing for extravasation of cancerous cells and metastasis. However, upregulation of lamin B2 was associated with metastasis in colorectal cancer, hepatocellular carcinoma and non-small cell lung cancer (Dong et al., 2021; Kong et al., 2020; Zhang et al., 2020a).

Lamin B2 generally tends to promote malignant transformation and disease progression by promoting cellular proliferation through several mechanisms, including but not limited to the upregulation of the methylation of histones (Zhang et al., 2020a), promoting the activity of MCM7 involved in DNA replication (Ma et al., 2017), activation of CDCA3 involved in the initiation and termination of mitosis (Ji et al., 2022) and silencing of p21 tumour suppressor protein (Dong et al., 2021). Furthermore, upregulation of lamin B2 has been associated with increased infiltration of immune cells in hepatocellular carcinoma (Kong et al., 2020).

1.4.2. Nuclear Lamins in Haematological Malignancies

Investigation into the published literature surrounding the expression of nuclear lamins in solid tumours is extensive, however less is known about the expression of nuclear lamins in transformed cells of the circulatory systems.

1.4.2.1. Lamin A/C

To discuss lamin expression in malignant cells, it is important to consider their expression in healthy cells. Dynamic changes in the protein expression of lamin A/C have been observed in immune cells during differentiation, activation and migration

(Contu et al., 2018; González-Granado et al., 2014; Rocha-Perugini & González-Granado, 2014; Yabuki et al., 1999). Earlier studies show that lamin A was below the limit of detection in terminally differentiated myeloid and lymphoid cells (Gerner & Sauermann, 1999), however other studies have shown that lamin A expression is only expressed during the later stages of lymphoid development (Guilly et al., 1990; Röber et al., 1989). Some immune cells such as macrophages are known to increase their expression of lamin A/C during differentiation (Gieseler et al., 1991; Peters et al., 1991; Saez et al., 2020). This is expected as lamin A/C expression is thought to be a marker of differentiation (Constantinescu et al., 2006; Röber et al., 1989). In contrast, neutrophil differentiation is associated with downregulation of lamin A/C (Manley et al., 2018; Olins et al., 2001, 2009; Saez et al., 2020).

Studies of haematological malignancies showed that lamin A and C protein expression were downregulated in acute lymphoblastic leukaemia (ALL) compared to peripheral blood T-lymphocytes isolated from healthy donors (Guilly et al., 1990; Stadelmann et al., 1990). In addition, lamin A/C has been shown to be downregulated in acute myeloid leukaemia (AML) (Wang et al., 2019a). In the case of lymphomas, lamin A/C protein expression has been found to be upregulated in T-cell lymphoma (Tsai et al., 2020) and Hodgkin lymphoma (Contu et al., 2018; Jansen et al., 1997). In the case of non-Hodgkin lymphoma, Stadelmann and colleagues note the presence of lamin A/C in peripheral blood lymphocytes upon activation, but an absence in lamin A/C expression in non-Hodgkin lymphoma cell lines, as determined by Northern Blot analysis (Stadelmann et al., 1990). This suggests, as with solid tumours, the subtype of disease is important when discussing the effect of lamin A/C on malignant transformation.

In addition, extensive knowledge has been documented about the expression of lamin A/C in T-cells and their contribution to T-cell activation (Rocha-Perugini & González-Granado, 2014), but little is known about the expression of lamin A/C in B-cells (Saez et al., 2020). In LMNA^{-/-} mice, the lack of lamin A/C was shown to result in striking defects in T- and B-cell development, however these defects were attributed to the indirect effects of the loss of lamin A/C in non-immune cells (Hale et al., 2010). With respect to B-cells, lamin A/C expression was shown to be undetectable in resting B-lymphocytes

from human blood (Contu et al., 2018; Paulin-Levasseur et al., 1989; Röber et al., 1989) and mice (Thiam et al., 2016), however lamin A/C expression increased upon treatment with lipopolysaccharide (Contu et al., 2018). Furthermore, lamin A/C is present in some B-cell lymphomas (Contu et al., 2018; Jansen et al., 1997; Saez et al., 2020) and it has been recently shown that different subtypes of diffuse large B-cell lymphoma (DLBCL), a very aggressive B-cell malignancy, showed different migration behaviours which correlated to different expression levels of lamin A/C (Sherif et al., 2024).

1.4.2.2. Lamin B1

B-type lamins are present within haematopoietic progenitor cells (Gerner & Sauermann, 1999; Guilly et al., 1990; Rober et al., 1990) while in leukaemias, the downregulation of lamin B1 has been shown to lead to poor prognosis in chronic myeloid leukaemia (Klymenko et al., 2018). The expression of lamin B1 has also been shown to be downregulated at a transcriptional and translational level in AML (Klymenko et al., 2018; Reilly et al., 2022). Conversely, an earlier study published in 1990 found that the mRNA expression of B-type lamins was upregulated in ALL, whereas B-type lamins were absent in normal resting peripheral blood samples, as determined by Northern Blotting however, this study did not differentiate between lamin B1 and lamin B2 (Stadelmann et al., 1990). With respect to lymphomas, primary lymphoid tumours were shown to have a decreased expression of lamin B1 compared to normal reactive lymph node samples (Klymenko et al., 2018). The downregulation of lamin B1 has also been shown correlated with disease progression in follicular lymphoma (FL) (Klymenko et al., 2018).

1.4.2.3. Lamin B2

The expression of lamin B2 has not been extensively explored in haematological malignancies. One publication by Meier and colleagues published in the late 1990's found two protein bands using a lamin B2 antibody with Western Blotting (WB) techniques (Meier et al., 1997). The authors denote the two bands as lamin B2 and lamin B2A. It was found that the expression of lamin B2A, the higher molecular weight protein, was greater in ALL and AML samples compared to peripheral blood

mononuclear cells (PBMC). No other known studies have identified the presence of a second lamin B2 protein band using WB techniques in both transformed and non-transformed cells, which may indicate that both lamin B1 and lamin B2 were identified through the use of non-specific lamin B-type antibodies in Meier's study, as both proteins are similar in molecular weight at 66 kDa for lamin B1 and 68 kDa for lamin B2. If this is true, the higher molecular weight protein identified in Meier's experiments would be representative of lamin B2, and therefore the results would show a higher expression of lamin B2 protein in ALL and AML samples compared to PMBCs. This is supported by the increase in the mRNA expression of B-type lamins found in ALL samples compared to resting PBMCs (Stadelmann et al., 1990).

Conversely, a modified or mutated form of lamin B2 may have been present within the acute leukaemia samples examined. In Meier's study, the lamin B2A band was of a higher molecular weight than the presumed native lamin B2 band, which may indicate a post-translationally modified form of lamin B2. Two separate lamin B2-specific antibodies were used in the study, which were developed by hybridoma techniques, and results obtained from both separate antibodies indicated the presence of two separate protein bands via immunoblotting methods. The use of multiple antibodies suggests that the presence of a modified form of lamin B2 within the samples is not inconceivable, as the likelihood of both antibodies being non-specific is low but not impossible. To investigate this possibility further, the protein bands could have been excised from an SDS-PAGE gel and sequenced to identify any post-translational modifications.

1.4.3. Nuclear Lamins in Malignancies of Activated B-cells

One of the main knowledge gaps in cancer research is the role of nuclear lamins in the pathogenesis of B-cell malignancies. There are several types B-cell malignancies which are classified dependent on the level of maturation of the B-cell upon transformation (Alaggio et al., 2022). This thesis will focus on Burkitt lymphoma (BL) and multiple myeloma (MM), which are discussed below.

Lymphoma is the collective term for cancers arising from lymphocytes and these cancers are classified into B-cell or T-cell lymphoma and further classified into Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (nHL) dependent on the presence of specific cells known as Reed-Sternberg cells, found only in HL (Aggarwal & Limaïem, 2025). BL is a rare, aggressive form of nHL caused by the cancerous transformation of activated B-cells within the GCs (López et al., 2022). Conversely, MM is characterised by the abnormal proliferation of plasma cell within the bone marrow and the production of monoclonal Igs from these malignant plasma cells (Monteith et al., 2023) (see Section 1.3.1 for more information on B-cell maturation stages). MM is categorised into different subtypes based on the type of light and heavy chain construction of the overproduced Igs (see Section 1.3.1 for more information). The most common subtype of MM is IgG-heavy chain and kappa light chain producing MM (Hemminki et al., 2021; Nandra et al., 2022).

Previous literature has shown that examination of nuclear lamin expression in MM is limited to their role as senescence and apoptotic markers when studying the efficacy of therapeutic agents (Guo et al., 2024; Joseph et al., 2003; Qiang et al., 2018, 2016). In terms of the influence of lamin expression on MM disease pathology, a thorough literature review found that no previous studies have investigated the protein expression of nuclear lamin isoforms in terms of their use as cancer biomarkers. With respect to BL, studies have shown an increase in lamin A/C in B-cells upon infection with the Epstein-Bar virus (EBV), with EBV infection being a common cause of B-cell transformation to BL (Banati et al., 2017).

In context to other B-cell malignancies, it has been previously shown that lamin B1 expression decreases upon B-cell activation (Klymenko et al., 2018) and decreases in lamin B1 were shown to correlate with poor progression-free survival and overall survival in chronic lymphocytic leukemia (CLL) patients. In addition, the downregulation of lamin B1 correlated with disease progression of FL to DLBCL (Klymenko et al., 2018). Since CLL and FL are malignant transformations of antigen-activated B-cells (Damle et

al., 2002; Jaffe, 2013; Klein et al., 2001), the authors theorized that the reduction of lamin B1 expression during B-cell activation may be a hallmark of activated B-cell cancers. Therefore, this thesis aims test this theory by comparing the expression of nuclear lamin isoforms, particularly lamin B1, in BL and MM.

1.5. Nuclear Lamins in Autophagy

Autophagy is an evolutionarily conserved cellular mechanism in which degradation of long-lived, dysfunctional or unnecessary components of the cell and other substances within the cytoplasm occurs in a controlled manner. This process allows for the recycling of cellular components and is essential for maintaining cellular homeostasis and operates at a continuous baseline level to ensure the disposal of cellular components which may accumulate as a result of normal cellular functioning (Klionsky & Emr, 2000). Macroautophagy is the main type of autophagy used to recycle cytoplasmic components of the cell such as damaged organelles and proteins. The initial step in macroautophagy is isolation of these components from the rest of the cell by encapsulating them within a double-membrane vesicle known as an autophagosome. Once the components are encapsulated, the autophagosome fuses with a lysosome, an organelle which contains hydrolytic enzymes known as lysozymes, to create an autolysosome. The lysozymes help degrade the contents of the autolysosome. During the process of macroautophagy, autophagy genes (denoted by the acronym ATG (Klionsky et al., 2003)), are activated.

Atg7 is an autophagy-related protein transcribed from the *ATG7* gene. This protein is crucial for several autophagy-related processes (Collier et al., 2021). Knock-out of Atg7 is not embryonically lethal, however *ATG7*^{-/-} mice die shortly after birth (Komatsu et al., 2005). Defects in autophagy and aberrant Atg7 expression have also been linked to progeroid laminopathy diseases such as Hutchinson-Gilford progeria syndrome (HGPS) in which a de-novo mutation in the *LMNA* gene leads to the accumulation of prelamin A, and the researchers theorised that prelamin A induces premature ageing through impairments in the autophagy process (Pan et al., 2020). Additionally, Osanai and colleagues found that autophagy was impaired in transgenic mouse models of chronic intracellular acidosis due to decreases in Atg7, which coincided with an aged phenotype including hair greying, reduced organ mass and decreases in lamin A/C (Osanai et al., 2018).

With regards nuclear lamins, several previous studies have demonstrated that autophagy is a mechanism of lamin B1 nuclear disassembly (Dou et al., 2015a, 2015b; Lenain et al., 2015). Considering Klymenko and colleagues' findings that decreases in lamin B1 are associated with B-cell activation (Klymenko et al., 2018), it was theorised that autophagy may be involved in the removal of lamin B1 during B-cell activation. This thesis aims to explore the relationship between Atg7 expression and the protein expression of nuclear lamins, and how this may change upon B-cell activation. This will be achieved using an *ATG7*-knockout mouse model.

1.6. Aberrant Expression of Lamin B1

Since nuclear lamins are involved in a wide range of cellular processes, their aberrant expression can have significant consequences on nuclear morphology, gene expression and malignant transformation, as discussed previously. This section will explore the roles of lamin B1 and consequences of *LMNB1* knock-out and discuss the upregulation of lamin B1 through gene duplication in neurodegenerative disease autosomal dominant leukodystrophy (ADLD).

1.6.1. Knock-Down Studies of Lamin B1

Several models of lamin B1 knock-down have been developed and utilised to examine the role of lamin B1 in development and differentiation. These studies have shown that knock-down of lamin B1 is not embryonically lethal, however mice die soon after birth due to developmental defects within the central nervous system (CNS) and cardiac and skeletal muscles (Coffinier et al., 2011; Sullivan et al., 1999; Vergnes et al., 2004).

Several knock-down studies in murine models have confirmed that B-type lamins play an important role in development and differentiation, especially within the CNS (Coffinier et al., 2011; Evangelisti et al., 2022; Giacomini et al., 2016; bin Imtiaz et al., 2021; Kim, 2023; Kim et al., 2011; Kim et al., 2013; Koufi et al., 2023; Mahajani et al., 2017; Shimi et al., 2011; Vergnes et al., 2004). In addition, loss of lamin B1 can affect neural and epicardial cell migration (Tran et al., 2016; Young et al., 2012) and supporting studies have linked this irregular migration pattern as a result of lamin B1 loss to nuclear membrane ruptures, accompanied by DNA damage and cell death (Chen et al., 2019). This corroborates previous literature surrounding the role of lamin B1 in mechanosensing and nuclear contractility (see Section 1.2 for more information).

Unsurprisingly, as a result of the B-type lamins' critical role in CNS development, mutations in *LMNB1* and *LMNB2* genes have serious biological consequences and are linked to several CNS diseases. These include ADLD, ataxia telangiectasia, microcephaly, neural tube closure defects and Alzheimer's disease (Evangelisti et al., 2022). However,

despite the confirmed negative consequences of lamin B1 loss on the CNS and cardiac and skeletal muscle development, studies on other cell types such as keratinocytes and hepatocytes seem to suggest that an absence of lamin B1 does not affect cellular proliferation or tissue development (Yang et al., 2011a, 2011b). Interestingly, reduction of lamin B1 is found in aging human skin (Dreesen et al., 2013) and in the brains of Alzheimer's disease patients (Frost et al., 2016) but the cause of this reduction and its impact on the function of specific organs is not fully understood.

Previous studies have suggested a partial compensatory mechanism between the two B-type lamins. Lee and colleagues developed reciprocal *LMNB1 LMNB2* knock-in mice that, despite having a very short lifespan of a few hours, demonstrated a potential compensatory mechanism to increase lamin B2 expression in the complete absence of lamin B1, but this observation was not seen in mice absent of lamin B2 (Lee et al., 2014). In addition, the compensatory mechanism did not prevent developmental defects associated with the loss of either B-type lamin isoform which confirms that both proteins play unique and important roles in brain development. More evidence to suggest some type of compensatory role between lamin B1 and lamin B2 was shown in Jung and colleague's publication, confirming that the farnesyl lipid anchor is essential for the function of lamin B1 but not for lamin B2, as shown by knock-in mice expressing a non-farnesylated version of lamin B1 displaying neuronal migration defects and abnormalities however, knock-in mice expressing a non-farnesylated version of lamin B2 did not show any signs of pathology (Jung et al., 2013). Furthermore, partial loss of B-type lamins in *Lmn1^{+/-} Lmn2^{+/-}* mice were shown to be free of developmental defects, survived development, and were even fertile (Kim et al., 2011; Lee et al., 2014). This implies a threshold of B-type lamin expression is necessary for survival. In confirmation of the findings of Lee and colleagues (Lee et al., 2014), Chen and colleagues also found that overexpression of lamin B2 in lamin B1 knockout mouse models does not fully compensate for the loss of lamin B1 as overexpression of lamin B2 did not eliminate nuclear membrane ruptures in lamin B1 knockout mouse models (Chen et al., 2019).

With respect to the immune system, lamin B1 is thought to play a role in thymus development and aging. Yue and colleagues (Yue et al., 2019) showed that of the three nuclear lamin isoforms, only lamin B1 is required in thymic epithelial cells, and is involved in the development and maintenance of different biological compartments of the thymus. In addition, downregulation of lamin B1 has been shown to occur upon activation of B-cells and initiation of SHM (Klymenko et al., 2018). This downregulation was theorised to be associated with the release of SHM-specific genes from their binding with nuclear lamins, and subsequent gene transcription. Several other studies have reported that B-type lamins are important for chromatin organisation and gene expression (Belmont et al., 1993; Gigante et al., 2017; Tang et al., 2008). As a result, this thesis aimed to investigate the expression of each nuclear lamin isoform in *LMNB1* knockout mouse models during B-cell activation by comparing protein expression in activated B-cells and other cells within the GCs.

1.6.2. Autosomal Dominant Leukodystrophy (ADLD)

Autosomal dominant leukodystrophy (ADLD, OMIM #169500) is a rare, slowly progressive, fatal neurological disorder characterised by symmetrical widespread myelin loss within the CNS. ADLD is caused by lamin B1 overexpression which is most commonly caused by *LMNB1* gene duplication (Padiath, 2019; Padiath et al., 2006), however lamin B1 overexpression in the absence of *LMNB1* gene duplication or other mutation has also been documented (Giorgio et al., 2014; Mezaki et al., 2018; Raininko et al., 2021). All documented mechanisms of lamin B1 overexpression led to increased levels of the wild-type protein, and as such, there is no abnormal gene product related to ADLD.

A number of clinical studies have assessed the symptoms present in ADLD patients (Brunetti et al., 2014; Brussino et al., 2010; Chen et al., 2022; Coffeen et al., 2000; Dai et al., 2017; Eldridge et al., 1984; Finnsson et al., 2015; Marklund et al., 2006; Meijer et al., 2008; Melberg et al., 2006; Mihashi et al., 2024; Molloy et al., 2012; Potic et al., 2013; Quattrocchio et al., 1997; Sandoval-Rodríguez et al., 2017; Dos Santos et al., 2012;

Schuster et al., 2011; Sundblom et al., 2009; Terlizzi et al., 2016; Zhang et al., 2019). Collectively, these studies report a myriad of autonomic, motor, and neurological symptoms present with ADLD. Autonomic symptoms include bladder, bowel and erectile dysfunction, and postural hypotension while motor symptoms are usually slow progressive and involve the legs, arms and face (Finnsson et al., 2015).

Neurological symptoms usually occur after the onset of autonomic symptoms, occur later in the disease process and may include ataxia (a group of conditions that affects co-ordination, balance and speech), heat intolerance and abnormal sweating, personality changes, depression and loss of consciousness. Other neurological symptoms and cognitive impairments may occur as a result of damage or dysfunction of the brain or spinal cord and may include weakness, twitching and spastic tendencies. Later disease symptoms may include difficulty swallowing, difficulty or loss of articulation and mild dementia (Brussino et al., 2009; Dos Santos et al., 2012; Finnsson et al., 2015; Sandoval-Rodríguez et al., 2017; Schwankhaus et al., 1994). Worsening of neurological symptoms has been documented during periods of infections or fever, which is reversible when patients recover (Chen et al., 2022; Finnsson et al., 2015; Schuster et al., 2011). With respect to infection, dry skin and recurrent urinary tract infections are common in ADLD patients (Finnsson et al., 2015; Schuster et al., 2011).

Few studies have found deviations to this pattern of disease onset, being initiation of autonomic symptoms in early disease which precedes or occurs together with motor and neurological symptoms (Finnsson et al., 2015; Mezaki et al., 2018; Potic et al., 2013; Zhang et al., 2019). Differences in the genetic causes of lamin B1 overexpression in ADLD may lead to differences in symptom presentation, as Mezaki and colleagues showed that patients with genomic deletions upstream of *LMNB1* exhibited relatively earlier symptom onset, more significant cognitive impairment and fewer autonomic symptoms than patients with *LMNB1* gene duplication (Mezaki et al., 2018). In contrast to other hereditary leukodystrophies, which have an early onset and are autosomal recessive or X-linked, ADLD is autosomal dominant and disease onset usually occurs

when the patient is in their forties or fifties (Schiffmann & van der Knaap, 2004) however, the most recent clinical publication of autosomal leukodystrophy related to lamin B1 overexpression suggests that not all cases develop after 40 years of age and can even develop in patients in their twenties (Mihashi et al., 2024). Patients with *LMNB1* duplication are usually asymptomatic in their twenties, with no brain atrophy or white matter alterations (Terlizzi et al., 2016), however MRI abnormalities have been shown to precede symptoms by 10 years in ADLD (Sundblom et al., 2009).

The exact prevalence of ADLD is unknown, however the literature states that over 30 families with more than 70 individuals have been reported. ADLD is commonly misdiagnosed as other neurodegenerative disorders such as Alzheimer's-type dementia, multiple sclerosis and neuronal intranuclear inclusion disease (NIID) due to the overlap in clinical findings and involvement of the CNS white matter (Beary & Conway, 2013; Chen et al., 2022; Coffeen et al., 2000; Eldridge et al., 1984; Meijer et al., 2008; Sandoval-Rodríguez et al., 2017; Schwankhaus et al., 1994). Since the ADLD diagnosis is not always straightforward, and evidence suggests cases may be misdiagnosed with other neurodegenerative disorders, there may be an underdiagnosis of ADLD patients and prevalence may be much more than is reported in the literature. Individuals with ADLD usually survive for 10-20 years after the onset of symptoms due to the slow progressive nature of motor symptoms (McKusick & Kniffin, 2021).

Currently, there is no disease-specific treatment option available for ADLD, and treatment focusses on the management of symptoms to improve quality of life. Research into treatment options have investigated the possibility of allele-specific silencing through RNA interference (RNAi) to downregulate mutant allele transcripts in patients with a *LMNB1* single nucleotide polymorphism (SNP) in one allele (Giorgio et al., 2019). While this approach may be a therapeutic option for some patients, different approaches may be necessary for patients lacking the *LMNB1* SNP. Furthermore,

studies into small molecules able of suppressing the levels of lamin B1 protein are also being explored (Nmezi et al., 2020).

ADLD is characterized by the widespread yet symmetrical demyelination of the CNS with progressive loss of white matter within the brain and spinal cord, and presence of vacuoles within the myelin (Coffeen et al., 2000; Finnsson et al., 2015; Melberg et al., 2006; Potic et al., 2013; Quattrocchio et al., 1997; Schwankhaus et al., 1994; Sundblom et al., 2009) leading to the myriad of symptoms described above. Demyelination of the brain and spinal cord can be seen early in the disease (Padiath & Fu, 2010) and is considered one of the most significant aspects of ADLD, contributing to both autonomic and neurological symptoms. Despite the considerable influence of the demyelination process on ADLD pathogenesis, the mechanism by which lamin B1 influences this process is still not clearly understood.

Within the CNS, oligodendrocytes are responsible for the myelination of axons (Sherman & Brophy, 2005) and as such, they have been considered to have a significant role in ADLD. Oligodendrocytes are a type of glial cell which support neuronal development and maintenance. Additionally, signals derived from astrocytes and neurons are essential in promoting myelin sheath formation and maintenance (Stadelmann et al., 2019). Considering the influence of lamin B1 in neurodevelopment, mice lacking functional lamin B1 protein survive embryonic development but exhibit neurodevelopmental defects and die shortly after birth (Vergnes et al., 2004). Lamin B-null mice suffer defects in mitotic spindle organisation in neuronal progenitor cells (Kim et al., 2011). Furthermore, conditional knock-out mouse models with a deletion of lamin B1 had a reduced cerebellum size and a reduced number of neurons (Coffinier et al., 2011). Lamin B1 is also required for normal dendrite development in primary mouse cortical neurons (Giacomini et al., 2016). As a result, the literature has explored the role of the myelin-producing glial cells in ADLD, and how the overexpression of lamin B1 relates to demyelination within the CNS. The leading theories surrounding the

effect of lamin B1 on cells within the CNS are reviewed by Neri and colleagues (Neri et al., 2023).

To study ADLD, several mouse models aiming to replicate the human ADLD phenotype have been developed with the aim of investigating the specific involvement of myelin in ADLD, the mechanisms involved in the late-age disease onset, and how these are linked to the expression of lamin B1. Heng and colleagues created a bacterial artificial chromosome (BAC) transgenic mouse model of ADLD. The BAC transgenic mouse model was produced by genomic insertion of a 177 kb sequence containing the entire mouse *Lmnb1* locus which resulted in the approximately 4-fold higher expression of lamin B1 protein and 3.5-fold higher expression of *LMNB1* mRNA than wild-type littermates (Heng et al., 2013). Studies on the BAC model suggested that overexpression of lamin B1 in oligodendrocytes, but not in neurons or astrocytes, was sufficient enough to produce late-age onset motor dysfunction seen in ADLD (Heng et al., 2013). Furthermore, recent work has shown that overexpression of lamin B1 in oligodendrocytes resulted in age-dependent epigenetic modifications and transcriptional downregulation of genes involved in lipid synthesis, overall resulting in a significant reduction in myelin-enriched lipids (Rolyan et al., 2015). Since myelin has a high lipid content, it has been hypothesised that lipid loss may be driving the demyelination process seen in ADLD, which may partially explain the late age onset of disease and cell-type specificity observed (Padiath, 2016). In addition, transgenic mice overexpressing lamin B1 in oligodendrocytes showed age-dependent somatic motor dysfunction but no autonomic dysfunction (Lo Martire et al., 2018; Rolyan et al., 2015), which is a nearly universal feature of ADLD (Guaraldi et al., 2011; Padiath & Fu, 2010), suggesting that oligodendrocyte dysfunction alone may not be the underlying cause for the range of symptoms seen in ADLD.

Both mouse models and human ADLD patients show the presence of vacuoles within the white matter but no changes in the proliferation or survival of oligodendrocytes (Melberg et al., 2006; Rolyan et al., 2015; Sundblom et al., 2009). This suggests that the

overexpression of lamin B1 is not lethal to oligodendrocytes, and that demyelination is not the result of decreases in the number of oligodendrocytes. Conversely, in ADLD patients, loss of neuronal axons was shown to be minimal while in mice models, axonal damage and neuronal loss was present (Melberg et al., 2006; Rolyan et al., 2015).

Studies investigating the role of astrocytes in the ADLD pathogenesis found that overexpression of lamin B1 resulted in a reduction in astrocyte survival (Ratti et al, 2021a, 2021b). In addition, astrocyte dysfunction in ADLD may reduce support to other cell populations involved in the myelination process (Ratti, et al., 2021a). Rolyan and colleagues hypothesised that, although ADLD is thought to be a non-inflammatory disease, inflammatory processes in astrocytes and microglia may contribute to the demyelination and axonal damage and subsequent neuronal loss seen in ADLD (Rolyan et al., 2015).

Since ADLD has been shown to affect specific cell types differently, this thesis aims to investigate the expression of nuclear lamins, particularly lamin B1, in activated B-cells and other GC cells in an ADLD mouse model compared to wild-type animals.

1.7. Proteomic Mass Spectrometry for Investigating Nuclear Lamins

To investigate the protein expression of nuclear lamins in different biological models and circumstances, a range of proteomic quantitation techniques have been previously used including Western Blotting, enzyme-linked immunosorbent assays (ELISAs), flow cytometry, immunohistochemistry, immunofluorescence and mass spectrometry. Antibody-based protein quantitation methods have several limitations including antibody cross-reactivity, non-linear signal responses and limited dynamic ranges (Solier & Langen, 2014). Proteomic mass spectrometry is often used for both relative and absolute quantitation of biomarkers in various types of biological samples as it offers higher sensitivity than antibody-based quantitation methods and can also be coupled with liquid chromatography and 2D-PAGE separation techniques to reduce sample complexity and increase sensitivity. This section will explore the concepts of liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS), the development of targeted mass spectrometry methods, and the quantitation of nuclear lamins by mass spectrometry.

Bottom-up proteomics is the most common method for protein identification and characterization by MS (Lenčo et al., 2022; Yates, 2013). This technique is based on the enzymatic digestion of proteins into peptides followed by the subsequent MS analysis of these peptides. The detected peptides are used to infer the abundance of the proteins of interest in the original sample (Picotti & Aebersold, 2012). Bottom-up proteomics usually also involves a separation technique, such as liquid chromatography (LC) to separate analytes in complex mixtures. When electrospray ionisation (ESI) is used in combination with these analytical techniques, it is referred to as liquid chromatography coupled electrospray ionisation tandem mass spectrometry assay (LC-ESI-MS/MS).

1.7.1. Liquid Chromatography-coupled Electrospray Ionisation Tandem Mass Spectrometry (LC-ESI-MS/MS)

Liquid chromatography electrospray ionisation coupled tandem mass spectrometry (LC-ESI-MS/MS) is one of the most popular methods used in proteomics to detect and quantify peptide and protein biomarkers (Aebersold & Mann, 2003; Ho et al., 2003). There are seven main stages of LC-ESI-MS/MS. These include sample injection; chromatographic separation by liquid chromatography; electrospray ionisation; transmission and focussing of ions using ion optics; mass analysis and ion selection and filtering using tandem mass analysers; ion detection; data representation and analysis (Ho et al., 2003). These will be described in the context of proteomic analysis.

In bottom-up proteomic analysis of biological samples, there are several steps involved in sample preparation before LC-ESI-MS/MS analysis can occur. The steps for sample preparation of cells include cell lysis, protein extraction; reduction; alkylation; enzymatic digestion; desalting (Duong & Lee, 2023). The initial step of sample preparation of cells includes the breakdown of cell membranes by use of a lysis solution. These solutions often contain buffers to control the pH and maintain the stability of the cellular components; salts to maintain osmolarity; detergents to disrupt cell membranes and solubilise the proteins; protease and phosphatase inhibitors to help preserve the physiological and biological state of the cells at the point of extraction. Sodium dodecyl sulphate (SDS) has become one of the most used ionic detergents for the solubilisation of biological materials due to its high affinity for proteins and quick mechanism of denaturation (Islam et al, 2017). In addition to the lysis solution, mechanical methods such as sonication can also be used to aid the solubilisation of cellular proteins.

Following protein solubilisation, a reducing agent is added to break disulphide bonds, which are important in the maintenance of the quaternary and tertiary protein structures. By cleaving these bonds, the proteins unfold from their complex macrostructures, allowing for more accessibility for enzymes during the digestion step.

Dithiothreitol (DTT) is a commonly used reducing agent and is considered a more favourable agent than previously used reducing agents such as β -mercaptoethanol and glutathione due to its greater stability and lower toxicity (Cleland et al., 1964; Muller & Winter, 2017). To prevent re-folding of the proteins and the formation of unwanted inter- and intra-protein disulphide bonds, thiol groups produced by the breakage of disulphide bonds are irreversibly alkylated by addition of an alkylating agent. Common alkylating agents include iodoacetamide (IAA) due to its high efficiency and specificity for reactions with thiol groups (Smythe et al., 1936).

After the proteins have been unfolded, enzymatic digestion of the proteins into short length peptides is necessary during bottom-up proteomic experimentation. The most common enzyme used in bottom-up proteomics is trypsin. Trypsin is an endopeptidase that cleaves exclusively at the peptide bond at the carboxy-terminal end of lysine (K) and arginine (R) amino acid residues, with the exception of lysine-proline (K-P) and arginine-proline (R-P) bonds (Olsen et al., 2004). Trypsin is one of the most widely used proteases in proteomic mass spectrometry experiments due to its stability, availability, and ability to produce relatively short peptides that create ions that are within the mass range of most mass spectrometers (Vandermarliere et al., 2013). The penultimate step of proteomic sample preparation is the removal of residual salts and contaminants from the sample before LC-ESI-MS/MS analysis via solid phase extraction (SPE). This is important as contaminants such as detergents, salts and enzymes used during digestion can interfere with LC performance and affect MS analysis by suppressing ESI of biomolecules and increasing chemical noise signals (Wang & Liang, 2012; Kachuk et al., 2015; Cavanagh et al., 2003). Finally, the peptides are resuspended in an LC-MS compatible solvent which is usually a mixture of water and an organic solvent such as methanol or acetonitrile. The addition of formic acid is necessary to enhance protonation in the case of positive mode LC-ESI-MS/MS (Núñez & Lucci, 2014). The samples are then transferred to specialised glass vials prior to LC-MS analysis.

In LC-ESI-MS/MS, sample injection is most often performed by an autosampler, which can be temperature controlled to enhance sample stability for particularly labile samples such as proteins and peptides (Steiner & Paul, 2019). Liquid chromatography (LC) is used to separate target peptides of interest based on their hydrophobicity (Finoulst et al., 2011). For proteomics applications, reversed-phase LC is usually performed (Lenčo et al., 2022; Yates, 2013) (see Section 1.7.1.1 for more information). Next, the peptide analytes are ionised by electrospray ionisation (ESI), to become peptide ions (Liuni & Wilson, 2011) (Section 1.7.1.2), which are then analysed by the MS instrument and quantified based on their mass-to-charge (m/z) ratio (Shuken, 2023) (Section 1.7.1.3). The output of this analysis is in the form of a chromatogram, showing the retention time of peptide analyte ions, that is the time it takes for the analytes to pass through the MS analytical system and reach the detector, relative to the intensity of the ion signal received by the detector, in the form of a peak. A mass spectrum is also provided which gives information on the m/z value of the analytical ions. For MRM-based MS assays, the peptide ions of interest are selected during experimental design and filtered during MS analysis, and therefore a full mass spectrum is not recorded. The chromatographic data is displayed as “peak area”, which is the measurement of the area of the chromatographic peak produced from the detection of a specific analyte ion over a period of time. The area of the chromatographic peak directly relates to the abundance of the analyte ion of interest, and thus correlates to the amount of the target peptide and therefore amount of target protein in the original sample. Separate peaks are formed for the detection of each specific analyte of interest, based on each analyte’s unique m/z value (Shuken, 2023). These instruments in tandem are complimentary for the separation and analysis of proteolytic peptides for quantitation purposes during bottom-up proteomics (Zhang et al., 2020b).

Some of the events involved in LC-ESI-MS/MS analysis take place at different air pressures. Sample injection, chromatographic separation, electrospray ionisation and data visualisation and analysis all occur at atmospheric pressure, while mass analysis and ion detection occur at vacuum pressure. Ion transmission and focussing occurs at

the transition between atmospheric and vacuum pressure within a medium pressure environment. The need for vacuum pressure during mass analysis and detection is to avoid signal loss from analyte ions colliding with residual background gas species and to prevent electrical arcing during the application of various electrical potentials as part of the MS analysis process (Screech, 2015). Figure 1-5 illustrates the seven stages of LC-ESI-MS/MS and the different instrumentation required.

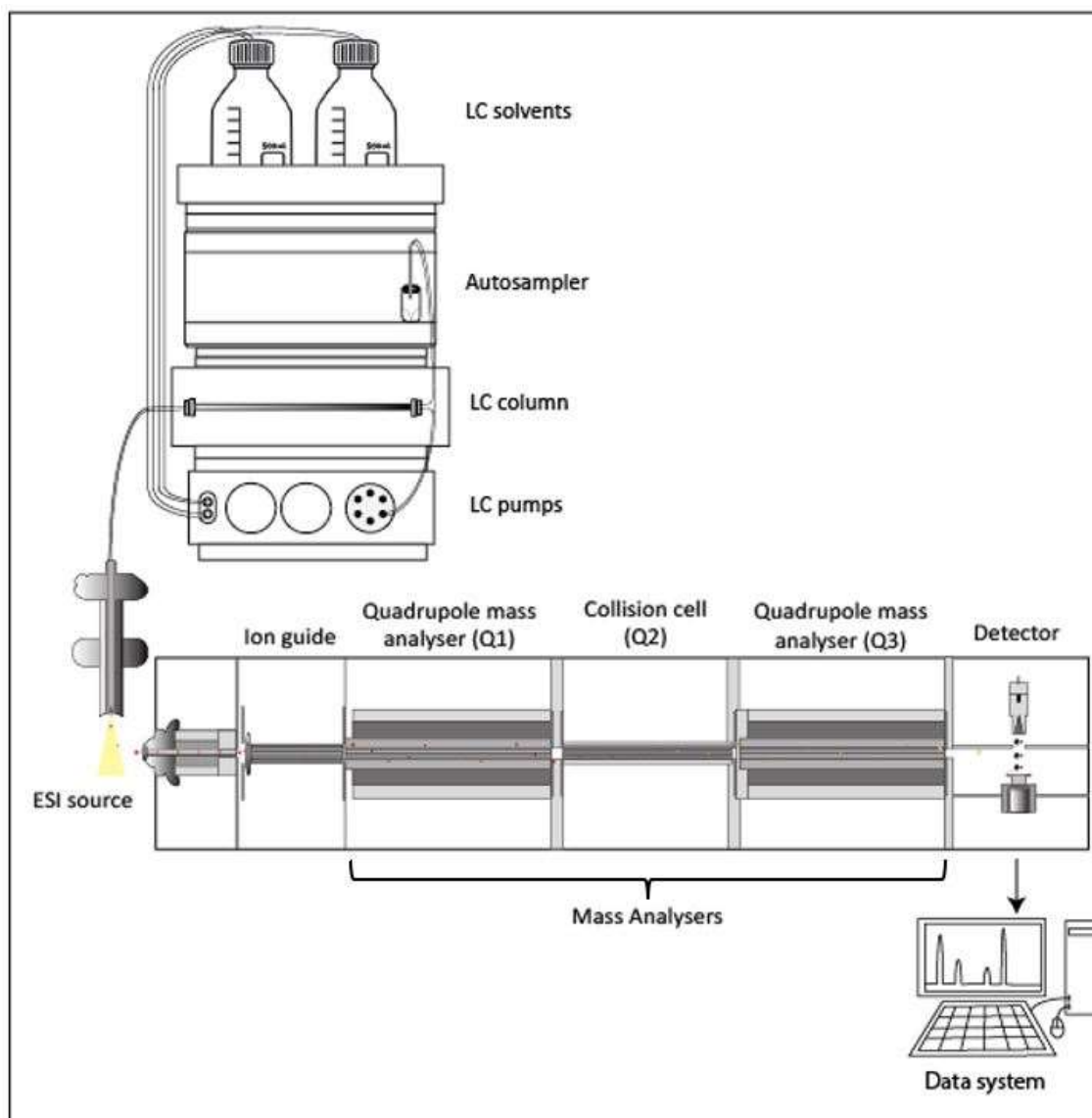


Figure 1-5: Diagram of an LC-ESI -MS/MS system and the instrumentation involved.

Yellow indicates the electrospray plume, red dots indicate the precursor ions, while orange dots represent the product ions as a result of CID within Q2.

To understand the process of targeted MRM-based LC-ESI-MS/MS analysis, an overview of the main events will be discussed.

1.7.1.1. Concepts of Liquid Chromatography

Liquid chromatography (LC) is an analytical technique used to separate specific components in complex samples. This process works by using high pressure pumps that

deliver a mixture of solvents, known as the mobile phase, through a system of tubing. The sample is introduced into this system, either via an autosampler or by manual injection, and combines with the mobile phase before being delivered to an analytical column which is filled with solid particles of adsorbent material, known as the stationary phase. Each component in the sample interacts differently with the stationary phase, resulting in different compound elution rates. These different rates lead to separation of the analytes as they elute from the column and into a mass spectrometer, as is the case with LC-MS (Figure 1-5). The data output is a chromatogram, which is a graphical representation of the signal intensity versus time, showing peaks which represent different components of the sample. Each sample appears in its respective time, called its retention time, and peak area is proportional to the amount of the target analyte within the sample (Laxman & Thange, 2022).

Often in proteomics, reversed-phase liquid chromatography is the method of choice. Reversed-phase liquid chromatography is a type of affinity chromatography which involves a non-polar, hydrophobic stationary phase and a polar mobile phase which allows separation of analytes based on the differences in hydrophobicity (Aguilar, 2004). For the separation of peptides, a gradient elution profile is used whereby the composition of the mobile phase changes over time, resulting in the elution of different peptide analytes over time. The analysis begins with peptide analytes suspended within a highly aqueous mobile phase composed of mostly water. The peptides enter the chromatographic column and bind to the stationary phase through hydrophobic interactions. The composition of the mobile phase then gradually changes and the percentage of organic solution, such as methanol or acetonitrile, within the mobile phase increases. When a specific composition of mobile phase is reached, the peptide analytes desorb from the stationary phase and elute from the column before being analysed via MS and detected, forming analyte-specific peaks in the resulting chromatogram. The solvent composition at the point where elution occurs depends on the specific peptide analyte's hydrophobicity properties (Aguilar, 2004). Manipulation of the mobile phase composition means the separation time of analytes can be controlled, leading to well-defined, sharper peak shapes which increases the accuracy

of peak area measurements and enhanced assay sensitivity and selectivity, which is critical in targeted MS assays such as MRM-based experiments. In addition, the use of water and methanol or acetonitrile as solvents is favourable as these solutions are compatible with the C18 adsorbent material usually used in proteomics, possess high solubility for peptides, and have a low viscosity which ensures high ionization efficiency during the electrospray ionisation process (Kageyama et al., 2019) (see Section 1.7.1.2 for further details).

As mentioned above, the most popular adsorbent material for reversed-phase chromatography for peptide separation are carbon-based compounds such as C18, but C4 and C8 are preferred for proteins and larger, hydrophobic peptides (Capriotti et al., 2011). These stationary phases are formed by the chemical attachment of silica to an organosilane molecule to create a hydrophobic surface. In the case of C18, the polar 18-carbon chain octadecylsilane is reacted with the polar silanol group on the surface of a spherical silica molecule. This creates a hydrophobic surface that interacts with non-polar and moderately polar analytes through several different types of bonds depending on the peptide analyte, leading to their retention on the column.

In addition to the type of adsorbent used, there are several other physical factors that should be considered when selecting a reversed-phase LC column. These influence the selectivity and resolution of analyte separation and include particle size, pore size, column length, and column diameter (Finoulet et al., 2011). Spherical silica particles containing pores are used to bind the C18 stationary phase to the internal surface of the column. Smaller particles of silica mean a greater surface area for peptide analytes to bind to the C18, leading to an enhanced capacity for adsorption to the column. In addition, a smaller particle size also means more complex paths for analytes to travel, leading to higher resolution in the form of sharper chromatographic peaks. On the other hand, a smaller particle size requires lower solvent flow rates or pumping systems that can operate at higher pressures, such as UPLC systems (Plumb et al., 2007). Secondly, an increase in the number of pores within each silica particle

increases the surface area where the peptides can adsorb to, as a smaller pore size leads to the increased number of pores per particle. Pore size may also control selectivity, due to undigested proteins and other larger particles being unable to penetrate the pores. The caveat to using small pores is a potential decrease in peak shape and resolution resulting from moderately sized peptides being unable to penetrate the pore and adsorb to the C18, and slower release from inside the pore upon elution, leading to a wider chromatographic peak (Godinho et al., 2020; Pearson et al., 1982). Column length and diameter play a crucial role in separation efficiency and analysis time. Narrower bore columns operate at low flow rates which results in a higher concentration of target peptide molecules passing through the column and binding to the C18 at any one time, which ultimately increases assay sensitivity. Additionally, narrow bore diameter columns have been shown to exhibit up to a 7-fold increase in the sensitivity compared to standard analytical columns (Ye et al., 2001). Longer columns provide higher resolution in the form of increased surface area for adsorbate-adsorbent interactions. The disadvantage to the use of longer and narrower columns is slower analysis times due to decreased flow rates.

As with all instrumentation, compromises must be made when considering factors that affect assay sensitivity, selectivity, instrument time and sample volumes. For the reasons discussed above, the LC column used in this study was a C18 column of 2.7 μm particle size, 2.1 x 150 mm internal diameter and length respectively, and 120Å pore size. The column of 150 mm and 2.1 mm internal diameter allows for a large surface area which contributes to increased resolution with a reasonable solvent flow rate. A moderate particle size of 2.7 μm and pore size of 120Å was used for the detection of small to medium-sized peptides of up to 20 amino acids in length.

1.7.1.2. Concepts of Electrospray Ionisation

Electrospray ionisation (ESI) is a technique used in mass spectrometry which uses an electrical field that is applied to a liquid sample containing analytes of interest to generate charged, gas-phase analyte ions that enter the mass spectrometer for analysis

(Ho et al., 2003). In LC-ESI-MS/MS, once the analytes within the liquid sample are elute from the chromatographic column, they enter the ion source of the mass spectrometer (Figure 1-5) where they undergo ESI to acquire a positive or negative charge. The idea of ESI was first proposed by Malcolm Dole at the International Symposium on Macromolecular Chemistry in Tokyo back in 1966, and these theories led to a publication, released in 1968 (Dole et al., 1968; Fenn, 2002). Dole's work involved the characterisation of synthetic polymers by mass spectrometry, and initiated research into electrospray as a concept of ionisation. Soon after, work by Beuhler and colleagues in 1974 expanded on the principles of ESI by use of rapid heating to vaporise peptides without decomposition (Beuhler et al., 1974). The first reports on the use of ESI came in 1984, with research from both John Fenn and Masamichi Yamashita (Yamashita & Fenn, 1984), and Lidia Gall's research group (Alexandrov et al., 2008) independently contributing to the advancement of research in this field. Ultimately, Fenn was awarded the Nobel Prize in Chemistry in 2002 for the use of ESI coupled with mass spectrometry for the analysis of biological macromolecules. English translations of Gall's 1984 findings were not available until 2008, which led to the lack of awareness of her work during previous years.

ESI is one of the most popular ionisation techniques able to be coupled with liquid chromatography tandem mass spectrometry (LC-MS/MS) for MS-based omics analysis as it is compatible with a wide range of sample matrices and biomolecules such as proteins, peptides, lipids and metabolites (Banerjee & Mazumdar, 2012). ESI is ideal for high throughput proteomics applications such as clinical biology studies on biomarkers and drug discovery (Ho et al., 2003; Ma, 2022; Williams et al., 2023). As an ionisation technique able of operating under ambient conditions, ESI is both compatible with LC systems and suitable for analysis of highly polar, less volatile or thermally unstable compounds, which is what makes it a particularly attractive ionisation method for the analysis of proteins and peptides that are denatured by high temperatures. In addition, ESI is considered a soft ionisation technique which allows for weak, non-covalent interactions to be preserves upon ionising the peptide biomolecules of interest, leading to minimal fragmentation upon ionisation (Przybylski & Glocker, 1996).

There are three main stages in ESI, which all happen in the atmospheric pressure region of the MS apparatus: (1) production and dispersal of charged droplets from tip of the high voltage ES capillary; (2) solvent evaporation and shrinkage of the charged droplets, leading to the formation of very small, highly charged droplets capable of producing gas-phase ions of charged analytes; (3) a mechanism by which the gas-phase analyte ions are produced (Bruins, 1998). Since peptides are often analysed in positive ionisation mode, the mechanism of ESI will be explained for positive ionisation of analytes.

In the first stage of positive mode ESI, a continuous stream of solvent containing analytes of interest passes through a stainless-steel capillary which is maintained at a high voltage (2.5-6kV) relative to the wall of the surrounding chamber (Ho et al., 2003). As the analyte solution passes through the highly charged capillary, hereby known as the nebuliser, a positive potential is applied to the nebuliser tip. This positive potential is referred to as the capillary voltage. The electric field is the highest near the tip of the nebuliser, which causes polarisation of the solvent near the meniscus of the liquid, with an enrichment of positive ions near the surface of the meniscus and negative ions further away from the meniscus. The effects of surface tension and polarisation cause the meniscus to assume a conical shape at the tip of the nebuliser and the subsequent formation of a jet stream of liquid. This conical shape known as the Taylor cone (Taylor, 1964). This formation occurs because the conical shape of the Taylor cone can hold more charge than the spherical shape of a meniscus. The jet stream then splits into droplets that are charged with an excess of positive ions. The positively charged droplets migrate away from the nebuliser and pass down a pressure gradient and potential gradient toward the analyser region of the mass spectrometer before entering the vacuum regions within the ion optics and mass analyser (Ho et al., 2003). This migration is also aided by the addition of a nebulising gas, usually composed of nitrogen, which flows outside of the nebuliser tip in the same direction of the sample flow to help guide the analytes into the mass spectrometer. The interface to the mass spectrometer is either a very small orifice or a heated capillary. In the case of the

instruments used in this study, the Agilent Ultivo QQQ and Agilent 6420 QQQ, this is a heated aluminium capillary. The ions that enter the heated capillary may be clustered with solvent molecules and other additives present within the sample, therefore the heat from the capillary helps the analyte ions undergo thermal declustering prior to analysis (Kearle & Verkoren, 2009). Figure 1-6 below shows a diagram of a Taylor cone produced during ESI.

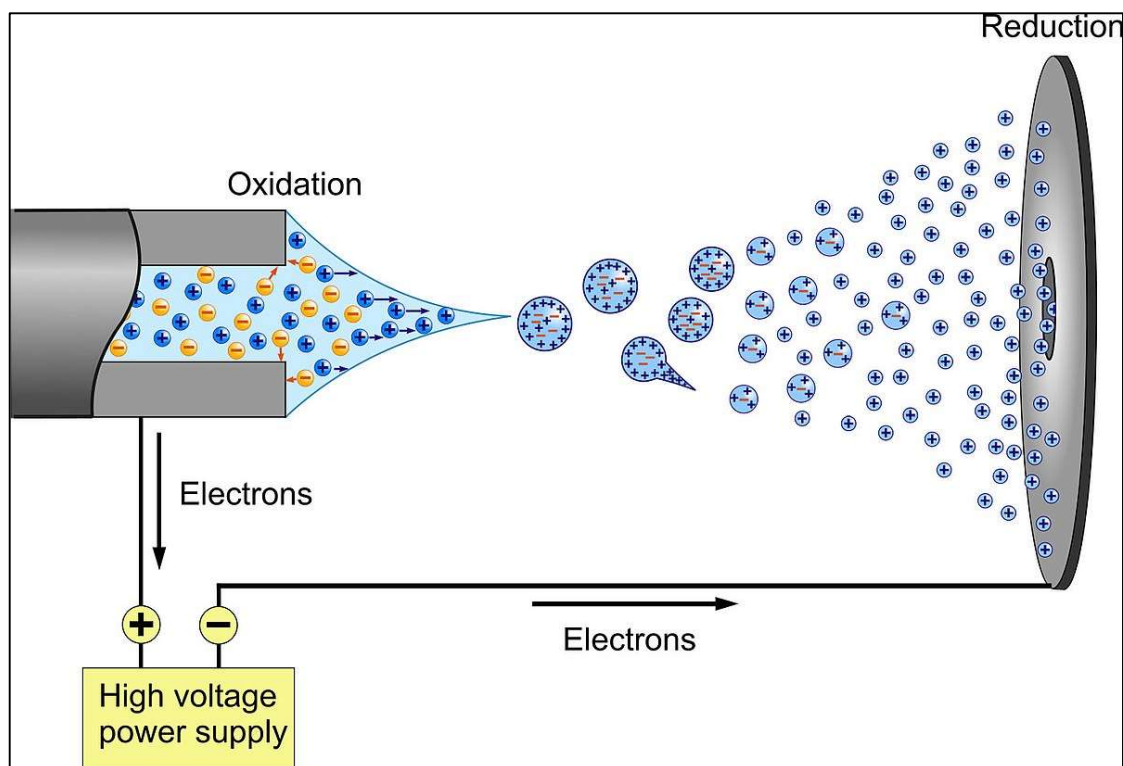


Figure 1-6: Diagram of a Taylor cone formed during electrospray ionisation in positive ionisation mode. A Taylor cone is formed at the tip of the capillary, from which a spray of charged droplets is expelled due to an applied voltage. Diagram from (Dahlin, 2015).

The second stage of ESI is where solvent evaporation of the positively charged droplets occurs, leading to the formation of gas-phase analyte ions. Evaporation of the solvent droplets is aided by a stream of heated drying gas which is henceforth known as the desolvation gas, which flows in the opposite direction to analytes entering the mass spectrometer. Manipulation of the desolvation gas temperature and flow rate allows for optimisation of the desolvation process. The addition of a sheath gas in some newer systems such as the Agilent Ultivo QQQ also columnates the sample spray at the front of the mass analyser and aids in solvent evaporation and migration of the analyte droplets to the MS inlet. Both of these gases are usually composed of nitrogen (Mordehai & Fjeldsted, 2009).

As the solvent droplets undergo evaporation and desolvation, they shrink in size and the distance between the positively charged analyte ions within the same droplet is reduced. This increases the repulsive forces between these positive charges at the surface of the droplet. At a critical limit known as the Rayleigh instability limit, the repulsion between these ions overcomes the surface tension of the droplet which destabilises the droplet and causes fission events known as Coulombic explosion events (Rayleigh, 1882). These events release charged progeny droplets which then undergo further desolvation and Coulombic explosions. Repeated evaporation and fission events take place until free gaseous-phase analyte ions are formed which occurs in the third and final stage of the ESI mechanism (Kearley & Verkerk, 2009).

The exact mechanism of gaseous ion formation is still under debate however, several hypotheses have been suggested. The two most popular theories on how this process occurs with peptide ion analytes are the ion evaporation model (IEM) and the charged residue model (CRM). The IEM was proposed by Iribarne and Thomson (Iribarne & Thomson, 1976; Thomson & Iribarne, 1979). In this theory, when the radii of the droplets shrink to less than 10 nm, instead of the droplets undergoing another round of Coulombic fission, the analyte ions are directly emitted from the droplets. This theory is supported by both experimental results and theoretical calculations and assumes that the expelled ion is one of the ionic charges at the surface of the droplet. The ion is expelled by the repulsive forces between itself and the remaining positive charges within the droplet. It is thought that IEM is more likely to occur during analysis of small molecular weight compounds (Iribarne & Thomson, 1976; Nguyen & Fenn, 2007). Conversely, the CRM, proposed by Dole in 1968, assumes that a series of Coulombic fission events results in the formation of a droplet containing a single analyte molecule accompanied by the positive charges on the surface of the droplet. Solvent evaporation from this droplet leads to a positively charged ion in the gaseous phase. This mechanism of gas-phase ion formation is thought to most likely occur during analysis of larger molecular weight compounds (Dole et al., 1968).

There is a third, lesser-known theory known as the chain ejection model (CEM), which has also gained support over the last decade. This model was first described by Ahadi and colleagues in 2012 (Ahadi & Konermann, 2012) and suggests that large analyte ions containing hydrophobic residues migrate to the surface of the droplet. Once at the surface, one chain terminus is partially ejected from the droplet into the gas phase. This is followed by further ejection of the rest of the analyte, which results in a highly charged gas phase ion which has exited the solvent droplet (Mora et al., 2000; Konermann et al., 2013; Metwally et al., 2018). This mechanism is thought to most likely occur during the ionisation of large analyte ions such as unfolded proteins. A schematic illustration of the proposed mechanisms of the formation of gaseous-phase ions is shown in Figure 1-7.

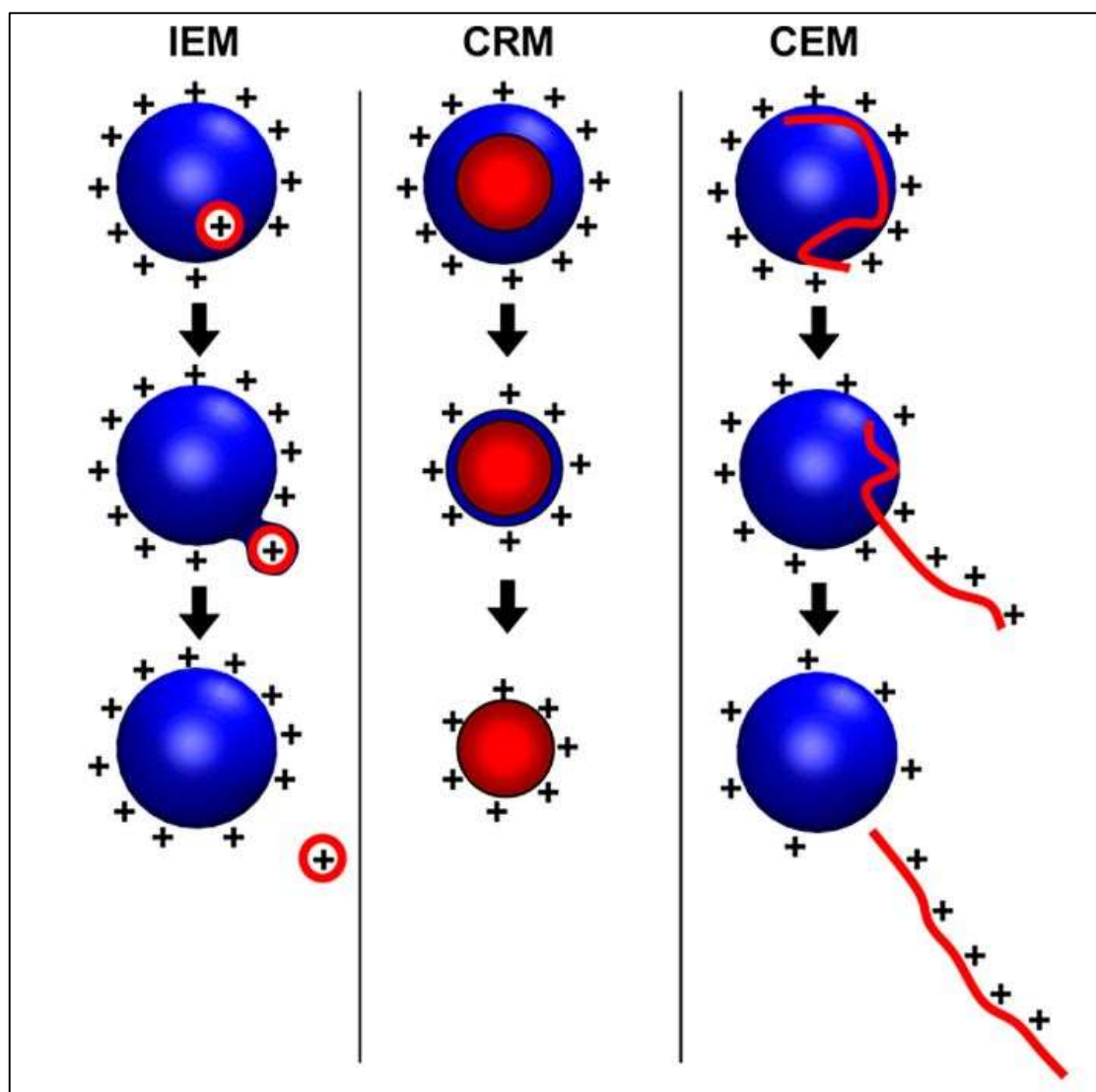


Figure 1-7: Schematic representation of the three proposed mechanisms of ESI. The ion evaporation model (IEM) is where ions are emitted from droplets which shrink during evaporation of the solvent until the electric field strength at the surface of the droplet is large enough for the ions to be expelled. The charged residue model (CRM) explains how a droplet containing a single analyte evaporates, transferring the residual charge to the analyte. The chain ejection model (CEM) is where a polymer, such as a protein, is partially ejected from the solvent droplet and the charge is retained on the exposed portion of the protein. The protein is ejected further from the droplet until its complete release. Diagram from (Konermann et al., 2013).

In summary, the IEM is experimentally well supported for small ions. However, this model may not apply for very large ions such as proteins, as CRM or CEM are more plausible (Kearle & Verkoren, 2009; Konermann et al., 2013). Molecular dynamics simulations have been used to uncover the mechanism of ESI by modelling the behaviour of highly charged nano-droplets; however, the outcome of these simulations is heavily dependent on certain assumptions and parameter settings (Hanifi et al., 2024). A review by Kerbale and Verkoren thoroughly explains the theory behind ESI (Kearle & Verkoren, 2009), while a publication by Crotti and colleagues expands on this knowledge to include how the size and charge state of droplets affects ion mobility and thus ESI measurements and ion suppression (Crotti et al., 2011).

1.7.1.3. Concepts of Mass Spectrometry

Mass spectrometry is a highly versatile technique used to precisely measure the molecular mass of biologically important molecules such as lipids, metabolites, proteins and peptides. The mass spectrometer is comprised of three basic components: the ion source; the mass analyser; the detector (Figure 1-5). The gas-phase analyte ions are produced in the ionisation chamber where the ion source, for example an ESI source, is kept. The ions are then focussed via several ion optics into an ion beam, allowing the analyte ions to enter the mass analyser. The mass analyser filters and separates the ions according to their mass to charge ratio (m/z value), which is determined by measuring the trajectory of each ion in response to electric and magnetic fields, depending on the mass analyser. The separated ions are transferred to the detector system which measures their abundance, and the results are displayed on a mass spectrum which shows the m/z and abundance of the detected ions. Several different gases and voltages are applied to the analyte ions to aid the generation and movement of the ions from the sample capillary into the mass analyser and to the detector.

In LC-ESI-MS/MS, after gas-phase ions have been produced during the ESI, the ions enter the orifice of a heated capillary, which is usually set to a temperature of 100°C to

300°C and kept in the interface between atmospheric pressure and high vacuum. Once the ions have entered the heated capillary, the heat causes the complete desolvation of the ions passing through. The combined use of desolvation gases and the heated capillary reduce the formation of cluster ions which can affect the analysis of individual analyte ions, therefore increasing the system's robustness (Anacleto et al., 1992). The ions then pass through a region of collision-induced dissociation (CID) where an electrical potential known as the fragmentor voltage (FV) is applied. This electrical potential helps to focus the analyte ions and accelerate them through the sampling cone, also known as a skimmer, before entering the mass analyser for analysis (Ho et al., 2003). The acceleration of ions into the mass analyser is also aided by the pressure gradient between the ion source (held at atmospheric pressure), the low-pressure chamber in which the capillary and ion optics are held, and the high vacuum chamber housing the mass analyser.

There are many types of mass analysers (de Hoffmann & Stroobant, 2007). Commonly used mass analysers include quadrupole (Q), time of flight (TOF), linear ion trap (LIT), Orbitrap and FTICR, which can be used alone or in tandem. The use of tandem mass analysers, which is the use of multiple mass analysers in succession, allows for fractionation of analyte ions and is particularly useful in peptide identification. One type of tandem mass analysed commonly used for targeted MRM quantitation is a triple quadrupole mass analyser (QQQ), which consists of a linear series of three consecutive quadrupole mass analysers (Zhang et al., 2014). Quadrupole mass analysers are made of four parallel conducting metal rods spaced about a central axis along which the analyte ions travel. The rods are usually hyperbolic or cylindrical in shape and each opposing rod pair is connected electrically, with radio frequency (RF) and direct current (DC) voltages applied (de Hoffmann & Stroobant, 2007), with adjacent rods supplied with opposite RF and DC voltages. For a given set of RF and DC voltages, only ions of a certain mass-to-charge ratio (m/z) will have a stable trajectory within these oscillating electric fields and will travel down the quadrupole between the rods. All other ions collide with the rods and become neutralised (Ho et al., 2003;

Smith, 2013). This is how the quadrupole can select ions based on a particular mass-to-charge ratio determined by the operator (de Hoffmann & Stroobant, 2007).

In MRM-based assays, a QQQ instrument selects for an analyte ion, known as a precursor ion, of a specific user-defined m/z (Q1), fragments the parent ion into smaller ions known as product ions (Q2), and selectively transmits a user-defined product ion (Q3) to be detected (Ho et al., 2003). Fragmentation of the precursor ions entering Q2 occurs by collision of the analyte ion selected during the filtering process in Q1 with an inert gas such as nitrogen via CID. CID is a commonly used technique in MS/MS analysis to fragment gas-phase ions by colliding them with a neutral target gas. This collision results in the transfer of kinetic energy to the ions to induce fragmentation of the original precursor ion into fragment ions known as product ions (Shukla & Futrell, 1994). With respect to peptide ions analysed by QQQ instruments within this thesis, the fragmentation pattern of different peptide precursor ions (that is, which bond of the peptide chain is broken during fragmentation) is highly dependent on several variables (Wells & McLuckey, 2005) (more information on peptide fragmentation patterns can be found in Section 3.4.2). One of these variables is the energy applied to each ion as it enters Q2, which is known as the collision energy (CE). The selection of a specific precursor ion and product ion pair is known as a transition.

The advantage of using MRM-based assays is the increased selectivity compared to full scan MS modes, where no filter is applied at Q1 or Q3, leading to all precursor and product ions being transmitted and detected. MRM-based assays allow only user-defined m/z ions to be transmitted through the quadrupoles to be detected, leading to increased assay selectivity and sensitivity by the removal of background ions with different m/z values compared to the user-defined selected m/z of interest. Other targeted assays can be performed on QQQ instruments such as a product ion scan (Q1 is set to a filter ion of a selected m/z while Q3 is set to scan the entire m/z range of product ions) and precursor ion scan (Q3 is set to select ions of a specific m/z while Q1 scans the entire m/z range) (Ho et al., 2003). The arrangement of three quadrupoles

was first developed in the mid-1970s by Jim Morrison of La Trobe University (Australia) for the purpose of studying the photodissociation of gas-phase ions (Morrison, 1991), however it was Yost and Enke at Michigan State University who developed the first the triple-quadrupole configuration into a mass spectrometer (Yost & Enke, 1978). Figure 1-8 below shows the process of ion selection during MRM analysis using a triple quadrupole mass analyser.

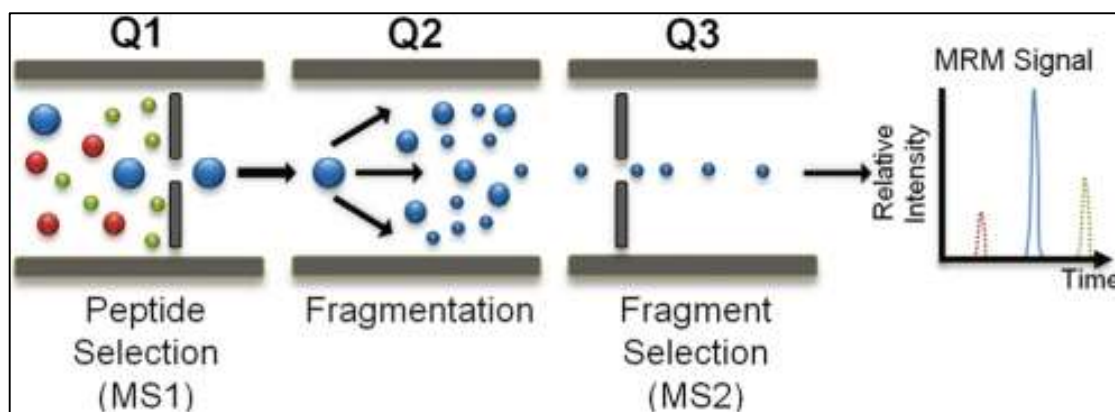


Figure 1-8: Process of ion selection and filtering during multiple reaction monitoring (MRM) experimentation using a triple quadrupole (QQQ) mass spectrometer.

Diagram from (Boja & Rodriguez, 2011).

Once the ions of interest have been transmitted through the QQQ mass analysers, the ions are detected. The most commonly used detection system in QQQ mass spectrometry is the use of a continuous-dynode electron multiplier. This detection system works by using a horn-shaped funnel made of glass which is thinly coated in a semi-conductive metal substance. A single electron collides with the metal-coated funnel and produces 1 to 3 electrons via a process known as secondary emission. By applying an electrical potential within the funnel, the emitted electrons bounce off the metal plate and accelerate to the other side of the funnel, inducing more secondary emissions and creating more electrons. This process is repeated several times, resulting in a large number of electrons which are collected by a metal anode at the end of the funnel. As part of MRM data presentation via LC-ESI-MS/MS, the detector records the

number of electrons collected by the metal anode and therefore calculates the abundance of each user-defined product ion. The ion intensity is represented as an ion count, which is calculated as the number of ions that have reached the detector with the effects of the electron multiplier taken into consideration. Therefore, the ion signal is proportional to the analyte concentration (Ho et al., 2003). The data is presented as a chromatogram which displays the target analyte ion abundance along the y-axis and retention time, this is the time it takes for the target analyte ions to reach the detector, along the x-axis.

The ionisation mode affects the observed masses displayed on the spectra. If the analytes are ionised in positive electrospray ionisation mode, the observed ions on the spectra will often be 1 Dalton greater than the neutral mass to account for a single protonation during ESI, whereas in negative ionisation mode, deprotonation leads to a signal one Dalton less than the neutral mass. In addition, multiply charged ions resulting from positive mode ESI will give the same retention time as their singly charged counterparts as all originate from the same analyte but with different numbers of protonated atoms, which will result in different m/z values of analyte ions depending on the number of protonations. In addition, all product ions of the same precursor ion will have the same retention time. Moreover, knowledge of the sample preparation process can also inform of any adducts or chemical impurities that may be bonded to the analytes of interest, which may affect their measured m/z value and decrease assay sensitivity due to being filtered out in Q1. Salts are particularly disruptive to LC-MS/MS analysis, not only due to their effect on the m/z of target analytes but also due to their crystallisation on the nebuliser tip, causing inefficient sample spray shape, misshapen Taylor cone conformations and poor ionisation of sample analytes.

1.7.2. Proteomic Investigations of Nuclear Lamins by LC-MRM-MS/MS

Targeted MRM-based LC-MS assays have rarely been used in previous studies to quantify nuclear lamins, although this method has been used by a small number of research groups. Aljada and colleagues used LC-MRM-MS/MS to identify the expression of lamin A/C, lamin C, lamin A $\Delta 10$ and lamin A $\Delta 50$ splice variants of the *LMNA* gene in cell lines (Al-Qahtani et al., 2019). This quantitative method measured three transitions related to one peptide precursor for each transcript variant, and a heavy labelled version of that peptide for quantitation purposes. Lamin A/C transcripts were quantified in the MCF7 breast cancer cell line and the U937 human myeloid leukaemia cell line. Later, the same research group used this method with the same single peptide to quantify the expression of lamin A/C transcripts in human cell lines of breast cancer, ovarian cancer, neuroblastoma, osteosarcoma and a non-tumourigenic epithelial cell line (Galal et al., 2021b). Additionally, Chen and colleagues quantified the lamin A isoform in the plasma of pregnant women and identified lamin A as a prenatal biomarker of congenital heart defects by MRM analysis. Their method used two peptide sequences for MRM-based quantitation of protein expression, which were different from the sequence used by Aljada's research group (Chen et al., 2016).

With respect to B-type lamins, although multiple studies have detected both lamin B1 and B2 by untargeted mass spectrometry in various sample types, an extensive literature search failed to identify any publications in which a targeted MRM-based mass spectrometry method was used to quantify lamin B1 and lamin B2. Murray-Nerger and Cristea comment on the use of targeted MRM-based mass spectrometry to quantify post-translational modifications in nuclear lamins in their 2021 review and noted the lack of literature in this field (Murray-Nerger & Cristea, 2021).

As part of this thesis, a targeted LC-ESI-MRM-MS/MS method was developed to detect the abundance of the four main nuclear lamin isoforms described previously. The method design included the *in-silico* tryptic digestion of the amino acid sequences of

each nuclear lamin isoform in *Homo sapiens* and *Mus musculus* species. Nine independent MS instrument parameters were optimised for the detection of these peptides in biological samples, and the optimised method was then used in subsequent experiments to quantify the expression of each nuclear lamin isoform in human cell lines and mouse splenocyte samples.

1.8. Thesis Aims and Objectives

The overarching aim of this study was to be able to detect, quantify and differentiate between the four main nuclear lamin isoforms in biological samples in order for lamins to be utilised as biomarkers of disease. To achieve this there were several objectives, the first of which was to examine the expression of different nuclear lamin isoforms by commonly used proteomic methods, including Western Blot analysis and untargeted LC-MS^e. The next objective was to design and optimise a targeted proteomic method that could provide high specificity between different nuclear lamin isoforms and provide high sensitivity for detection of nuclear lamins in cell types with low protein expression due to the differential abundance of these target proteins within cell types. The final objective was to use this optimised targeted MS method to examine the expression of nuclear lamin isoforms in biological samples of human and mouse origin to elucidate the role of nuclear lamins in health and disease. The human samples analysed included cell lines representative of different B-cell cancers, cell lines representative of different tissue stiffnesses, and in a B-cell cell line in the absence of the AID protein, which is involved in B-cell activation during the immune response. These samples were chosen to explore the role of nuclear lamins in B-cell activation and malignancy, and in mechanosensing. Mouse samples chosen for analysis included a mouse model of the lamin B1-overexpressing neurodegenerative disease ADLD and two gene knock-down mouse models (*LMNB1* and *ATG7*) to explore the consequences of gene knockout on the protein expression of nuclear lamins. As a result of this study, it is hoped that scientific community will gain a better understanding of the role of nuclear lamins in the pathogenesis of ADLD and B-cell malignancies, which may aid in the diagnosis and treatment of these diseases. In addition, this study aims to expand upon the roles of nuclear lamins in mechanosensing, B-cell activation and autophagy.

1.9. Thesis Organisation

This thesis will firstly describe the proteomic evaluation of nuclear lamin isoform expression in secondary cell lines by Western Blot and LC-MS^e analysis (Chapter 2). This includes a range of cell lines with different stiffnesses, and malignant B-cell cell lines, and the BL-2 cell line in the presence and absence of AID expression. The second results chapter, Chapter 3, will describe the optimisation of a targeted mass spectrometry assay to evaluate the protein expression of different nuclear lamin isoforms in a semi-quantitative manner. The third results chapter, Chapter 4, will examine the nuclear lamin protein expression in the same cell lines investigated in Chapter 2 using the optimised targeted MS method developed in Chapter 3. The final results chapter, Chapter 5, will investigate the expression of different nuclear lamin isoforms in splenocytes from a variety of mouse models by using the optimised LCMRM-MS/MS method developed in Chapter 2. These include a mouse model of autosomal dominant leukodystrophy, a disease of overexpressed lamin B1; a mouse model of lamin B1 knock-down and a mouse model of Atg7 knock-down will also be analysed individually to explore the expression of different nuclear lamin isoforms in mice deficient of lamin B1 and explore the role of nuclear lamins in autophagy. A final chapter, Chapter 6, will collectively discuss the methodology and experimental results of the thesis and provide concluding remarks, highlight study limitations and explore future studies in the quantitation of nuclear lamin isoforms and their potential as disease biomarkers.

2. Proteomic Investigation of Nuclear Lamin Expression in Cell Lines

2.1. Introduction

As discussed in Chapter 1, nuclear lamins have a diverse role in several cellular processes, including mechanosensing (Section 1.2), malignant transformation and progression (Section 1.4), and roles within the immune response (Section 1.3). This chapter will explore the role of nuclear lamins in mechanosensing, and in B-cell activation and malignancy.

With respect to mechanosensing, Section 1.2 explores the roles of nuclear lamins in regulating cellular and nuclear stiffness, and nuclear elasticity and deformation. It is thought that lamin A regulates cortical and nuclear stiffness and migration (Lammerding et al., 2004, 2006), while lamin B1 regulates nuclear morphology and stiffness (Vahabikashi et al., 2022b), and lamins B2 and C are involved in modulating nuclear elasticity and deformation (Vortmeyer-Krause et al., 2020). Furthermore, A-type lamin expression has been shown to be higher in cells residing in stiff tissues and lower in cells within softer biological microenvironments, while the expression of B-type lamins shows minimal variation across different tissue stiffnesses (Swift et al., 2013).

Regarding B-cell malignancies in Section 1.4.3, Klymenko and colleagues found that lamin B1 expression is reduced during B-cell activation, and low levels of lamin B1 were poor prognostic indicators in CLL patients. Additionally, low lamin B1 expression was associated with disease progression of FL to DLBCL (Klymenko et al., 2018). Since CLL and FL are both malignant transformations of activated B-cells, the authors theorized that the reduction of lamin B1 expression during B-cell activation may lead to constitutively low levels of lamin B1 in cancers arising from activated B-cells, such as BL

and MM. Therefore, this thesis aims test this theory by comparing the protein expression of nuclear lamin isoforms in BL and MM.

Furthermore, the involvement of nuclear lamins in B-cell activation comes from studies showing the decreased expression of lamin B1 upon B-cell activation (Klymenko et al., 2018). In addition, the binding of lamin B1 to LADs within the genome has been shown to regulate gene transcription (Guelen et al., 2008). In B-cells, shRNA knockdown of lamin B1 resulted in spontaneous SHM and aberrant expression of Ig light chain proteins (Klymenko et al., 2018) (Section 1.3.3). The influence of lamin expression on AID, a key regulating enzyme involved in SHM and antibody diversification in B-cells, is yet unknown. Knockout of lamin B1 in the malignant B-cell cell line BL-2 suggested that there was no change in AID expression compared to untreated BL-2 cells. Since lamin B1 expression does not seem to affect AID expression, it was theorised that the expression of AID during SHM may cause the downregulation of lamin B1, leading to the re-localisation of genes involved in SHM away from the nuclear periphery and subsequent expression of genes involved in SHM. The first step to this hypothesis was to investigate if AID expression decreases the expression of lamin B1. The aim of this section was to investigate if knockdown of AID in the BL-2 cell line resulted in the upregulation of lamin B1. Additionally, the expression of other nuclear lamin isoforms upon AID knockdown was also examined.

To summarise, this chapter will examine the protein expression of the most common isoforms of nuclear lamins (lamin A, B1, B2, and C) in different cell lines. These include a range of cell lines that represent different tissue stiffnesses to study the influence of each isoform on cellular mechanosensing. Additionally, the protein expression of these four lamin isoforms will be investigated in cell lines representative of different B-cell malignancies, including BL and MM IgG and IgE subtypes to investigate the potential for nuclear lamins as biomarkers within these cancers. Finally, the expression of the main nuclear lamin isoforms will be examined in the BL cell line BL-2, where the expression of AID has been transcriptionally silenced by *AICDA* gene knock-out to

investigate if AID expression affects the expression of nuclear lamin isoforms, namely lamin B1, in activated B-cells.

2.2. Aims and Objectives

The aim of this chapter is to explore nuclear lamin dynamics in the mechanosensing, in different B-cell cancers, and during B-cell activation. This was performed by Western Blot and LC-MS^e analysis

This will be tested through three objectives:

1. Investigate the protein expression of nuclear lamins in BL-2, HEK293T and SW1353 cell lines. These cell lines are representative of cells found within a range of soft and stiff tissue microenvironments and will be assessed by Western Blot (WB) and LC-MS^e analysis.
2. Investigate the protein expression of nuclear lamins by WB and LC-MS^e analysis in cell lines representative of B-cell malignancies of BL (BL-2) and different subtypes of MM (OPM-2 and U266).
3. Investigate the protein expression of nuclear lamins in the BL-2 cell line with and without the gene activation of AID (BL-2 AID^{+/+} and AID^{-/-} respectively) by WB and LC-MS^e analysis.

2.3. Materials and Methods

2.3.1. Cell Culture

The Burkitt lymphoma cell line BL-2 and the BL-2 AID^{-/-} cell line were maintained in suspension culture in RPMI 1640 media containing L-glutamine, supplemented with 20% foetal bovine serum (FBS) and 50 U/mL penicillin and streptomycin (PS). The OPM-2 and U266 multiple myeloma (MM) cell lines were maintained via suspension culture in RPMI 1640 containing L-glutamine and supplemented with 20% FBS, 50 U/mL PS, 5% non-essential amino acids (Merck Life Sciences UK Limited, Gillingham, UK) and 5 M sodium pyruvate. BL-2, OPM-2 and U266 cell lines were grown in T25 cell culture flasks in suspension culture. The HEK293T epithelial cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 50 U/mL PS. SW1353 chondrosarcoma cells were cultured in DMEM supplemented with 10% FBS, 142 µM L-ascorbic acid (Merck Life Sciences UK Limited, Gillingham, UK) and 50 U/mL PS. HEK293T and SW1353 cells were cultured in T75 or T175 cell culture flasks in adherent cultures. BL-2, BL-2 AID^{-/-} and OPM-2 cell lines were maintained at a concentration of 0.5-1x10⁵ cells per mL. After 48-72 hours of continued growth of the BL-2 and BL-2 AID^{-/-} cell lines, a single cell suspension was made, and cells were sub-cultured with fresh media. For OPM-2 and U266 cell lines, continuous growth was performed for 72-96 hours before sub-culturing in fresh media from single cell suspensions. For HEK293T and SW1353 cell lines, after 72-96 hours of continued culture, cells were sub-cultured at a 1:3-1:8 ratio. All cell lines were cultured at 37°C, 5% CO₂ with 95% relative humidity (RH). Subculturing of HEK293T and SW1353 cell lines consisted of removing and discarding cell culture media and washing cells in phosphate buffered saline (PBS). 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) solution was added to each cell culture flask and cells were incubated for 3-5 minutes at 37°C, 5% CO₂, 95% RH. Fresh cell culture media was then added to the flask to neutralise the trypsin-EDTA solution. The cell mixture was then transferred to a 50 mL tube and centrifuged at 1,000 rpm for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in fresh cell culture media before being transferred to new cell culture flasks. Cell culture medias RPMI 1640 media and DMEM (both

supplemented with L-glutamine) FBS, PS, PBS, sodium pyruvate and trypsin-EDTA were all purchased from Gibco at Thermo Fisher Scientific UK Ltd (Loughborough, UK). For downstream analysis, adherent cell lines (HEK293T and SW1353) were washed in PBS and then harvested via trypsinisation as described above. For suspension cultures, no trypsinisation was required, and cells were instead transferred directly from the culture flask to a 50 mL tube, centrifuged at 800 rpm for 3 minutes with a low deceleration speed before the supernatant was discarded and cell pellet resuspended in fresh cell culture media. Once a cell line was suspended in fresh culture media, the cell suspension was mixed at a 1:1 v/v ratio with Trypan Blue exclusion dye (Invitrogen by Thermo Fisher Scientific UK Ltd, Loughborough, UK), and 10 μ L of this mixture was loaded onto a 0.1mm x 1/400 mm² Neubauer Improved haemocytometer (Hawksley, Sussex, UK). The dye is excluded from live cells as they possess an intact membrane however, dead cells have a compromised membrane and so take up the dye and are therefore stained dark blue, which is visible under the microscope. The number of live and dead cells in the sample were counted and an average cell count was determined by the mean live cell count of two 1 mm² areas. The total number of cells per mL cell suspension was calculated using the following equation:

$$\text{Cell count per mL} = \text{Mean cell count} \times \alpha \times 10^4$$

Equation 1-2-1: Calculation to determine cell count per mL during in-vitro culture of cell lines, where α represents the dilution factor with Trypan Blue.

Once the number of live cells per mL was calculated, the number of cells required for further analysis was transferred to a fresh centrifuge tube, pelleted, washed twice with PBS, placed on ice, and collected by centrifugation at 13,000 rpm for 2 minutes at 4°C. The cell pellet was stored at -80°C after the supernatant was removed.

2.3.2. Cell lysis

Cell lysates were prepared through whole-cell lysis or subcellular fractionation, depending on the requirements of the downstream analysis.

2.3.2.1. Subcellular fractionation

Immediately after collection, cell pellets were resuspended in hypotonic lysis buffer (HLB) (20 mM HEPES, 10 mM KCl, 2 mM MgCl₂, 1 mM EDTA, pH 7.4) containing 1x protease and phosphatase inhibitor cocktail (PIC) (Merck Life Sciences UK Limited, Gillingham, UK).

Cell suspensions were incubated on ice for 15 minutes before being passed through a 27-gauge needle connected to a 1 mL syringe ten times. After incubation on ice for a further 20 minutes, samples were centrifuged at 3,000 rpm for 5 minutes at 4°C. The supernatant containing the cytoplasmic and mitochondrial fractions was transferred to a separate Eppendorf tube. Pellets containing nuclear fractions were resuspended in HLB containing 1x PIC and passed through a 25-gauge needle ten times using a 1 mL syringe. Nuclear fractions were centrifuged at 3,000 rpm for 10 minutes at 4°C and the supernatant was discarded, leaving the pellet containing the nuclear fraction. The nuclear fractions were resuspended in Tris-buffered saline (TBS) containing 0.1% sodium dodecyl sulphate (SDS) and then sonicated using a probe sonicator for 3 seconds. The cytoplasmic and mitochondrial fraction previously isolated was centrifuged at 8,000 rpm for 5 minutes at 4°C and the supernatant containing the cytoplasmic fraction was transferred to a separate Eppendorf. The mitochondrial pellet was discarded, and the cytoplasmic and nuclear fractions were stored at -80°C.

2.3.2.2. Whole cell lysis

Whole cell lysates were prepared by resuspension of the cellular pellet in lysis buffer (0.5 M triethylammonium bicarbonate (TEAB), 0.05% SDS, 1 x PIC) with manual shearing through a 26-gauge needle connected to a 1 mL syringe ten times. Samples were sonicated by use of a probe sonicator at 40% power for a total of 30 seconds, with a 15-second incubation on ice after the first 15 seconds of sonication, or use of a sonicating water bath where samples were sonicated for 30 seconds and then incubated on ice for 30 seconds, which was repeated ten times. Samples were

centrifuged at 13,000 rpm and the supernatant containing the protein lysate was stored.

2.3.3. Protein quantification

Protein quantification was performed by either using the Qubit Protein Assay Kit (Thermo Fisher Scientific UK Ltd, Loughborough, UK) using a Qubit 4 fluorometer, as per the manufacturer's instructions or by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific UK Ltd, Loughborough, UK) using a 1:10 dilution of each sample in lysis buffer without PIC and performed as per the manufacturer's instructions.

2.3.4. Western Blotting (WB)

2.3.4.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein lysates were prepared by mixing with 4x LDS loading buffer and 2.5% β -mercaptoethanol. Samples were heated at 95°C for 5 minutes before loading onto a 4-12% Bis-Tris polyacrylamide gel along with a 10-250 kDa or 11-245 kDa protein ladder (New England Biolabs, Herts, UK). A 1x MOPS or MES SDS running buffer was added to the electrophoresis tank and electrophoresis was conducted at 100 V until the dye front reached the end of the gel.

2.3.4.2. Protein Transfer

Transfer of proteins to 0.45 μ m polyvinylidene difluoride (PVDF) membrane was carried out by a wet transfer method using a Tris-Glycine based transfer buffer containing 20% methanol at 100 V for 1 hour or at 10 V overnight, at 4°C. The wet transfer was prepared by activating the PVDF membrane in 100 methanol and the assembling the transfer stack, with the SDS-PAGE gel and membrane situated between two pieces of filter paper and one sponge on either side. Alternatively, the protein transfer was carried out using a semi-dry transfer system (BioRad, Watford, UK) using a TransBlot Turbo transfer system and kit as per the manufacturer's instructions, at 25 V for 10 minutes.

Loading buffer, SDS-PAGE gels, SDS running buffer and PVDF membrane were all purchased from Invitrogen by Thermo Fisher Scientific UK Ltd (Loughborough, UK). Ponceau S stain (Merck Life Sciences UK Limited, Gillingham, UK) was used to check the success of the transfer.

2.3.4.3. Antibody Incubation

Once the Ponceau S stain had been removed and the PVDF membrane has been washed three times for 5 minutes each in Tris-buffered saline (20 mM Tris, pH 7.5, 100 mM NaCl) (TBS), the membrane was blocked for 1 hour in 5% milk TBS containing 0.1% Tween-20 (TBS-T). Primary antibody diluted in 5% milk in TBS-T was added to the membrane and left to incubate for 4 hours at room temperature or overnight at 4°C, with gentle rolling. Table 2-I below provides details of the primary antibodies used.

Table 2-I: Antibodies and their respective dilutions used in Western Blot experiments.

Antibody	Host	Estimated Molecular Weight (kDa)	Dilution	Manufacturer
Lamin A/C	Rabbit	70 and 61	1:500 – 1:1,000	Cell Signaling Technology (Leiden, The Netherlands)
Lamin B1	Rabbit	68	1:1,000	Abcam (Cambridge, UK)
Lamin B2	Rabbit	68	1:1,000 – 1:5,000	Proteintech (Manchester, UK)
α -tubulin	Mouse	42	1:5,000	Invitrogen by Thermo Fisher Scientific (Loughborough, UK)
GAPDH	Mouse	37	1:10,000	Merck Life Sciences UK Limited

After incubation, the primary antibody solution was removed and the membrane was washed 5 times for 5 minutes per wash in TBS-T. Secondary LI-COR antibodies (IRDye 680RD and 800 CW Goat anti-Mouse and Goat anti-Rabbit) (LI-COR Biotechnology, Cambridge UK) at a 1:10,000 concentration prepared in 5% milk in TBS-T were added to the membrane and left to incubate in the dark for 1 hour at room temperature with gentle rolling. After incubation, a further 3 washes in TBS-T were performed before the fluorescent signal was detected using a LI-COR Odyssey Imaging System and accompanying software (ImageStudio v3.1). The lamin A/C antibody was designed to detect both lamin A and C and resulted in two bands in WB analysis. The higher molecular weight band was representative of the expression of lamin A at around 70 kDa, while the lower molecular weight band was representative of the expression of lamin C at around 61 kDa.

2.3.4.4. Densitometry analysis

Densitometry analysis was performed by quantifying the fluorescent protein signal for the target protein and housekeeping protein using ImageJ version 1.54 software. Images were loaded as .tiff files into ImageJ and converted to 8-bit black and white images within the software. The signal was measured for each target and housekeeping protein for each cell line, and area measurements were exported to .csv files before conversion to .xlsx files. Microsoft Excel was used to perform the densitometry calculations and normalisation of the target protein expression to the expression of the housekeeping protein. Firstly, the background signal of the PVDF membrane was subtracted from the signal of each individual protein band (housekeeping protein or target protein). For housekeeping proteins, the lane normalisation factor was determined by dividing the value of each observed signal by the highest observed housekeeping protein signal on the membrane. To calculate the normalised signal of each target protein band, the observed signal intensities of each experimental target band were divided by the lane normalisation factor. The normalised target protein signal was then plotted for each cell line. Bar charts were produced, and statistical analysis performed in GraphPad Prism version 9.2 or 10.0.

2.3.5. Liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS)

2.3.5.1. In-Gel Digestion

Protein lysates were loaded onto a 4-12% SDS-PAGE gel and protein electrophoresis conducted as per Section 2.3.4.1. The gel was then stained with a Coomassie-based stain (Imperial Protein Stain) (Thermo Fisher Scientific UK Ltd, Loughborough, UK) overnight at 4°C with gentle rocking. The gel was washed in MilliQ 18mΩ ultrapure water, and the protein bands of interest (between 58 and 100 kDa) were excised from the gel using a scalpel. Gel pieces were de-stained by washing twice in de-staining solution (25 mM ammonium bicarbonate (ABC) in 50% acetonitrile (ACN)) at 37°C for 30 minutes per wash, with shaking at 700 rpm. Samples were reduced through incubation in 3 mM 1,4-dithiotritol (DTT) (Merck Life Sciences UK Limited, Gillingham, UK) at 60°C for 10 minutes. Alkylation of samples was then performed by addition of 2.6 μM iodoacetamide (IAA) (Merck Life Sciences UK Limited, Gillingham, UK) and incubation at room temperature in the dark for 1 hour. Samples were then washed again twice in de-staining solution. 100% ACN was added to gel pieces and left to incubate for 15 minutes at room temperature before allowing samples to air-dry for 10 minutes. Samples were trypsinised in 20 ng/μL Sequence Grade Modified Trypsin (Promega, Hampshire, UK) and incubated at room temperature for 15 minutes. 100 mM HEPES was added to the digestion mixture and samples left to incubate overnight at 30°C with shaking at 700 rpm. The digestion mixture was transferred to a separate Eppendorf and 1% trifluoroacetic acid (TFA) was added to the gel pieces. After a 5-minute incubation, extracted peptides in TFA were combined with the peptides within the digestion mixture and stored at -80°C. Samples were then desalted using 200 μL C18 + Carbon TopTips (Glygen, Maryland, USA) as per the manufacturer's instructions, and eluted in 70% ACN containing 0.1% TFA. Samples were lyophilised by vacuum centrifugation before resuspension in 25 μL 2% ACN with 0.1% formic acid (FA) and transferred to low-volume LC-MS vials (Waters Corporation, Wilmslow, UK).

2.3.5.2. Liquid chromatography

LC-MS/MS was performed using a Waters Acquity H Class UPLC system coupled to a Waters Xevo G2-XS quadrupole time-of-flight (QToF) mass spectrometer (Waters Corporation, Wilmslow, UK). 5 μ L of each sample was injected using an autosampler which was set to a constant temperature of 10°C. Peptide separations were performed on an Acquity UPLC Peptide CSH C18 Column (130Å 1.7 μ M 2.1x100 mm column (Waters Corporation, Wilmslow, UK) set to 45°C. Liquid chromatography was performed with solvent A (100% Milli-Q water with 0.1% FA) and solvent B (100% ACN with 0.1% FA). Peptides were eluted at 0.2 mL/min, with the chromatography method shown below in Table 2-II.

Table 2-II: Liquid chromatography method for LC-MS^e analysis of whole cell lysates of cell lines.

Time (mins)	% A	% B
Initial	97	3
61	5	95
62	2	98
64	2	98
64.1	97	3
66	97	3

2.3.5.3. Mass spectrometry

Electrospray ionization was performed in positive polarity mode with the following source settings: capillary voltage 3 kV; sampling cone 40 V; source offset 80 V; source temperature 100°C; desolvation gas temperature of 250°C and flow of 600 L/h; cone gas flow 50 L/h. An internal lockspray calibrant of 200 pg/ μ L leucine enkephalin of m/z 556.2771 was injected every 30 seconds to aid in data alignment. The mass spectrometer was operated in the data-independent MS^e mode to fragment all ions and detect all fragment ions within a mass range of 100 - 2000 m/z with a 0.2 second survey scan time, an MS/MS scan time of 1 second, and a parent ion transfer collision

energy alternating between 10 and 30 V. Raw mass spectrometry data was collected using MassLynx v4.1 software.

2.3.5.4. Data analysis

High-resolution, profile MS^e data was analysed using Progenesis Q1 for Proteomics (version 4.2) (Nonlinear Dynamics by Waters Corporation, Wilmslow, UK) and uploaded in the form of .raw files. All samples were aligned with a score of over 90% alignment with the software-selected reference sample from the sample set. Peptides were identified via a limited database analysis method whereby a FASTA file containing the canonical and isoform sequences of human lamin A/C, lamin B1 and lamin B2 (Uniprot accession number P02545, P20700 and Q03252 respectively) were used to match m/z values to human nuclear lamin peptide sequences, with trypsin as the digestion reagent and up to two missed cleavages allowed. A maximum protein mass of 250 kDa was chosen with peptide and fragment tolerances set to less than 10 ppm and the false discovery rate (FDR) set to less than 4%. Variable modifications included oxidated methionine, while fixed modifications included carboxymethylated cysteine. Ion matching requirements included three fragments per peptide, seven fragments per protein, and one peptide per protein. Only peptides with charge states of 2+ or more and which were quantifiable in all samples analysed were included in the final analysis. Relative quantitation using non-conflicting peptides was chosen as the quantitation method, as there are common peptide sequences within the different human nuclear lamin isoforms. The ion abundance measurements of peptides relating to the target protein were used to quantify the expression of the target protein in each sample.

2.4. Results and Discussion

2.4.1. Protein Expression of Nuclear Lamins in Cells of Different Tissue Stiffnesses

2.4.1.1. Western Blot Analysis

With respect to the protein expression of nuclear lamin isoforms in cell lines representative of different tissue stiffnesses, the previous literature suggests that cells residing within tissues with a greater stiffness expressed more lamin A/C whereas B-type lamin expression remained similar across different tissue stiffnesses (Swift et al., 2013). WB analysis was performed on whole cell lysates of cell lines representative of cells within different tissue stiffnesses to investigate the protein expression of A-type and B-type lamins. The BL-2 cell line was derived from the malignant transformation of activated B-cells which reside within the secondary lymphoid tissues of the body such as the lymph nodes and spleen, and these tissues are considered soft and spongy in nature. HEK293T cells are a human embryonic kidney cell line and are representative of cells residing within the human kidney, which is comprised of soft tissue with a fibrous renal capsule. The SW1353 cell line is a chondrosarcoma cell line, which is the cancerous growth of cartilage cells. Cartilage is generally considered a hard and stiff type of connective tissue.

Figure 2-1 shows the densitometry results of analysing lamin A and C protein expression in cell lines representative of different tissue stiffnesses and representative images of the lamin A and lamin C protein bands produced by WB analysis.

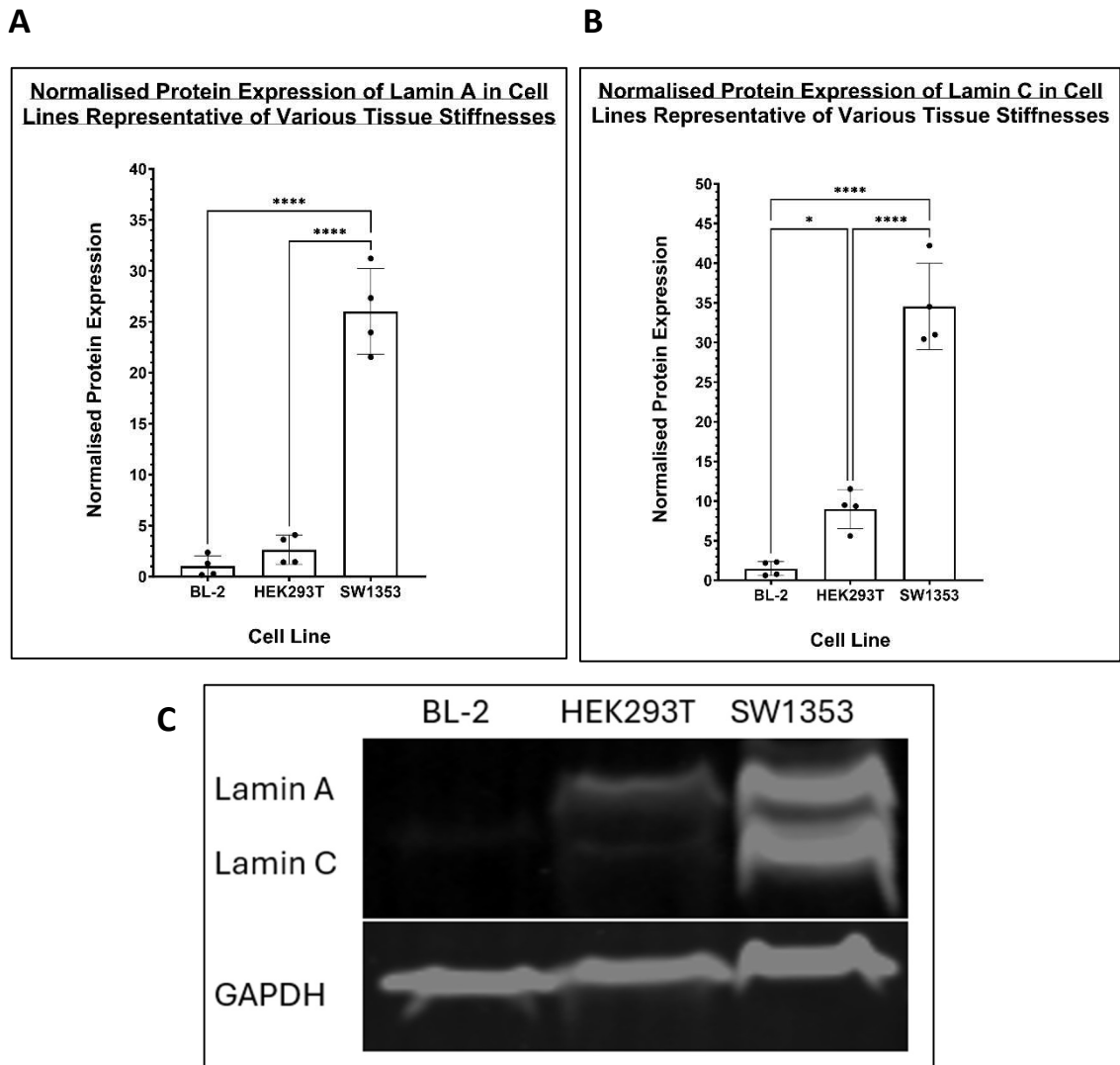


Figure 2-1: Densitometry analysis of the protein expression of A) lamin A and B) lamin C in cell lines representative of various cell stiffnesses (n=4). C) Representative protein bands resulting from Western Blot analysis of lamin A and lamin C.

Expression of each target protein band was normalised to the expression of GAPDH housekeeping protein. Error bars indicate standard deviation (SD). Lamin A (A): One-way ANOVA $p < 0.0001$. Tukey's multiple comparisons test. BL-2 vs HEK293T $p = 0.6713$. BL-2 vs SW1353 $p < 0.0001$. HEK293T vs SW1353 $p < 0.0001$. Lamin C (B) One-way ANOVA $p < 0.0001$. Tukey's multiple comparisons test: BL-2 vs HEK293T $p = 0.0335$. BL-2 vs SW1353 $p < 0.0001$. HEK293T vs SW1353 $p < 0.0001$.

The data in Figure 2-1A and Figure 2-1B shows the expression of both lamin A and lamin C in BL-2 cells is barely detectible, with moderate expression observed in

HEK293T cells, and high expression seen in the SW1353 cell line. Statistical analysis via a one-way ANOVA with Geisser-Greenhouse correction of the results showed a significant increase in the protein expression of both lamin A and lamin C across all three cell lines ($p < 0.0001$ for both isoforms). Post-hoc analysis via Tukey multiple comparisons test showed there was a significant difference in the expression of lamin A when comparing the SW1353 cell line with both the BL-2 and HEK293T cell lines, whereas there was no significant difference in the expression of lamin A when comparing BL-2 cells to HEK293T cells. Conversely, Tukey's multiple comparison test of lamin C expression suggests a significant difference in the expression of lamin C when comparing BL-2 cells to HEK293T cells. There was also a significant difference in the expression of lamin C when comparing the SW1353 cells to both the BL-2 and HEK293T cell lines.

Early studies on nuclear lamin expression in haematopoietic cells showed the expression of A-type lamins is below the limit of detection in resting B-cells (Contu et al., 2018; Paulin-Levasseur et al., 1989; Röber et al., 1990), but increases upon activation (Contu et al., 2018). In the case of lymphomas, lamin A/C protein expression has been found to be upregulated in Hodgkin lymphoma (Contu et al., 2018; Jansen et al., 1997; Saez et al., 2020) but was considered undetectable in non-Hodgkin lymphoma (Stadelmann et al., 1990). BL-2 cells are representative of Burkitt lymphoma, which is the malignant transformation of activated B-cells and a type of non-Hodgkin lymphoma, and so the expression of lamin A and C in these cells was expected to be below the limit of detection. The results in Figure 2-1 show that lamin A and C were detected in these cells, albeit at low levels.

With respect to each A-type lamin isoform, lamin C appears to be slightly higher all three cell lines examined compared to lamin A. Lamin A is thought to play a more dominant role in influencing nuclear stiffness and mechanotransduction (Lammerding et al., 2004, 2006; Swift et al., 2013), while lamin C has been shown to correlate with cortical stiffness and cellular elasticity (González-Cruz et al., 2018a, 2018b; Vahabikashi et al., 2022b; Vortmeyer-Krause et al., 2020). This suggests that lamin C is more involved in the stiffness of the cytoplasm, while lamin A influences the stiffness of the

nucleus. Despite this, both lamin A and C follow the same pattern of expression across these cell lines.

The results of Figure 2-1 support previous findings that cells within stiffer tissue microenvironments express more lamin A/C, while cells within softer tissues express less lamin A/C (Swift et al., 2013). The expression of lamin A/C in HEK293T cells is more similar to that of BL-2 cells, while expression in SW1353 cells is high compared to the other two cell lines. HEK293T has been described as a less differentiated cell line due to its embryonic origin (Piekarowicz et al., 2017) and studies have shown that lamin A/C levels are lower in undifferentiated cells and high lamin A/C expression is observed in more differentiated cell types (Constantinescu et al., 2006; Frock et al., 2006). Consequently, it is unsurprising that lamin A/C protein expression in HEK293T cells is similar to that of BL-2 cells. The study, upon which this experiment aimed to recreate, examined kidney cells isolated from mice and not an embryonic kidney cell line (Swift et al., 2013) and therefore this may explain why the results in Figure 2-1 differ slightly from previously reported findings.

Next, the protein expression of lamin B1 was examined. Figure 2-2 below shows the densitometry results and an image of the protein band produced by WB analysis of the expression of lamin B1 in the three cell lines representing cells within tissues of different stiffnesses.

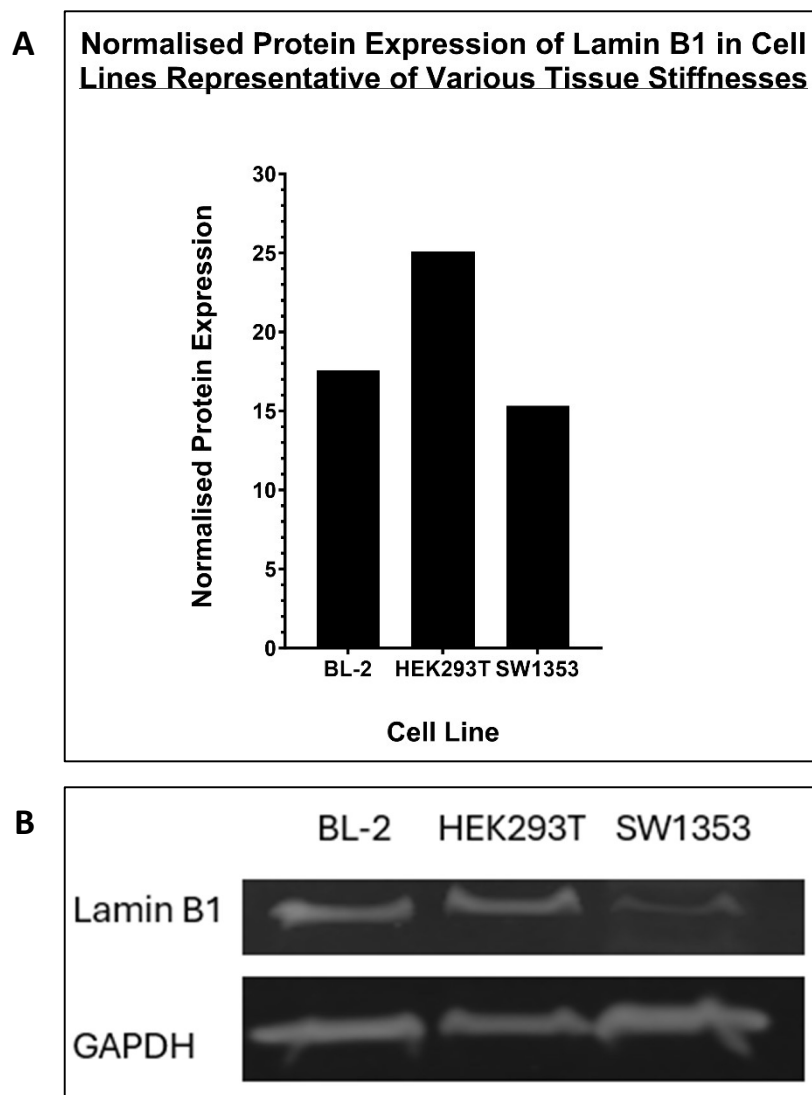


Figure 2-2: A) Densitometry analysis of the protein expression and B) protein bands resulting from Western Blot analysis of lamin B1 in cell lines representative of various cell stiffnesses (n=1). Expression of lamin B1 protein was normalised to the expression of GAPDH housekeeping protein.

The results in Figure 2-2 suggest that HEK293T may have the greatest protein expression of lamin B1 out of the three cell lines analysed. HEK293T cells are embryonic, which may explain their relatively high expression of lamin B1 compared to the two more mature cell lines, as previous literature suggests that lamin B1 protein expression is high in embryonic cells (Constantinescu et al., 2006). The expression of lamin B1 in BL-2 and SW1353 cell lines is similar, which supports previous findings that lamin B1 expression does not vary much across different cell lines. The slight variation between BL-2 and SW1353 cell lines with respect to lamin B1 expression may be

explained by their transformed nature, as both cell lines are representative of cancer cells, and the literature suggests that the expression of nuclear lamins can differ in malignant cells (Section 1.4). The variation also may be an artefact of the analysis of only one biological replicate. The results in Figure 2-2 are considered preliminary due to the low number of biological replicates analysed and thus, no statistical analysis was performed.

Finally, the expression of lamin B2 in cell lines representing cells within tissues of various stiffnesses was investigated via WB analysis. Figure 2-3 below shows the protein expression of lamin B2 in these three cell lines.

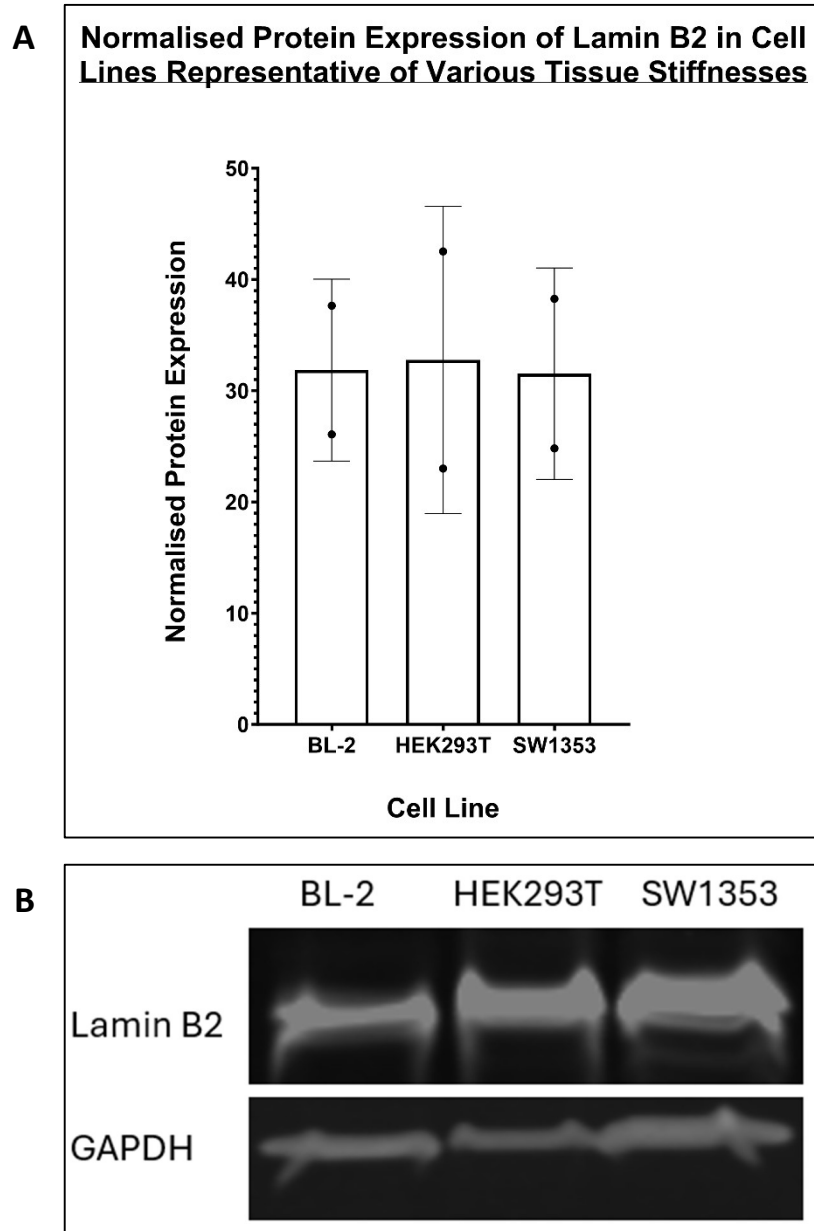


Figure 2-3: A) Densitometry analysis of the protein expression and B) representative protein bands resulting from Western Blot analysis of lamin B2 in cell lines representative of various cell stiffnesses (n=2). Expression of lamin B2 protein was normalised to the expression of GAPDH housekeeping protein. Error bars indicate standard deviation.

The results in Figure 2-3 suggest that there is no change in the expression of lamin B2 protein in cell lines representative of different tissue stiffnesses, in agreement with

previous literature showing that lamin B2 expression varies the least across different cell and tissue types (Swift et al., 2013). Similarly to the experiment measuring the expression of lamin B1 in these cell lines, no statistical analysis was performed on this data due to a low number of biological replicates.

2.4.1.2. LC-MS^e Analysis

To further support the WB examination of nuclear lamins in cell lines representative of different tissue stiffnesses, examination of the expression of nuclear lamin isoforms was performed using mass spectrometry. LC-MS^e analysis resulted in 6 fragment ions used to quantify lamin-A/C (Uniprot accession: P02545) (see Appendix III Table 8-II for more information on the peptides used in this quantitation analysis).

Figure 2-4 shows the abundance of lamin A/C protein in whole cell lysates of cell lines representative of different tissue stiffnesses measured by LC-MS^e analysis and represented as the raw abundance of ions counted for the tryptic peptides correlating to lamin A/C.

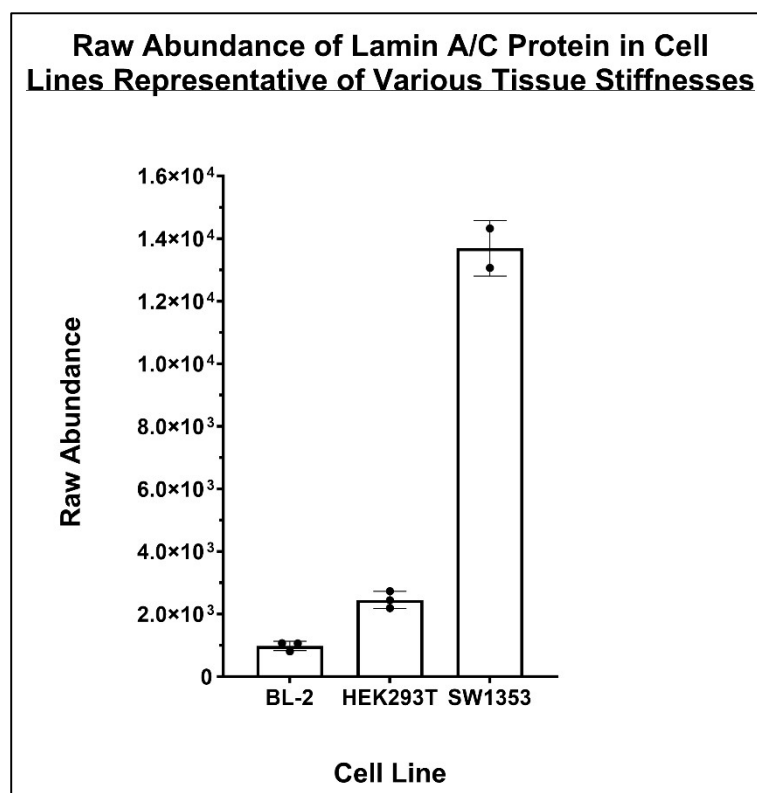


Figure 2-4: Raw abundance of lamin A/C protein in cell lines representative of different stiffnesses (BL-2 n=3, HEK293T n=3, SW1353 n=2). The mean of the replicates is plotted as bar charts with error bars indicating standard deviation.

The results in Figure 2-4 show a low abundance of peptide ions related to lamin A/C protein in BL-2 cells, and a greater amount observed in HEK293T cells. The greatest abundance of lamin A/C peptide ions was found in SW1353 cells. Due to a low number of technical replicates for the SW1353 cell line, statistical analysis could not be conducted. This suggests that increasing lamin A/C protein expression may scale with tissue stiffness, which would correlate with the WB results shown in Figure 2-1 and previously published data showing lamin A/C protein expression increases in tissues with increasing stiffnesses (Swift et al., 2013).

The LC-MS^e analysis did not detect the presence of the lamin B1 or lamin B2 isoforms in these cell lines. This may be due to ion suppression of the target peptide ions from other peptide ions within the complex whole cell lysate samples. The concentration of

protein analysed in this experiment may also be too low to accurately detect nuclear lamin isoforms. Additionally, the ion matching requirements and tolerance settings used to quantify proteins using Progenesis QI for Proteomics may be too stringent, suggesting changes to the software default settings, better instrument calibration, and better ionisation efficiency through optimising electrospray ionisation source settings may be necessary. Additionally, a more targeted mass spectrometry approach may be more suitable for increasing selectivity of peptide ions specific for quantitation of nuclear lamins.

2.4.1.3. Summary

The proteomic results show that the expression of nuclear lamins differs in cells of different tissue stiffnesses. WB and LC-MS^e data suggest that the expression of lamin A/C is relatively low in undifferentiated cells and in cells residing in softer tissues such as was observed in the HEK293T human embryonic kidney cell line and the BL-2 Burkitt lymphoma cell line respectively, while the expression of A-type lamins in SW1353 chondrosarcoma cell line was high. This was expected due to the stiff cartilage tissue in which chondrocytes reside (Figure 2-1 and Figure 2-4 respectively). This correlates well with previous data in this field that shows cells residing in softer tissues have a lower protein expression of lamin A/C while cells within stiff tissues have a higher lamin A/C protein expression (Swift et al., 2013). When investigating each A-type lamin individually, the data seems to suggest that there is a slightly greater expression of lamin C than lamin A, however the same pattern of increasing expression with increasing cell stiffness is seen across the three cell lines examined (Figure 2-1).

Lamins B1 and B2 were undetectable by MS analysis, but WB analysis suggests that there is no notable change in the expression of lamin B2 lamins across cells of different tissue stiffnesses (Figure 2-3), while HEK293T cells had a higher lamin B1 expression than the other two cell lines, possibly due to their embryonic origin (Figure 2-2). Results obtained for the expression of lamin B2 across cells from different tissue stiffnesses agree with previous publications from this field which report no significant change in protein expression across different tissue types (Swift et al., 2013).

2.4.2. Protein Expression of Nuclear Lamins in Malignant B-Lymphocytes

2.4.2.1. Western Blot Analysis

As a result of Klymenko and colleague's findings which showed decreases in lamin B1 expression upon B-cell activation, and suggested that low lamin B1 expression plays an important role as a prognostic biomarker in CLL (Klymenko et al., 2018), the hypothesis for this study was that BL and MM cells all have a similarly low lamin B1 expression, and low lamin B1 expression is a hallmark of cancers arising from previously activated B-cells that have been locked in a low-lamin B1 state after B-cell activation. The expression of lamin A/C and lamin B2 may also be similar across these cell lines, however there is currently no literature on this topic. WB analysis was performed on whole cell lysates of cell lines representative of BL and MM. These cell lines include the BL cell line BL-2, and two MM cell lines, OPM-2 and U266, representative of IgG lambda and IgE lambda MM respectively. Figure 2-5 below shows the protein expression of lamin A/C in these cell lines as determined by WB analysis.

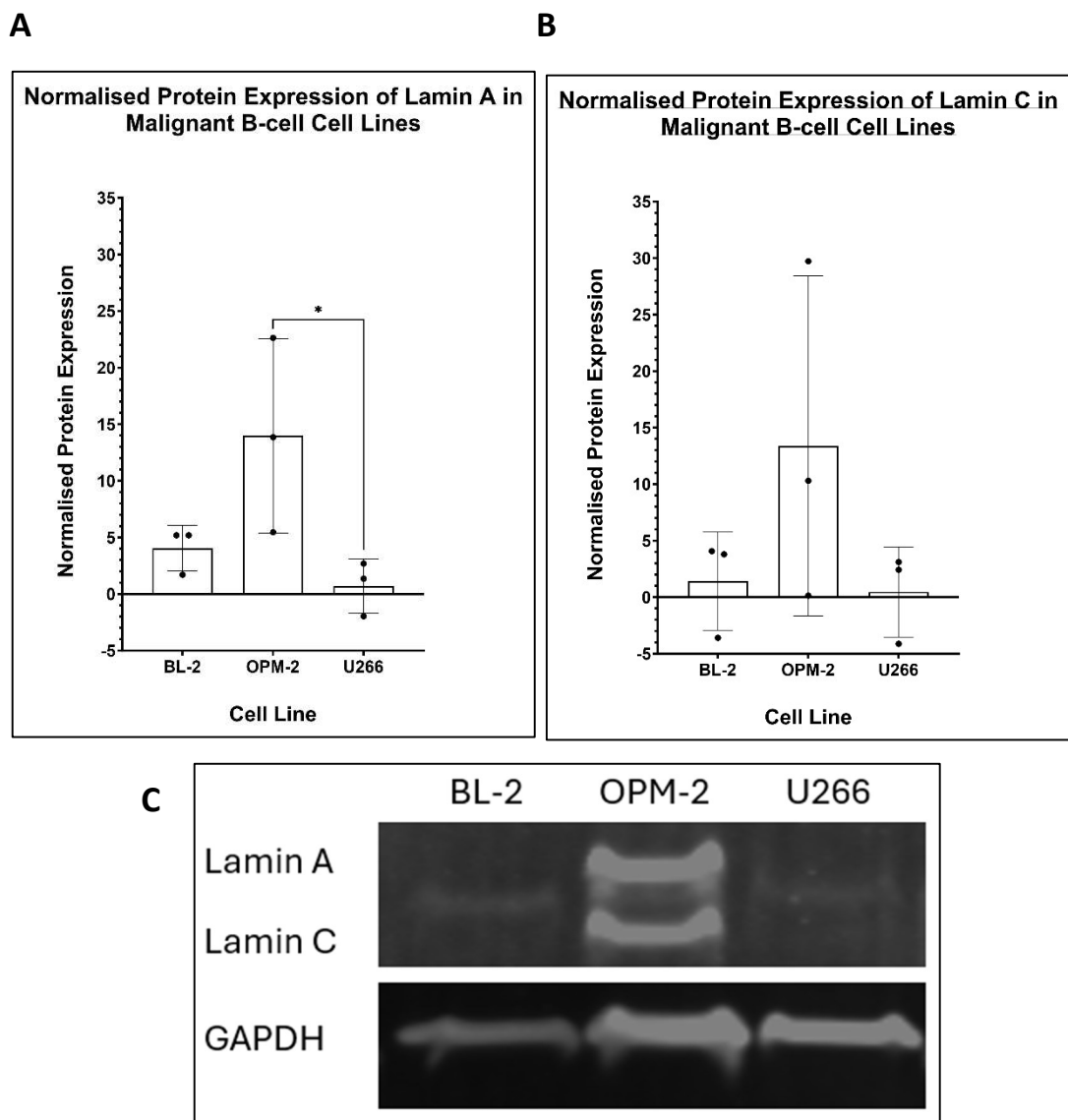


Figure 2-5: Densitometry analysis of the protein expression of A) lamin A and B) lamin C in malignant B-lymphocyte cell lines (n=3). C) Representative protein bands resulting from Western Blot analysis of lamin A and lamin C. The expression of lamin A and lamin C was normalised to the expression of GAPDH. Error bars indicate standard deviation. (One-way ANOVA of lamin A expression, $p=0.0499$, *. Tukey's multiple comparisons post-hoc BL-2 vs OPM-2 $p=0.1300$. BL-2 vs U266 $p=0.7312$. OPM-2 vs U266 $p=0.0490$. One-way ANOVA of lamin C expression, $p=0.2460$. Tukey's multiple comparisons post-hoc BL-2 vs OPM-2 $p=0.3269$. BL-2 vs U266 $p=0.9913$. OPM-2 vs U266 $p=0.2811$).

The results shown in Figure 2-5A and Figure 2-5B and Figure 2-5C suggest that the protein expression of lamin A and lamin C is greater in OPM-2 MM cells compared to BL-2 lymphoma cells and U266 MM cells. This finding is particularly interesting, as all cell lines are representative of malignant, activated B-cells and even between different subtypes of MM, the expression of both A-type lamins varies greatly. Figure 2-5C shows a protein band of a molecular weight which is between lamin A and lamin C. This band is the residual signal of lamin B1 (molecular weight 68 kDa) which was probed previously on the same PVDF membrane, and this signal therefore does not indicate or contribute to the protein expression of lamin A or lamin C which have a molecular weight of 70 and 61 kDa respectively.

Statistical analysis via one-way ANOVA of the results shown in Figure 2-5A suggests there was a significant change in lamin A protein expression in cell lines representative of B-cell malignancies ($p=0.0499$). Tukey's multiple comparisons post-hoc analysis suggested there was a significant difference in the expression of lamin A when comparing the OPM-2 to the U266 cell line. Considering lamin C, Figure 2-5B suggests there is no significant difference across the three cell lines ($p=0.2460$). This is possibly due to the large error bars observed between biological replicates.

It has been shown that, unlike in solid tissues where the expression of A-type lamins is often scales with tissue stiffness (Swift et al., 2013), the expression of A-type lamins in haematopoietic cells varies much less (Shin et al., 2013). This may be the case for healthy haematopoietic cells however, this does not seem to be the case for malignant haematopoietic cells. Furthermore, as discussed in Section 1.4, a review of the literature suggests that it is important to consider the cancer subtype when examining the expression of nuclear lamins. The OPM-2 cell line was derived from the peripheral blood of an MM patient with IgG lambda subtype in leukaemic phase (Katagiri et al., 1985). Studies have shown that the most common type of heavy chain produced in myeloma is IgG (Bal et al., 2022). Conversely, the U266 cell line was established from the peripheral blood of an MM patient with IgE lambda subtype (Nilsson et al., 1970). IgE is considered a rare subtype of MM, which has been documented to make up 0.1% of cases of MM (Hejl et al., 2018), however the Canadian Cancer Society reports that

IgE is the most aggressive type of MM and is known to progress to plasma cell leukaemia (PCL) or spread outside the bone marrow quickly (Canadian Cancer Society, 2024). This ability to spread outside the bone marrow may be influenced by the significantly lower expression of lamin A/C found in this cell line compared to the other MM cell line, as metastasis has been linked with low lamin A/C expression in multiple cancer types, as discussed in Section 1.4. In addition, IgE subtype MM is shown to have a higher rate of disease progression to PCL of all MM subtypes (Galakhoff et al., 2020; Jákó et al., 1997). IgE is also associated with poor prognosis (Macro et al., 1999), however researchers have discussed the delayed diagnosis of rarer MM subtypes may result in a more aggressive clinical course. Therefore, the question of whether IgE MM has poorer prognosis is a current subject of debate (Jiménez & Conejero, 2022).

With respect to AML, data from The Cancer Genome Atlas indicated that the expression of *LMNA* was downregulated in malignant cells compared to healthy cells (Wang et al., 2019a). Müller and colleagues found that most ALL cells expressed B-type lamins but only 5.8% of ALL cells expressed lamin A/C (Müller et al., 1994), suggesting that A-type lamin expression is low or undetectable in lymphoma. Conversely, A study by Tsai and colleagues measured the expression of lamin A/C in T-cell ALL compared to T-cell lymphoma and found that T-cell lymphoma samples had a significantly higher expression of lamin A/C than that observed in T-cell ALL blasts and in background normal T-cells (Tsai et al., 2020). Current literature suggests that inactive T- and B-cells express barely detectable amounts of lamin A/C (Contu et al., 2018; González-Granado et al., 2014), however the expression of lamin A/C increases upon lymphocyte activation (Contu et al., 2018; González-Granado et al., 2014; Rocha-Perugini & González-Granado, 2014). In addition, there are mixed results pertaining to the expression of lamin A/C in Hodgkin and non-Hodgkin lymphoma (Contu et al., 2018; Jansen et al., 1997; Stadelmann et al., 1990). This gives more support to the idea that the cancer subtype significantly affects the expression of nuclear lamins.

Moreover, since the expression of A-type lamins is commonly associated with differentiation, it would be expected that MM cell lines, derived from transformed plasma cells which are terminally-differentiated activated B-lymphocytes, would

express the highest amount of A-type lamins while the BL-2 cell line derived from malignant activated B-lymphocytes which have not yet transformed to plasma cells, would have a lower expression of A-type lamins than that seen in MM cells. This could suggest that lamin A/C downregulation in U266 cells compared to OPM-2 cells promotes a more aggressive disease subtype, as some cancers become less differentiated to avoid immune evasion which can lead to drug resistance and disease relapse (Li & Stanger, 2020). This de-differentiation has been observed previously in MM cells co-cultured with osteoclasts and lead to an apoptosis-resistant phenotype (Yaccoby, 2005).

Next, WB analysis was conducted on whole cell lysates of BL and MM cell lines to investigate the expression of B-type lamins. Figure 2-6 below shows the protein expression of lamin B1 in BL-2, OPM-2 and U266 cell lines.

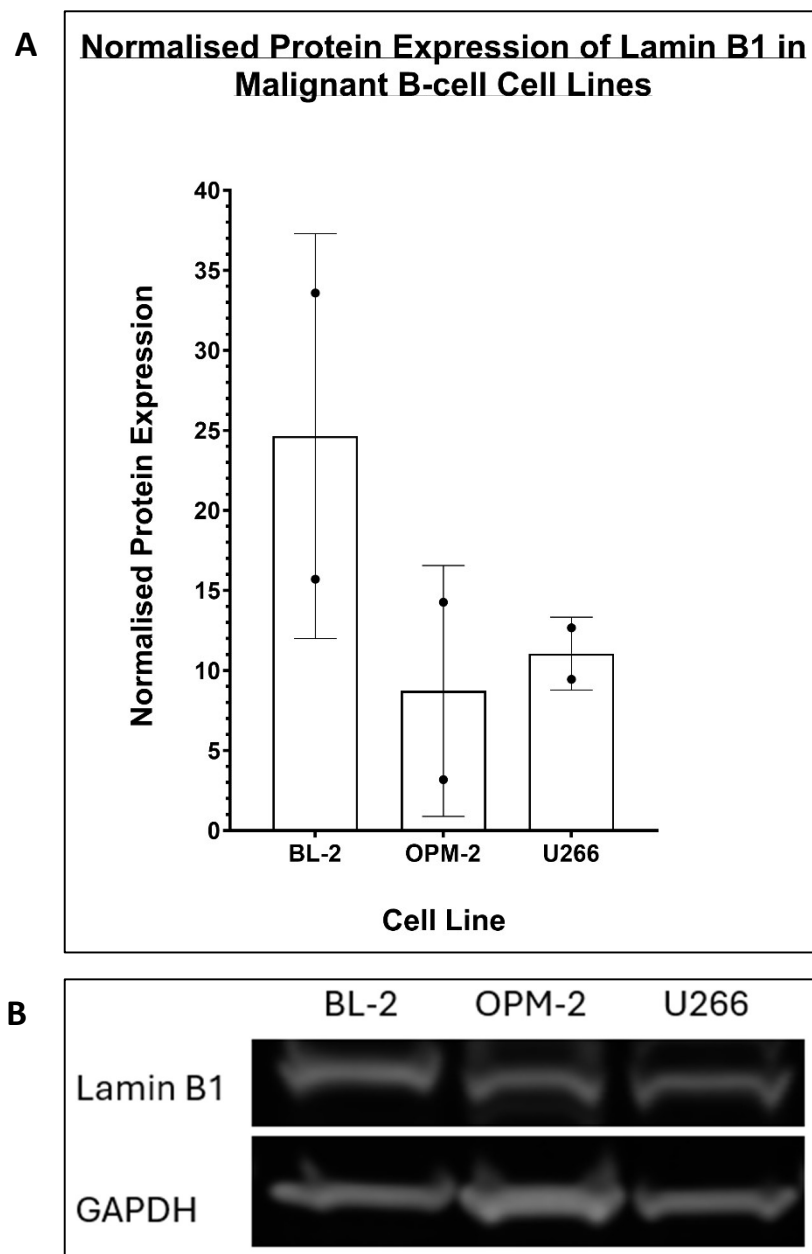


Figure 2-6: A) Densitometry analysis of the protein expression and B) representative protein bands resulting from Western Blot analysis of lamin B1 in malignant B-cell cell lines (n=2). The expression of lamin B1 was normalised to the expression of GAPDH. Error bars indicate standard deviation.

The results in Figure 2-6 suggest that the expression of lamin B1 is lower in MM cell lines compared to the BL-2 cell line. Both MM cell lines have a lower protein expression of lamin B1 than that seen in the BL-2 cell line. Additionally, the U266 cell line has a lower expression of lamin B1 than the OPM-2 cell line.

With respect to B-type lamins, Stadelmann investigated the mRNA expression of B-type lamins in ALL patients. Northern blot analysis found that B-type lamins were present in all ALL patient samples but were undetectable in normal resting peripheral blood lymphocytes (Stadelmann et al., 1990). Since the 1990s, the limits of detection have improved and the ability to differentiate between different B-type lamin isoforms has also improved. In addition, it has been shown that, unlike in solid tissues where the expression of B-type lamins is often constitutively expressed, or weakly scales with tissue stiffness (Swift et al., 2013), the expression of B-type lamins in haematopoietic cells can vary greatly (Shin et al., 2013).

In malignant lymphocytes, immunohistochemical analysis conducted by Klymenko and colleagues found a decrease in lamin B1 expression in lymphoid and non-lymphoid malignancies, including follicular lymphoma (FL) and chronic lymphocytic leukaemia (CLL), compared to cells within the intrafollicular areas of normal human reactive lymph nodes (Klymenko et al., 2018). Biopsies from 43 FL patients before and after FL transformation showed a significant decrease in lamin B1 protein expression in transformed FL biopsies compared to untransformed biopsies, as determined by immunohistochemical analysis (Klymenko et al., 2018). Furthermore, gene expression profile analysis of peripheral blood lymphocytes isolated from 337 previously untreated CLL patients were analysed by Q-RT-PCR and showed patients with lower *LMNB1* mRNA expression had a poorer prognosis compared to patients with higher *LMNB1* expression (Klymenko et al., 2018). These findings suggest that the downregulation of lamin B1 is associated with the progression of malignant transformation in B-lymphocytes and poor patient prognosis. Furthermore, there has recently been discussion about the loss of lamin B1 in the development of myelodysplastic syndrome (MDS) and AML (Papapetrou, 2022). A recent study by Reilly and colleagues showed a loss of lamin B1 expression in MDS and AML resulting from the loss of *LMNB1* gene due to deletion of chromosome 5q (Reilly et al., 2022).

The presence of lamin B1 has been detected in other MM cell lines, RPMI 8826 and OCI-MY5, by WB analysis, with seemingly slight variance in expression between the two cell lines (Guo et al., 2024). Although direct densitometry comparison between the

two cell lines was not displayed, Guo and colleagues' publication investigating the expression of lamin B1 in MM cell lines upon inhibition of EZH2 shows that different MM cell lines transfected with empty vectors have a slightly different expression in lamin B1, with RPMI 8226 cells expressing more lamin B1 protein than OCI-MY5 cells (Guo et al., 2024). RPMI 8226 is an IgG lambda light chain MM cell line similar to the OPM-2 cell line, while OCI-MY5 is an IgA lambda subtype of MM. This information suggests that IgG lambda subtype MM cells express higher levels of lamin B1 than IgA-secreting MM cells. Although IgG-secreting MM is considered the most common subtype (Bal et al., 2020; Kehl et al., 2022), the IgA subtype of MM is thought to have worse prognosis (Macro et al., 1999; Wang et al., 2018). In addition, the discovery that MM cell lines express less lamin B1 than BL cell lines could be due to MM cells being derived from a more mature B-cell, being transformed plasma cells, compared to BL cells which are a result of transformed, activated B-lymphocytes which have not yet matured to plasma cells despite their antigen activation, as lamin B1 has been shown to decrease upon differentiation (Wesley & Levy, 2023). Both MM cell lines expressed less lamin B1 protein than BL-2 cells, which may indicate that malignant transformation of more mature B-cells results in reduced levels of lamin B1 when compared to less mature transformed B-cells.

Finally, the expression of lamin B2 was investigated by WB analysis in the malignant B-cell cell lines and the densitometry results and a representative image of the protein bands are shown below in Figure 2-7.

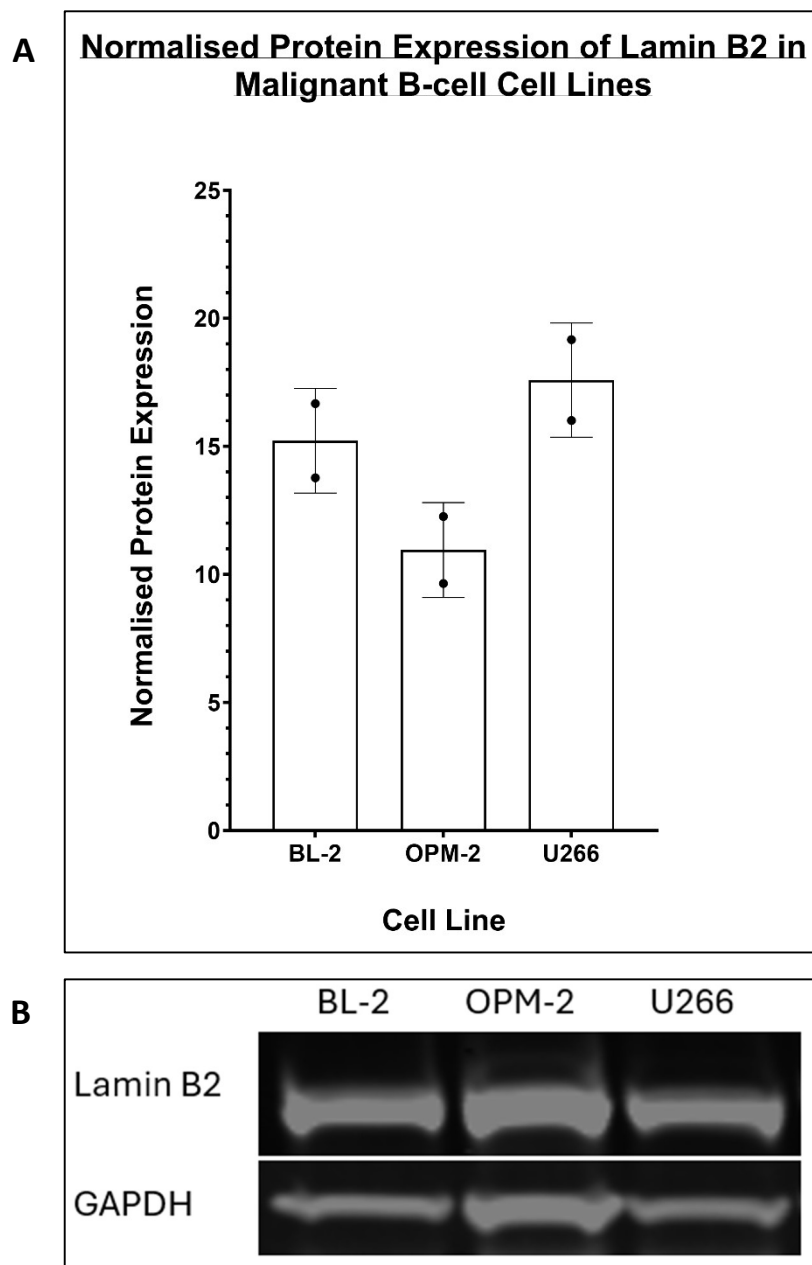


Figure 2-7: A) Densitometry analysis of the protein expression and B) representative protein bands resulting from Western Blot analysis of lamin B2 in malignant B-lymphocyte cell lines (n=2). The expression of lamin B2 was normalised to the expression of GAPDH. Error bars indicate standard deviation.

The result in Figure 2-7 suggest that, in contrast to the expression of lamin A/C and lamin B1 in MM cell lines, OPM-2 cells have the lowest lamin B2 expression while U266 cells have the highest lamin B2 expression.

U266 cells were shown to have the lowest expression of lamin A, lamin C and lamin B1 but the highest expression of lamin B2. It may be reasonable to suspect that an increase in lamin B2 in this cell line may be due to the cell's need to compensate the lack of other lamin isoforms to maintain nuclear integrity, as Figure 2-5 and Figure 2-6 showed that U266 cells has low lamin A/C and low lamin B1 expression respectively. This is supported by previous research that suggests that lamin B2, in addition to the other lamin isoforms, plays a role in the maintenance of the nuclear envelope and nucleolar morphology (Sen Gupta & Sengupta, 2017) and provide mechanical and structural integrity to the nucleus (Coffinier et al., 2011). This implies a fine balance between the expression of nuclear lamins is needed to provide proper nuclear structure and function.

Previous studies have suggested that lamin B2 follows lamin A/C-mediated nuclear mechanics (Vortmeyer-Krause et al., 2020), however this observation was not noted in BL and MM cell lines. In fact, the opposite effect was seen, with lamin B2 expression showing a somewhat inverse correlation to the expression of lamin A/C, particularly when examining the two MM cell lines. Considering that the U266 cell line is representative of a more aggressive subtype of MM, low expression of nuclear lamins might aid in nuclear deformability which may increase metastatic potential but also may influence the expression of oncogenes due to their role in gene expression (Dechat et al., 2008; Kim et al., 2019; Prokocimer et al., 2009; Shimi et al., 2008).

As mentioned for lamin B1, previous literature on solid tissues shows the expression of B-type lamins is often constitutively expressed, or weakly scales with tissue stiffness (Swift et al., 2013). In contrast, the expression of B-type lamins in haematopoietic cells can vary greatly (Shin et al., 2013). This variation is observed in Figure 2-7 with respect to differences in lamin B2 expression across cell lines representative of different B-cell cancers.

A previous study investigating the expression of lamin B2 in haematological malignancies identified two protein bands using two monoclonal antibodies, and found that that the expression of lamin B2A, the higher molecular weight protein, was

greater in ALL and AML samples compared to peripheral blood mononuclear cells. (Meier et al., 1997). No such second lamin B2 protein band was observed in this thesis (data not shown), which may suggest the previous literature experienced off-target antibody binding, or a second lamin B2 band was observed due to modification or fragmentation of the lamin B2 protein either during the experimental process or as a result of malignant transformation in the cell types analysed.

2.4.2.2. LC-MS^e Analysis

Further proteomics experiments in the form of LC-MS^e analysis was performed on whole cell lysates to examine the expression of nuclear lamin isoforms in cell lines representative of B-cell malignancies and support the WB data shown above. The LC-MS^e data analysis pipeline resulted 20 fragment ions used to quantify lamin A/C and 5 fragment ions used for lamin B1 quantitation (see Appendix III Table 8-III and Table 8-IV more information on the peptides used in this quantitation analysis). Figure 2-8 shows the raw abundance of lamin A/C protein expression in cell lines.

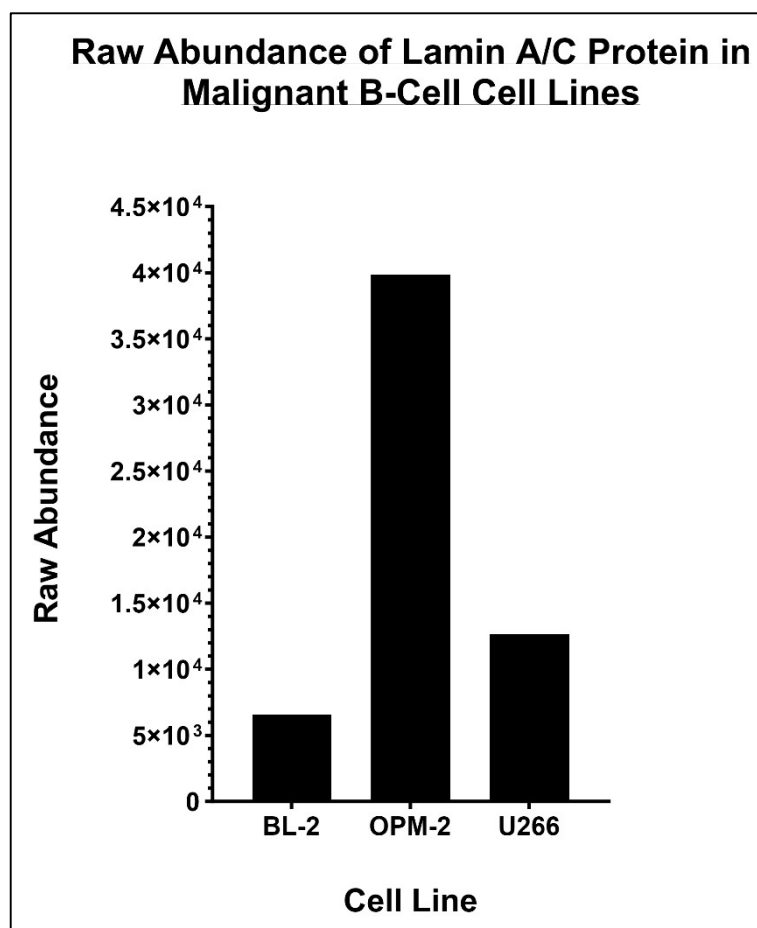


Figure 2-8: Raw abundance of lamin A/C protein in cell lines representative of B-cell malignancies (n=1).

Figure 2-8 shows the preliminary data obtained when investigating the ion abundance of lamin A/C peptides in the three B-cell cancer cell lines. Statistical analysis could not be performed due to the low number of biological replicates for this experiment. The expression of lamin A/C as determined by MS^e analysis is similar to the results obtained by WB analysis shown in Figure 2-5. Both experiments suggest that the expression of lamin A/C is the highest in the OPM-2 cell line, and lower in the BL-2 and U266 cell lines. More biological replicates are necessary to confirm the accuracy of this finding and provide statistical significance to the data.

To examine the expression of lamin B1 in these cell lines, Figure 2-9 below shows the raw abundance of lamin B1 in cell lines of B-cell malignancies.

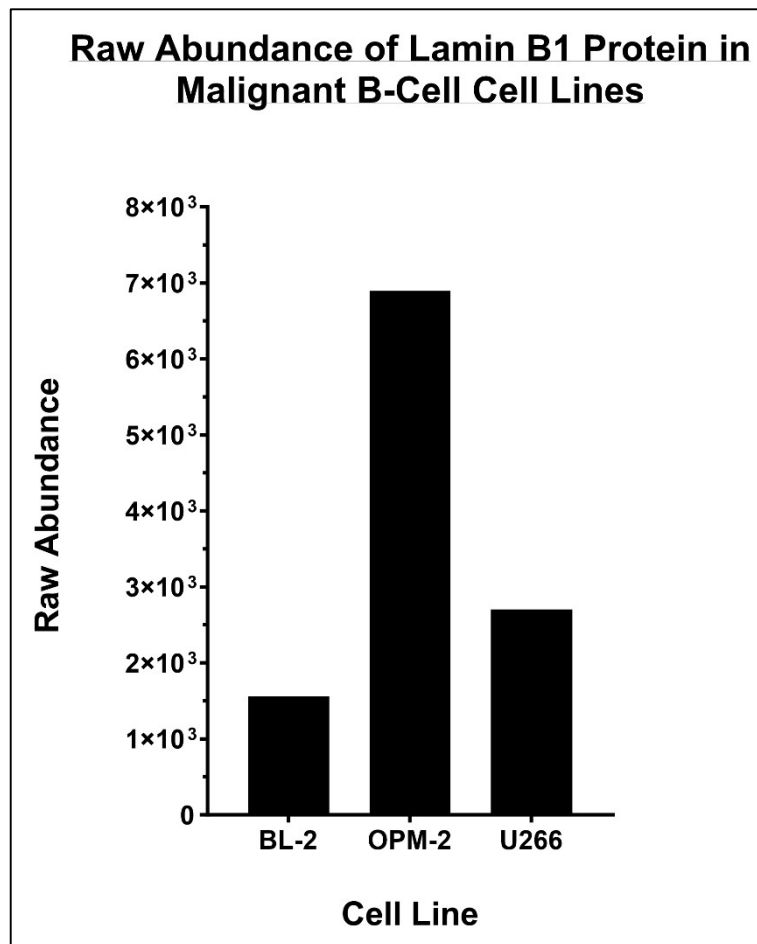


Figure 2-9: Raw abundance of lamin B1 protein in cell lines representative of B-cell malignancies (n=1).

The results in Figure 2-9 suggest a similar pattern of nuclear lamin expression than that observed for lamin A/C in these cell lines in Figure 2-8: that there is a high expression of lamin B1 in OPM-2 cells and a lower expression seen in BL-2 cells and U266 cells, albeit there is a greater expression of lamin B1 in both MM cell lines when compared to the BL cell line. Again, statistical analysis could not be performed due the low number of biological replicates for this experiment and more replicates are required to provide a more accurate result for the expression of lamin B1 in malignant B-lymphocytes.

The LC-MS^e data for the expression of lamin A/C and lamin B1 follow the same overall pattern, with high levels of both lamin A/C and lamin B1 seen in OPM-2 cells and less protein expression seen in BL-2 and U266 cells. Additionally, the LC-MS^e data for the expression of lamin B1 detected by LC-MS^e in these cell lines is not in agreement with the WB data acquired in Figure 2-6. This could be due to the low abundance of peptides detected and measured via this MS method. The results shown in Figure 2-8 and Figure 2-9 suggest a raw abundance of peptide ions used to measure lamin A/C and lamin B1 of as low as 1.5×10^3 (Lamin B1 in BL-2 cells) up to 4×10^4 (lamin A/C in OPM-2 cells). As mass spectrometry is a sensitive method of quantifying ions, the number of ions detected for these peptides may not be seen in high enough quantities to differentiate the peptide signals from the background noise, which may be electrical noise, residual background ions within the mass analyser, or a result of other ions within the complex sample analysed. Further to more experimental replicates of the MS data, normalisation of the ion measurements to a known housekeeping protein or internal standard normalisation peptides may be advisable. Since actin has been shown to be aberrantly expressed in MM (Witjes et al., 2020), its use as housekeeping protein for normalisation purposes may be unsuitable.

As with the results obtained in Section 2.4.1 for the LC-MS^e analysis of nuclear lamins in cell lines of different stiffnesses, the analysis of B-lymphocyte malignant cell lines via LC-MS^e analysis did not detect the presence of the lamin B2 isoform. As mentioned in Section 2.4.3, it would be better to use a more sensitive mass spectrometry method to successfully detect all nuclear lamin isoforms in somatic cells and to further support WB data in elucidating the expression of nuclear lamins in B-cell malignancies.

2.4.2.3. Summary

When investigating the protein expression of different nuclear lamin isoforms in cell lines of different B-cell malignancies, the WB and LC-MS^e results shown above revealed that there are different levels of nuclear lamin protein expression across different types of B-cell malignancies. Data shown in Figure 2-5 and Figure 2-8 show that the expression of A-type lamins is notably higher in the OPM-2 IgG lambda-secreting MM

cell line compared to the expression of lamin A/C observed in the BL cell line and the MM IgE-lambda-secreting U266 cell line.

When examining the expression of B-type lamins in B-cell malignancies, the expression of lamin B1 showed a lower expression in MM cell lines compared to the BL cell line, (Figure 2-6). Conversely, the LC-MS^e data showed that OPM-2 had the greatest expression of lamin B1 out of all 3 cell lines examined (Figure 2-9), however more technical replicates are necessary to confirm the findings of this investigation, and this LC-MS^e analysis would also benefit from normalisation methods. The protein expression of lamin B2 was shown to be the highest in the U266 MM cell line, and the lowest in the OPM-2 cell line, with BL-2 cells expressing an amount between the two MM cell lines (Figure 2-7). This is of significant interest as U266 cells contained the lowest concentration of A-type lamins and OPM-2 contained the highest concentration of A-type lamins, as determined by WB analysis (Figure 2-5) and previous studies suggested that lamin B2 follows a similar pattern of expression to lamin A/C (Vortmeyer-Krause et al., 2020). As a result, this leads to speculations that U266 cells may compensate for the lack of lamin A/C and lamin B1 by increasing their expression of lamin B2 to maintain nuclear integrity. On the contrary, OPM-2 cells may decrease their expression of lamin B2 to account for their overexpression of lamin A/C, as the thickness of the nuclear lamina may adversely affect their metastatic ability, as has been previously examined in melanoma cells by artificially increasing the stiffness of the nuclear lamina by overexpression of the $\Delta 50$ LMNA transcript (Ribeiro et al., 2014). Overall, it is speculated that a fine balance between the expression of nuclear lamins is needed to maintain nuclear integrity and prevent nuclear rupture. The mechanisms by which A-type lamins are overexpressed in the OPM-2 IgG-lambda MM cell line, or how lamin A/C is under-expressed in the U266 IgE-lambda MM cell line, are yet to be elucidated.

To date, there have been no specific investigations in the published literature pertaining to the collective expression of each of the nuclear lamin isoforms in transformed B-cells, therefore the novel data provided in this section provides intriguing insights to the roles of nuclear lamins in the malignant transformation of

activated B-lymphocytes and plasma cells. The expression of lamin B1 in haematological diseases has been explored (Klymenko et al., 2018), however the data pertaining to the expression of A-type lamins and lamin B2 provides evidence that A-type lamins differ considerably between different transformed activated B-cells and between different subtypes of MM, and that the expression of A-type lamins in plasma cell neoplasms is different to the expression seen in BL. The data represented in this chapter could lead to significant advancements in the treatment of MM patients based on their disease subtype, as the vastly increase lamin A/C expression observed in OPM-2 cells could result in its use as a potential biomarker of disease in IgG-type MM.

2.4.3. Protein Expression of Nuclear Lamins in Malignant B-Lymphocytes

2.4.3.1. Western Blot Analysis

When investigating the expression of nuclear lamins in AID knockout BL-2 cell lines, it was hypothesised that the expression of AID would negatively affect the expression of lamin B1. Therefore, the hypothesis was that BL-2 AID^{-/-} cell lines contained more lamin B1 than BL-2 cells which have been previously shown to express AID (Faili et al., 2002a; Klymenko et al., 2018). To examine the relationship between nuclear lamin expression and AID expression, WB analysis was performed on nuclear fractions resulting from the subcellular fractionation of the BL-2 wild-type (BL-2 AID^{+/+}) and BL-2 AID^{-/-} cell lines. Knockout of AID expression was achieved through *AICDA* gene knockout via homologous recombination (Faili et al., 2002a). The densitometry results and images of the protein bands are shown below in

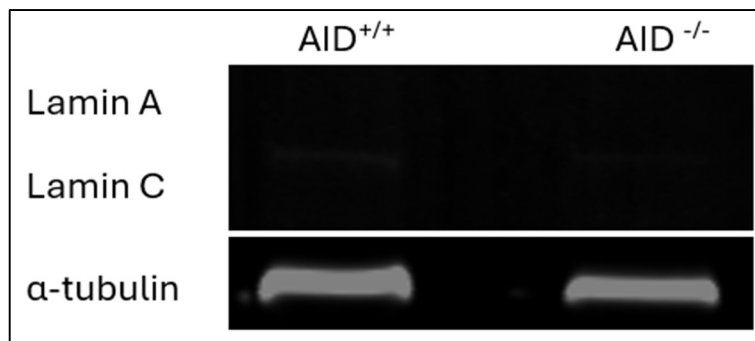


Figure 2-10.

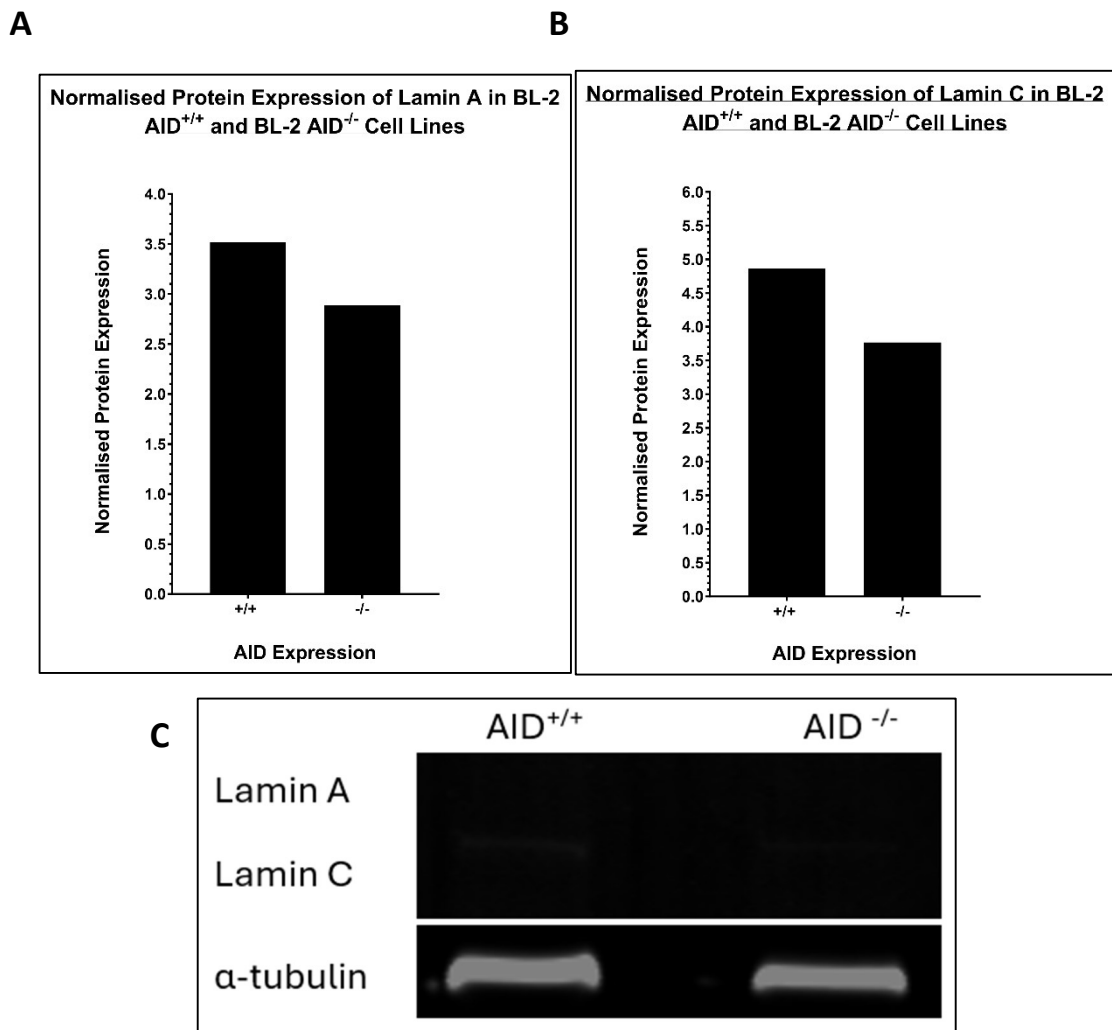


Figure 2-10: Densitometry analysis of the protein expression of A) lamin A and B) lamin C in BL-2 AID^{+/+} and BL-2 AID^{-/-} cell lines (n=1). C) Protein bands resulting from Western Blot analysis of lamin A and lamin C. The expression of lamin A and lamin C was normalised to the expression of α -tubulin.

The results in

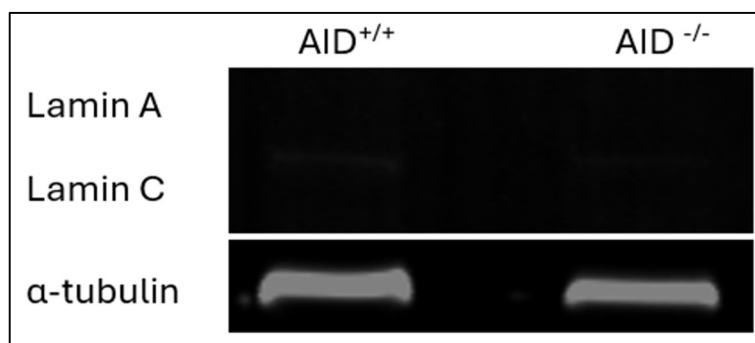


Figure 2-10 suggest that the expression of A-type lamins is barely detectible in BL-2 cells which do and do not express AID however, due to the low number of biological

replicates and very faint protein bands observed by Western Blot analysed, no conclusive remarks can be made.

Next, the expression of lamin B1 in BL-2 cells in the presence or absence of AID expression was investigated by WB analysis and the results are shown in Figure 2-11.

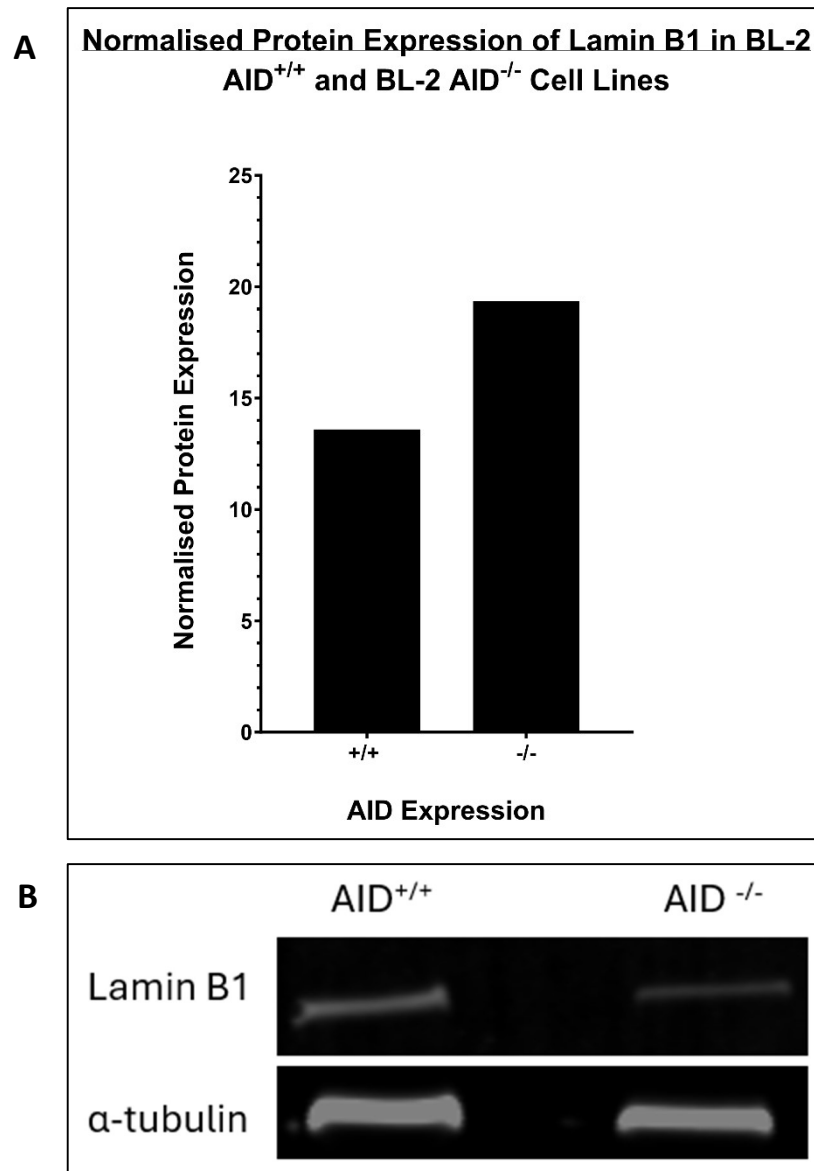


Figure 2-11: A) Densitometry analysis of the protein expression and B) protein bands resulting from Western Blot analysis of lamin B1 in BL-2 AID^{+/+} and BL-2 AID^{-/-} cell lines (n=1). The expression of lamin B2 was normalised to the expression of α-tubulin.

The results in Figure 2-11 suggest that a higher lamin B1 expression is observed in the BL-2 AID^{-/-} cell line compared to the BL-2-expressing cell line. The hypothesis of this study was that BL-2 AID^{-/-} cells would express more lamin A/C if increases in AID expression were in some way related to the decrease in lamin B1 observed in activated B cells, which has been reported in previous studies (Klymenko et al., 2018). The preliminary results in Figure 2-11 suggest this hypothesis may be true, however more biological replicates need to be assessed ~~in-order~~ to confirm this finding. The protein expression of lamin B2 was not investigated in these samples.

2.4.3.2. LC-MS^e analysis

Following WB analysis, LC-MS^e analysis was performed on whole cell lysate samples to examine the expression of nuclear lamin isoforms in the BL-2 cell line with and without gene expression of AID. Progenesis QI for Proteomics analysis and subsequent data filtering resulted in 2 fragment ions used for the quantitation of lamin B1 (see Appendix III Table 8-V for more information on these peptides). Lamin A/C and lamin B2 were not quantifiable in these samples by LC-MS^e analysis. Figure 2-12 shows the raw abundance of lamin B1 protein expression in these two cell lines, as measured by LC-MS^e analysis.

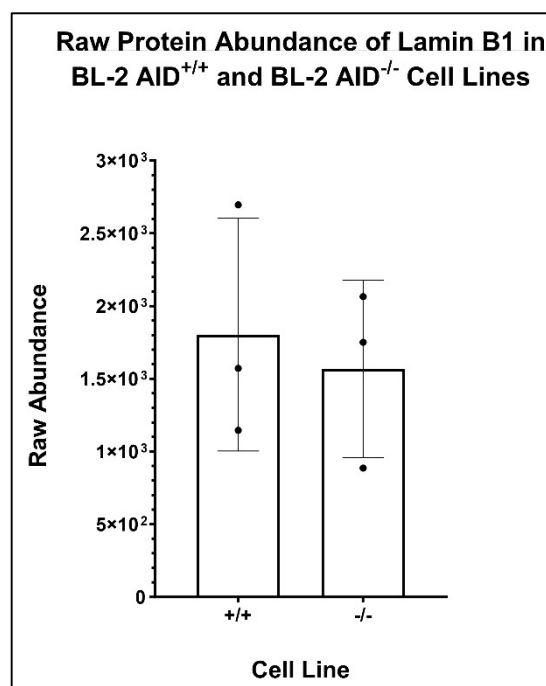


Figure 2-12: Raw abundance of lamin B1 protein in BL-2 AID^{+/+} and BL-2 AID^{-/-} cell lines (n=3). The mean of three technical replicates is plotted as bar charts with error bars indicating standard deviation. Unpaired T-test, p=0.7043.

The LC-MS^e results shown in Figure 2-12 suggest that there is no change in the expression of lamin B1 upon AID expression in the BL-2 cell line. An unpaired T-test showed that there was no significant difference in lamin B1 expression in BL-2 AID^{+/+} and BL-2 AID^{-/-} cell lines (p=0.7043).

2.4.3.3. Summary

When examining the expression of lamin A, B1 and C in the BL-2 cell line expressing AID and in the same cell line where *AICDA* gene expression was knocked out, the preliminary WB results suggest A-type lamin expression may decrease in the absence of AID expression (

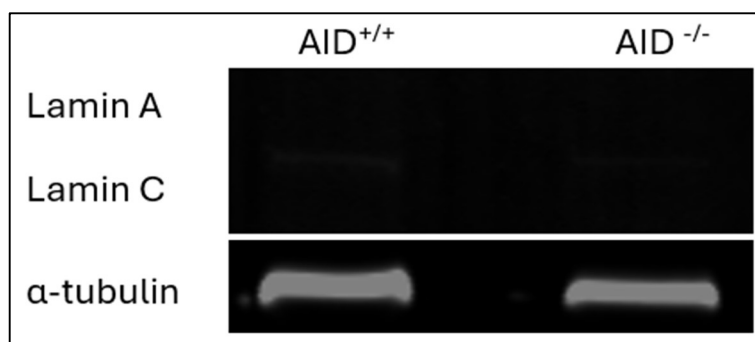


Figure 2-10) while lamin B1 may increase in expression (Figure 2-11). The results of the LC-MS^e analysis performed in with three biological replicate samples suggested that there was no significant difference in the protein expression of lamin B1 between these two cell lines (Figure 2-12). This finding rejects the hypothesis that AID expression results in the decreased lamin B1 expression observed in activated B-cells. It is important to consider that the BL-2 cell line is representative of transformed activated B-cells, and it is not known if this transformation affects the expression of AID, or other proteins involved in B-cell activation, when compared to non-transformed activated B-cells.

Furthermore, LC-MS^e analysis did not detect the presence of the nuclear lamin A/C or B2 isoforms, suggesting further sample processing steps are necessary to decrease the sample complexity, and analysis of a greater number of cells per sample may also increase the ion abundance of each isoform detected via this method. Additionally, amendments to the Progenesis QI for Proteomics search parameters and tolerances may be necessary to detect these isoforms in these samples.

2.5. Conclusion

The aim of this study was to investigate the protein expression of different nuclear lamin isoforms in human cell lines via WB and LC-MS^e analysis. This aim was not completely achieved in all studies due to a low number of biological replicates analysed. In addition, when conducting LC-MS^e analysis of whole cell lysates from a variety of cell lines in this chapter, lamin A/C was difficult to detect in studies involving the BL-2 cell line and other B-cell cell lines. Furthermore, lamin B2 was shown to be below the limit of quantitation in all LC-MS^e studies. This may be due to the rules used to quantify proteins when using Progenesis QI for Proteomics, whereby requirements for quantitation of a peptide ion included three fragments per peptide, seven fragments per protein, and one peptide per protein, a false discovery rate of 4% and a mass error of less than 10 ppm. If more lenient rules were applied during the data analysis stage, lamin B2 may be quantifiable however, caution must be taken during MS^e data analysis as increasing the false discovery rate and mass error of ions may lead to an increase in the number of false positive identifications of ions matched to peptide sequences.

When investigating nuclear lamin protein expression across different cell lines representative of different tissue stiffnesses (Section 2.4.1), the three cell lines examined were chosen as they were thought to collectively span a broad range of tissue stiffnesses. The results of this chapter aligned with previous literature in the field, suggesting cells residing within a stiff tissue microenvironment have a greater expression of lamin A/C while cells residing within soft tissues express less lamin A/C. Previous literature shows that lamin A increased 30-fold from soft to stiff tissue and scaled with tissue microelasticity while lamin B1 differed less than 3-fold across the same cell types (Swift et al., 2013). Considering B-type lamins, the relatively unchanged expression of lamin B2 across different cell lines examined in Section 2.4.1 supported previously published data (Swift et al., 2013). Conversely, lamin B1 protein expression was seemingly higher in HEK293T cells compared to BL-2 cells and SW1353 cells and may be explained by its undifferentiated nature.

When investigating the protein expression of nuclear lamin isoforms in B-cell malignancies in Section 2.4.2, it was found that the two MM cell lines expressed different levels of all nuclear lamin isoforms despite both being derived from transformed plasma cells. The OPM-2 IgG lambda-secreting MM cell line expressed high levels of A-type lamins compared to both BL-2 Burkitt lymphoma cells and U266 IgE lambda-secreting MM cells, highlighting this isoform as a potential biomarker of disease in this MM subtype. Conversely, the opposite expression pattern was observed when investigating the lamin B1 in these cell lines, with both MM cell lines expressed less lamin B1 protein than BL-2 cells, which could suggest that lamin B1 expression gradually decreases in malignancies of more differentiated B-cells. Lamin B1 protein abundance reported by LC-MS^e analysis seemed to show a similar expression pattern to lamin A/C, which could be an artefact of non-normalised protein loading. For more accurate normalisation, a mixture of peptides with known sequences can be spiked into the samples during processing to account for changes in the volume of sample and resulting peptide abundances can be normalised to the abundance of these spiked-in peptides. In addition, a pooled sample containing equal amounts of all samples can be processed alongside the experimental samples, which can be used as a reference sample during data analysis. Both approaches aim to provide better normalisation of data through accounting for sample losses during processing and instrumental variations between sample injections. The expression of lamin B2 in these cell lines showed variations across the different types of B-cell malignancies, possibly attributing to a compensatory mechanism of low lamin A/C and B1 expression, as was observed in U266 cells, or due to reasons yet unknown. Further investigation into the expression of lamin B2 in haematological malignancies may provide insights into its role in transformed cells.

Finally, the results reported in Section 2.4.3 suggest that lamin B1 protein expression is not affected by AID expression in BL-2 cells, as per the results of LC-MS^e analysis, and preliminary WB data may compliment this if more biological replicates were to be analysed. Preliminary WB data on lamin A/C protein expression in BL-2 AID^{+/+} and BL-2^{-/-} cells suggests this protein isoform is either not present or exists in very low

quantities in these two cell lines, but again, more biological replicates need to be analysed for conclusions to be made in this area.

Since the expression of nuclear lamins has been previously shown to affect the organisation of cytoplasmic proteins often used as housekeeping proteins such as α -tubulin and β -actin (Broers et al., 2004), and these two proteins are major components in maintaining cellular and tissue stiffness (Bunnell et al., 2011; McKenna et al., 2023), α -tubulin and β -actin were not used as housekeeping proteins for loading controls during Western Blot densitometry analysis. Instead, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping protein to normalise the expression of each nuclear lamin isoform in experiments where nuclear lamin A/C varied greatly between samples. GAPDH is a glycolytic enzyme involved in glucose metabolism and has not been shown to contribute to nuclear or cellular stiffness (Nicholls et al., 2012). As a result, this affected the number of technical replicates presented for these experiments. However, since the cell and tissue stiffness was not a main focus of the experiment which investigated the effect of AID expression on BL-2 cells (Section 2.4.3.1.), it was decided that α -tubulin could be used as a housekeeping protein for this experiment.

To conclude, this report has begun to investigate the expression of nuclear lamins in different cell types to elucidate the roles of nuclear lamins in mechanosensing, and in B-cell malignancies and B-cell activation. LC-MS^e analysis resulted in the limited detection of nuclear lamin isoforms across all human cell lines analysed. This suggests a need for a more sensitive method of nuclear lamin protein detection. More targeted methods should be used to investigate the expression of each nuclear lamin isoform in human cell lines. As a result, Chapter 3 will explore the development of a targeted mass spectrometry method to investigate the expression of each nuclear lamin isoform in biological samples of human and mouse origin.

2.6 Future Work

In this chapter, both whole cell lysates and nuclear fractions of cell pellets were used to investigate the expression of different nuclear lamin isoforms in cell lines to examine their roles in mechanosensing and in the malignant transformation and activation of B-cells. Unfortunately, the subcellular fractionation protocol was not reproducible, and future experiments could include optimisation of this method. Potential reasons for the lack of robustness and reproducibility with this method include the variations in the volume of lysis buffer necessary to disrupt the cell membranes of different cell types. In addition, the pH of lysis buffer seemed to have a significant effect on the effectiveness of cell lysis. Ideally, this protocol should be optimised for each cell line and for different amounts of cells collected per cell pellet. Optimisation of subcellular fractionation for different cell lines and cell numbers was attempted using the protocol described in Section 2.3.2.1 and using commercially available subcellular fractionation kits (data not shown), but yielded inconsistent results with limited success rates, therefore it was agreed that whole cell lysates were to be used for the remainder of the thesis. Additionally, more biological replicates are necessary for some experiments. Use of more sample replicates will provide more accurate and reliable data pertaining to the expression of nuclear lamins in these cell lines.

3. Development and Optimisation of an LC-MRM-MS/MS Method for the Relative Quantitation of Nuclear Lamins

3.1. Introduction

In Chapter 2, various proteomics techniques were used to measure the expression of nuclear lamin A, B1, B2 and C isoforms in a range of human cell lines. LC-MS^e analysis was used to determine the protein abundance of each isoform and resulted in the limited detection of some nuclear lamin isoforms. As a result, Chapter 3 will explore the development and optimisation of a targeted mass spectrometry (MS) assay to investigate the abundance of the four nuclear lamin protein isoforms. The targeted MS approach will be developed using a multiple reaction monitoring (MRM) assay performed using a liquid chromatography coupled with electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) system and will be designed for examination of different isoforms of nuclear lamins in biological samples of both human and mouse origin.

In Chapter 1 Section 1.7, the fundamentals of LC-ESI-MS/MS analysis are described in detail. Moreover, information on the different instrument parameters that affect sample ionisation and detection via MS are discussed. Optimisation of the targeted MRM-MS assay by adjusting these user-defined instrument parameters is imperative for increasing the assay sensitivity, which is important in detecting low abundant analytes. Previous literature has shown that optimisation of mass spectrometry operational parameters can result in significant increases in analyte response and data quality (Bieber et al., 2019; Cuykx et al., 2017; Marginean et al., 2009; Norton et al., 2006; Norton et al., 2005; Norton & Shamsi, 2007; Presser, 2009; Presser et al., 2014; Révész et al., 2023; Sherwood et al., 2011; Teh et al., 2011). The sensitivity of LC-MS/MS assays can vary due to multiple operational factors, including but not limited

to: mobile phase composition and flow rate; sample volume; nebuliser pressure; drying gas flow rate and temperature; fragmentor voltage; and collision energy (Bae et al., 2018). To optimize HPLC-MS/MS assay sensitivity, selecting the most important variables is necessary to avoid tedious experimentation and reduce cost and labour expenses. Considering this, there is a need to apply a systematic and comprehensive methodology to optimise the HPLC-MS/MS method for analysis of low abundant peptides in complex samples.

With respect to sample ionisation, the ESI source parameters are often selected according to instrument manufacturer's recommendations or previous user experience, rather than by careful consideration of the specific experimental conditions. In addition, the collision energies (CE) of target molecules, that is the energy provided to the ion upon entering the collision cell can be predicted using linear equations built into targeted MS software, however these calculations can be inaccurate and result in sub-optimal voltages for target ion detection. The fragmentor voltage (FV) is a parameter that is specific to Agilent Technologies instruments and is used in the ion optics region of the mass spectrometer. FV is an important factor in transmission of the target ions into the mass analyser and is often overlooked when developing targeted MS assays. Therefore, this study aims to meticulously analyse the influence of the CE, FV and ESI parameters on peptide ionisation and MS detection and optimise these factors for MS detection of nuclear lamin peptide targets.

Figure 3-1: Schematic of the Agilent 6420 triple quadrupole instrument, highlighting the main components of the ESI source and mass spectrometer. Figure 3-1 and Figure 3-2 below show representative schematics of the Agilent 6420 QQQ and the Agilent Ultivo QQQ mass spectrometers. The differences in the ESI source, mass analysers and detectors can be observed in these figures and are explained below.

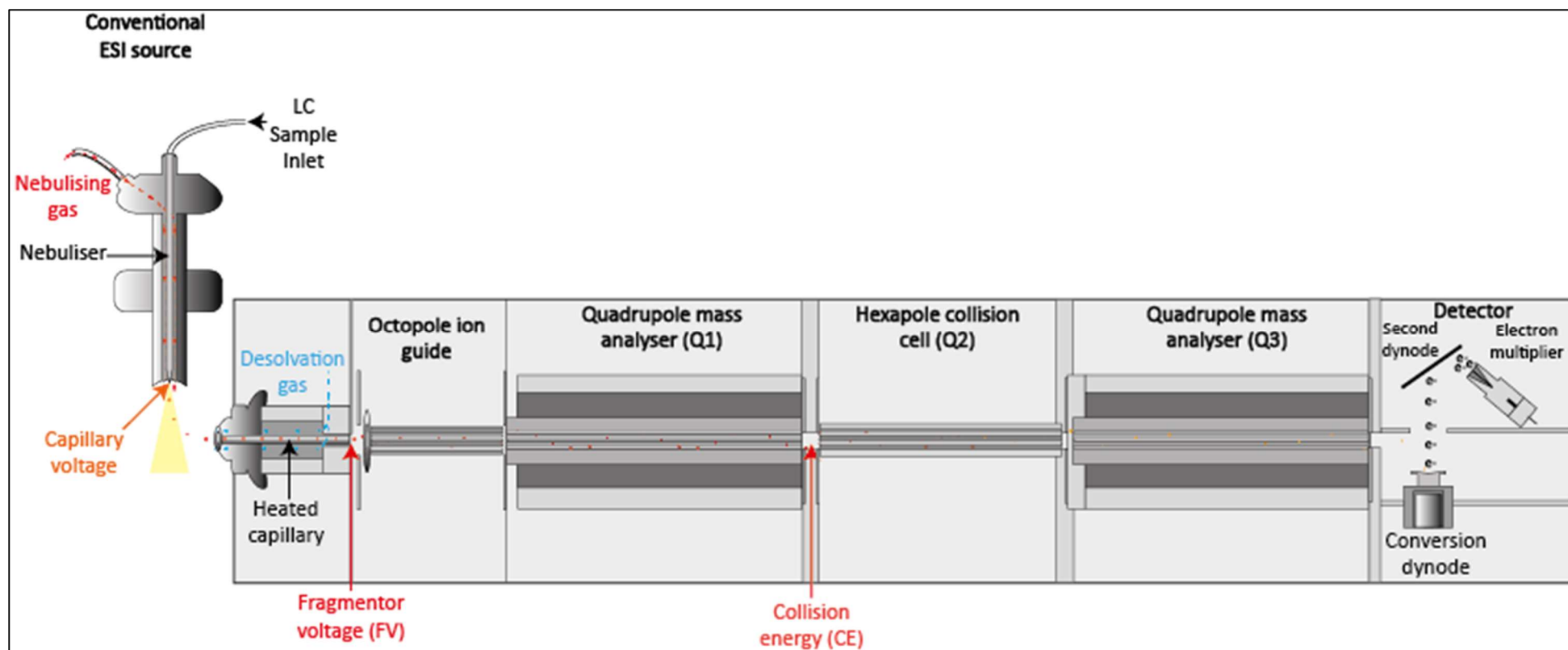


Figure 3-1: Schematic of the Agilent 6420 triple quadrupole instrument, highlighting the main components of the ESI source and mass spectrometer. Red dots indicate the precursor ions, while orange dots represent the product ions as a result of CID within Q2.

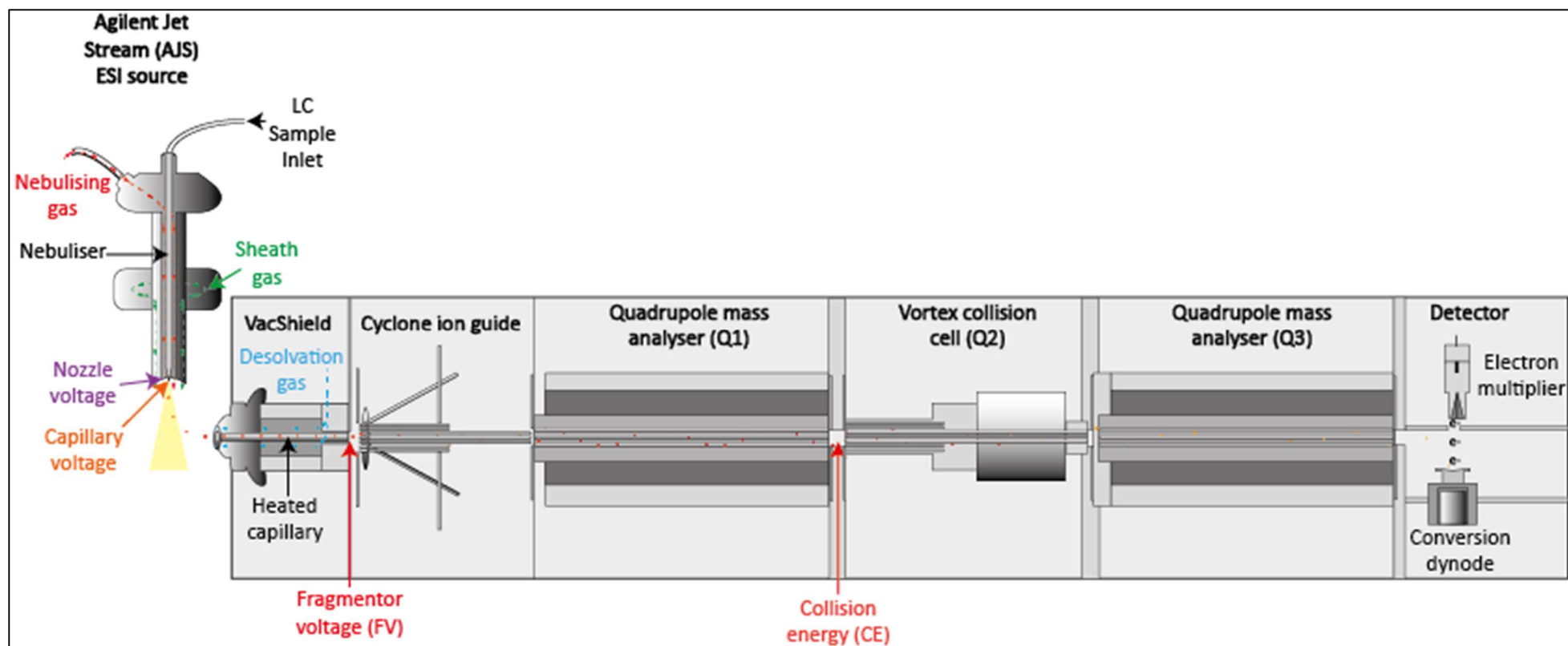


Figure 3-2: Schematic of the Agilent Ultivo triple quadrupole instrument, highlighting the main components of the ESI source and mass spectrometer. Yellow indicates the electrospray plume, red dots indicate the precursor ions, while orange dots represent the product ions as a result of CID within Q2.

There are several key differences in the hardware of the Agilent 6420 and the Agilent Ultivo QQQ instruments. Firstly, the Agilent Ultivo contains an Agilent Jet Stream (AJS) ESI source while the Agilent 6420 contains a conventional ESI source. The differences in these two ESI sources are the addition of a sheath gas and an adjustable nozzle voltage as part of the AJS ESI. The sheath gas is an additional flow of superheated nitrogen gas implemented into the AJS ESI system which flows in the same direction as the solvent flow and nebuliser gas to help confine the electrospray in order to more effectively desolvate the ions and concentrate them into a thermal confinement zone. This allows for a higher density of ions to be presented at the front of the MS inlet, allowing for a greater number of ions to be sampled by the MS and thus improving instrument sensitivity compared to conventional ESI sources. Furthermore, the nozzle voltage is the voltage applied to a metal ring around the end of the apparatus which houses the nebuliser. Adjusting this voltage leads to the widening or narrowing of the electrospray plume for efficient desolvation and to further concentrate the plume allowing for more ions present at the MS inlet (more information on the components of the AJS ESI source are explained in Section 3.4.5). The Agilent Ultivo also includes VacShield technology, which allows for the capillary to be removed for maintenance and cleaning without venting the instrument whereas this is not possible on the Agilent 6420, and the instrument must be vented to remove the capillary.

In addition, there are improvements to the ion optics hardware on the Agilent Ultivo compared to the Agilent 6420, where the Agilent Ultivo contains a Cyclone ion guide whereas the Agilent 6420 instrument has an octopole ion guide. The Cyclone ion guide is a proprietary technology made by Agilent Technologies and is comprised of a dual hexapole (also known as a dodecapole) at the entrance to the ion guide and a hexapole at the exit. The outer rods that comprise the additional hexapole to create the dual hexapole system improve the transmission of high mass ions by providing additional ion beam confinement as they enter and traverse the ion guide. Additionally, the dual hexapole and single hexapole components of the Cyclone ion guide are tapered and twisted. The tapered shape, whereby the ion guide is widest at the entrance and narrower at the exit, allows for maximum ion collection at the MS source and creates a

tightly columnated ion beam at the exit, as the ions enter the mass analyser. The twisted shape of these hexapoles enable a greater drop in pressure from the atmospheric source to the quadrupole mass analyser over a shorter distance than traditional ion guides while also reducing ion scatter or loss, which improves instrument sensitivity.

With respect to the collision cells, the collision cell contained within the Agilent 6420 instrument is a single hexapole collision cell whereas the Agilent Ultivo has an upgraded collision cell known as a Vortex collision cell. The Vortex Collision Cell is a proprietary Agilent Technologies technology and uses similar technology to the Cyclone ion guide, with a twisted and tapered dodecapole and hexapole configuration. This configuration allows for the optimal pressure for fragmentation in the front and higher pressure in the middle for cooling and ion beam collimation of the ion beam before entering Q3.

Finally, the Agilent 6420 contains an off-axis dual high energy dynode while the Agilent Ultivo contains an off-axis single high energy dynode detector. The off-axis design of the dynodes allows for neutral ions to pass through, reducing background noise and increasing instrument sensitivity. In addition to this, the Agilent Ultivo contains a detector horn with increased surface area within the electron multiplier which allows for collection of ions over a wider energy spread, allowing for maximum sensitivity of the detection system, resulting in a greater signal compared to previous models without the need of a second dynode. Further information on all technologies related to the Agilent 6420 and Agilent Ultivo can be found in the respective Concepts Guides provided by Agilent Technologies (Agilent Technologies Inc, 2017 & 2012).

Table **3-I** below shows a list of the MS parameters optimised in this study, and groups them based on whether they are solvent flow rate-dependent parameters or whether the optimal setting is dependent on the characteristics of the compound of interest.

Table 3-I: List of optimisation parameters used in the study, grouped based on their dependence on the solvent flow rate or type of analyte.

Flow rate-dependent parameters	Analyte-dependent parameters
Nebuliser pressure	Capillary voltage
Desolvation gas temperature	Fragmentor voltage
Desolvation gas flow rate	Collision energy
Sheath gas flow rate	Sheath gas temperature
	Nozzle voltage

3.2. Aims and Objectives

This aim of this chapter was to design and optimise a sensitive, selective and robust LC-MRM-MS/MS method to detect nuclear lamin peptides. This will be achieved through several objectives.

Firstly, the targeted assay will be designed by *in-silico* digestion of amino acid sequences of each nuclear lamin isoform found in *Homo sapiens* and *Mus musculus* species. The peptides selected for use in the assay design will be selected based on several criteria related to target molecule characteristics relevant for MS detection. Appropriate synthetic peptides corresponding to the nuclear lamin isoforms will be analysed on two different QQQ instruments to investigate the difference in sensitivity between the instrumentation. CE will be optimised for each transition on both instruments, and investigations into the effect of different instrument models on optimal CE will be conducted. After selection of the most sensitive instrument based on overall MS signal response, the instrument with the greatest sensitivity will be used for further optimisation of operational parameters. FV will be optimised for each peptide precursor ion, in addition to seven operational parameters related to the use of the ESI source. The significance of optimising each of these nine parameters, and factors affecting their optimal operational parameters, will also be discussed. The final optimised MRM method will be used in later chapters to measure the relative expression of nuclear lamin isoforms in biological samples of human and mouse origin.

3.3. Materials and Methods

3.3.1. Reagents

LC-MS grade water and acetonitrile (ACN) were purchased from Fisher Scientific Ltd (Loughborough, UK). >99% formic acid for LCMS was purchased from VWR International Ltd (Leicestershire, UK). Low volume LCMS vials were purchased from Waters Corporation (Wilmslow, UK).

3.3.2. Sample Preparation

All synthetic peptides of nuclear lamins (lamin A, lamin B1, lamin B2 and lamin C) were synthesised by and purchased from JPT Peptide Technologies GmbH (Berlin, Germany). Crude peptides were synthesised with an N-terminal free amine and a C-terminal lysine or arginine, with an approximate amount of 10-50 µg per peptide and freeze dried for delivery on dry ice. For peptides containing cysteine residues, all cysteine residues were substituted with carbamidomethyl-modified cysteine (analogous to using iodoacetamide). Peptides were resuspended in 5% ACN containing 0.1% formic acid.

3.3.3. LC-MRM-MS/MS

3.3.3.1. Liquid Chromatography (LC)

Liquid chromatography was performed as per the gradient described below in Table 3-II.

Table 3-II: Liquid chromatography gradient used in the analysis of lamin synthetic peptides.

Time (mins)	%A	%B	Flow Rate (mL/min)
0	95	5	0.3
15	50	50	0.3
17	10	90	0.3
18	10	90	0.3
20	95	5	0.3
25	95	5	0.3
Post time (5 mins)	95	5	0.3

Reversed-phase LC separation was performed using an Agilent AdvanceBio Peptide Mapping column, 2.7 μm , 2.1 x 150 mm, 120Å column (PN 653750-902) purchased from Agilent (Cheshire, UK). Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile.

3.3.3.2. Multiple Reaction Monitoring-based Mass Spectrometry (MRM-MS)

The MRM-LC-ESI-MS/MS experiments were performed on an Agilent Ultivo QQQ with an Agilent Jet Stream ESI source interfaced with an Agilent 1290 Infinity II LC system and an Agilent 6420 QQQ with a conventional ESI source coupled to an Agilent 1260 Infinity LC system. Nitrogen was used as the collision gas and nebulising gas for both instruments, while the desolvation gas and sheath gas used on the Agilent Ultivo AJS ESI was also nitrogen. Default ESI settings for each instrument are described below in

Table **3-III** and Table 3-IV.

Table 3-III: Default electrospray ionisation source parameters for Agilent 6420 QQQ.

Source Parameter	Value
Desolvation Gas Temperature (°C)	280
Desolvation Gas Flow Rate (L/min)	11
Nebuliser Pressure (psi)	35
Capillary Voltage (V)	+3500 to - 3000

Table 3-IV: Default electrospray ionisation source parameters for Agilent Ultivo QQQ.

Source Parameter	Default Value
Sheath Gas Temperature (°C)	250
Sheath Gas Flow Rate (L/min)	11
Desolvation Gas Temperature (°C)	300
Desolvation Gas Flow Rate (L/min)	7
Nebuliser Pressure (psi)	15
Capillary Voltage (V)	4000
Nozzle Voltage (V)	1500

MRM transitions used in this study were derived from the *in-silico* digestion of *Homo sapiens* and *Mus musculus* nuclear lamin isoform peptide sequences and experimental analysis of all transitions suitable for MRM assay design. MRM transitions were monitored using unit resolution in both Q1 and Q3 quadrupoles to maximize specificity. Each MRM transition had a minimum dwell time of 20 seconds and a detection window of 1 minute. Scheduled MRM, also known as dynamic MRM, was used to reduce the number of concurrent transitions and to maximize the dwell time for each transition. Data was acquired using Agilent MassHunter Data Acquisition version 1.2 software. MRM-MS/MS data was acquired in the form of .raw files.

3.3.3.3. Data Analysis

Data in the form of .raw files was uploaded into Skyline MS version 20.0 software. Manual inspection of each peptide precursor was conducted to examine peak shape, and peak boundaries were adjusted where necessary. Chromatographic peaks that gave a poor shape i.e. were not Gaussian or contained multiple peaks per analyte, were not included in the final data set. Information pertaining to peak area measurements for each transition during all experiments was exported to Microsoft Excel as .xlsx files for data analysis. Fold change was calculated as the measured peak area acquired using default settings divided by the measured peak area achieved when using the adjusted or optimised settings. For results showing the measured peak area of nuclear lamin peptide precursors, the sum of the measured peak area recorded for the top 4-5 transitions for each peptide was calculated. Chromatograms were visualised using Skyline MS software while the creation of bar charts, dot plots and violin plots and all statistical analysis was performed using Graphpad Prism version 9.2 or 10.0 software.

3.4. Results and Discussion

3.4.1. Selection of Suitable Precursor Ions

The aim of this study was to develop a targeted MRM-based LC-ESI-MS/MS assay to perform semi-quantitative analysis of different isoforms of nuclear lamins. As part of the study, it is imperative to be able to differentiate between the different protein isoforms. *Homo sapiens* lamin C sequence differs from the canonical prelamin-A/C sequence by the substitution of the residues GSHCSS to VSGSRR at residue 567-572, while residues 573-664 are missing from lamin C sequence (Fisher et al., 1986; Gerhard et al., 2004; McKeon et al., 1986). In the *Mus musculus* proteome, the lamin C sequence differs from the canonical prelamin-A/C sequence by substitution of the residues GSHCSG to VSGSRR at residue 569-574 and residues 575-665 are missing from the lamin C sequence (Carninci et al., 2005; Furukawa et al., 1994; Riedel & Werner, 1989). Therefore, to differentiate between lamin A and lamin C protein expression in both organisms, the lamin C-specific peptide VSGSR was included in the final peptide list. In biological samples of mouse origin, the lamin A-specific peptide GSHCSGSGDPAEYNLR, found at the C-terminal cleavage point, was also included in the final peptide list. The tryptic peptide at this cleavage point in humans is SVTVVEDEDEDGDDLHHHHGSHCSSSGDPAEYNLR which is 37 amino acids in length, from residue 546 – 582, therefore considered unsuitable for MRM analysis due to its length, so only the mouse lamin A-specific peptide at that cleavage point was considered for analysis. In addition, one prelamin A peptide (SYLLGNSSPR) and two C-terminal peptides that are cleaved from mature lamin A to form lamin C during post-translational processing in both humans and mice (TVLCGTCGQPADK and SVGGSGGGSGFDNLVTR) were also included in the final peptide target list. Appendix II Figure 8-3 to Figure 8-6 shows the *Homo sapiens* and *Mus musculus* amino acid sequences for the main four nuclear lamins and their sequence homology.

The Universal Protein Resource Knowledgebase (UniProt KB) protein sequence database (www.uniprot.org) was used to acquire FASTA sequences of human (*Homo sapiens*) and mouse (*Mus musculus*) prelamin-A/C (accession numbers P02545-1 and

P48678-1 respectively), lamin B1 (P20700 and P14733 respectively), lamin B2 (Q03252 and P21619-1 respectively) and lamin C (P02545-2 and P48678-2 respectively). All sequences were imported into Skyline v.20 (MacCoss Lab Software) and peptides were filtered according to Table 3-V below.

Table 3-V: Skyline MS peptide settings used for *in-silico* digestion of nuclear lamins in human and mouse protein sequences.

Peptide Setting	Parameter
Digestion: Enzyme	Trypsin [KR/P]
Digestion: Missed cleavages	No missed cleavages
Filter: Minimum and maximum peptide length	6-20 ¹
Filter: Exclude potential ragged ends	Yes ²
Filter: Exclude peptides containing	RP/KP sequences
Modification: Structural modifications	Carbamidomethyl cysteines (C)

After filtering the target peptide list as per Table 3-V, peptides that were not present in both human and mouse sequences simultaneously were excluded, along with any peptides that were not isoform-specific, with some exceptions. These exceptions include one pan-lamin peptide (LLEGEEER) and one pan B-type lamin peptide (LSPSPSSR). These peptides were included to examine the overall expression of nuclear lamin proteins, and the combined expression of the two B-type lamins respectively. To differentiate between lamin A and lamin C in mouse models, the lamin A-specific peptide GSHCSGSSDPAEYNLR and lamin C-specific peptide VSGSR were included in the final peptide target list. In addition, the two C-terminal peptides that are cleaved from mature lamin A to form lamin C, and one peptide found exclusively within prelamin A before post-translational modification to mature lamin A were also included in the peptide target list. Table 3-VI details the final candidate tryptic peptide target list for

¹ VSGSR, the only lamin C-specific peptide, was included.

² GSHCSGSDPAEYNLR, a mouse lamin-A specific peptide, was included.

development of the MRM-based assay to quantify the expression of lamin A, B1, B2 and C in biological samples derived from human and mouse origin. Several of these peptide sequences were identified when analysing cell lines via LC-MS^e analysis in Chapter 1 and these have been identified in Table 3-VI. The position of these target peptides within the amino acid sequences of each nuclear lamin isoform can be found in Appendix II Figure 8-3 to Figure 8-6.

Table 3-VI: Final candidate tryptic peptide target list for development of the MRM-based assay to quantify the protein expression of lamin A, B1, B2 and C in biological samples derived from human and mouse origin. * indicates peptides used for quantitation of each nuclear lamin isoform via LC-MS^e in Chapter 2.

Target Protein	Candidate Peptide
Lamin A/C	METPSQR
	SGAQASSTPLSPTR *
	EDLQELNDR
	LAVYIDR
	SLETENAGLR
	ITESEEVVSR*
	AAYEAE LGDAR *
	TLDSVAK
	DLEALLNSK
	EAALSTALSEK *
	TLEGELHDLR
	LEAALGEAK
	QLQDEMLR
	NIYSEELR *
	LVEIDNGK
	LADALQELR
	AQHEDQVEQYK
	NSNLVGAAHEELQQSR *

	IDSLSAQLSQLQK *
	DLED ~~S~~ LAR *
	MQQQLDEYQELLDIK *
	LALDMEIHAYR
	LSPSPTSQR
	SSFSQHAR
	VAVEEVDEEGK *
	TALINSTGEEVAMR
Lamin A (mouse only)	GSH <u>C</u> SGSGDPAEYNLR
Lamin A	TVL <u>C</u> GT <u>C</u> GQPADK
	SVGGSGGGSF ~~G~~ DNLVTR *
Prelamin A	SYLLGNSSPR
Lamin B1	LAVYIDK
	SLETENSALQLQVTER
	ALYETELADAR *
	ALDDTAR
	AEHDQLLLNYAK
	EYEAALNSK
	DAALATALGDK
	SLEGDLEDLK
	QLADETLLK
	<u>C</u> QSLTEDLEFR
	LVEVDSGR
	QIEYEYK
	LAQALHEMR
	EQHDAQVR
	EELEQTYHAK
	LSSEMNTSTVNSAR
	EELMESR
	IESLSSQLSNLQK *
	LALDMEISAYR

	NTSEQDQPMGGWEMIR
	IGDTSVSYK
	NQNSWGTGEDVK
	NSQGEEVAQR
Lamin B2	ALELENDR
	VLDETAR
	EGELTVAQGR
	DLESLFHR
	AEDGHAVAK
	SVFEEEVN
	LVEVDSSR
	QQEYDFK
	SQHDEQVR
	EQEMTEVR
	DQSLGNWR
Lamin C (mouse only)	VSGSR
All nuclear lamin isoforms	LLEGEEER
Pan B-type lamins	LSPSPSSR

3.4.2. Selection of Suitable Product Ions

In MRM-QQQ-MS, detection of the target analyte is based on collision-induced dissociation (CID) within Q2, in which the precursor ion fragments following collision with an inert gas and produces product ions (also known as fragment ions), as mentioned previously in Section 1.7.1.3. The abundance of product ions measured by the detector correlates to the abundance of a specific precursor ion and consequently, the abundance of the target protein in the original sample. Therefore, an essential feature to ensuring accurate, precise and robust results are achieved using MRM-based tandem mass spectrometry analysis is the controlled fragmentation of a selected precursor ion to produce specific fragment ions for each analyte of interest.

The peptide bond between two amino acids is the most prone to breakage during CID fragmentation of peptide ions (Cardoza et al., 2012). This creates b- and y-ions, where the b-ions are the product ion produced when the charge is retained on the N-terminus (i.e. beginning of the sequence) while y-ions are produced when the charge is retained at the C-terminus (i.e. at the end of the sequence). Other types of ions can be produced during the fragmentation process inside the collision cell, such as a- and x-ions, and c- and z-ions. A-, b- and c-ions are a result of retained charge on the N-terminal peptide chain, while x-, y- and z-ions have a retained charged on the C-terminal peptide chain. Figure 3-3 explains the nomenclature used when describing the production of fragment ions during CID fragmentation as part of MS/MS analysis of peptides.

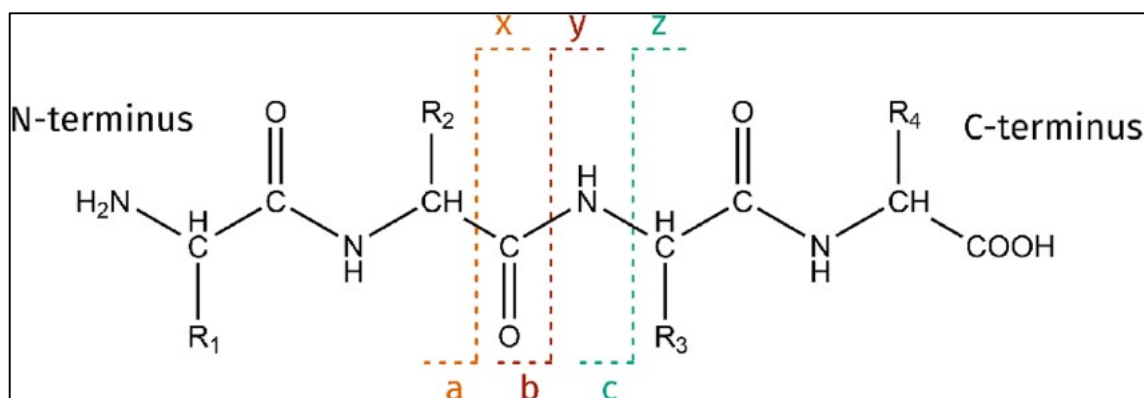


Figure 3-3: Nomenclature used for describing the different types of product ions produced by the fragmentation of peptides during CID events. Diagram from (Hale & Cooper, 2020).

It has been reported that y-type ions from CID fragmentation are more robust than b-type ions (Palzs & Suhal, 2005; Sherwood et al., 2009a), and are therefore more favourable during MRM assay development. Product ions produced by CID are also strongly dependent upon the instrument used and the sequence of the precursor ion undergoing fragmentation (Huang et al., 2005; Kapp et al., 2003; Khatun et al., 2007; Memboeuf et al., 2010; Sherwood et al., 2009a; Sherwood et al., 2011). The peptide backbone is usually fragmented at the weakest peptide bond, and notable sequence characteristics for increasing fragmentation probability include the presence of proline (Breci et al., 2003; Tabb et al., 2003) and histidine (Tabb et al., 2003). Careful selection of fragment ions is critical during MRM assay design, as fragmentation by CID may result in loss of some residues and therefore shorter fragments than expected during MRM, affecting reproducibility and accuracy of protein quantitation (Dupré et al., 2012).

When selecting suitable precursor and product ions to monitor for MRM-MS/MS-based analysis, transitions which provide the best signal response should be chosen for accurate quantitation of the analyte. Transitions for MRM assay design can be selected from libraries of previously collected MS/MS spectra, such as PeptideAtlas, GMPDB and PRIDE. It should be noted that these spectra are usually acquired from ion-trap,

Orbitrap and tandem time-of-flight instruments whereas MRM is usually conducted on triple quadrupole instruments. This is important as the product ions resulting from CID vary between instrument types, therefore the choice of instrument used is an important consideration when using spectral libraries to design MRM assays. Other methods of selecting suitable transitions are the use of computational tools to predict fragmentation. In addition, software tools such as ProtView (Puliasis et al., 2023) MRMAid, SRMAAtlas, MRMer and MaRiMba have been previously used to predict the fragmentation of user-inputted peptide sequences (Liebler & Zimmerman, 2013). This topic is discussed in detail by Wilburn and colleagues (Wilburn et al., 2021). The final and most accurate method of determining the best transitions for MRM-based targeted quantitation is through empirical analysis of synthetic peptide standards or through the production of recombinant proteins of interest. Empirical analysis of peptide standards allows the user to confirm the detection of selected transitions resulting from *in-silico* digestion and allows for the determination of chromatographic retention times simultaneously.

The number of transitions monitored per analyte in an MRM assay is another consideration of MRM design, as this determines the selectivity of the assay. Assays that monitor a single transition per peptide target may detect many signals that could be considered “false positives” for quantitation, as fragments from different precursor ions may have the same m/z , and the likelihood of this occurring increases with increasing sample complexity. Therefore, monitoring three or more transitions per peptide significantly increases the confidence in assay specificity. Previous reports suggest that y -ions resulting from CID fragmentation are more robust than b -ions (Palzs & Suhal, 2005; Sherwood et al., 2009a), therefore the transitions selected for MRM assay development in this chapter were limited to y -ions. In addition, the peptide backbone is usually fragmented at the weakest peptide bond, which is the peptide bond between proline and any adjacent amino acid on its N-terminus, due to its cyclical structural group which incorporates the N-terminal amino group.

During this study, empirical analysis of each synthetic peptide in single injections was initially performed. This allowed for the determination of each peptide's retention time which was necessary for development of a scheduled MRM (also known as dynamic MRM) method for multiplexing analysis of several target proteins within the same sample in a single injection. During a typical MRM analysis, all transitions for peptides derived from a small set of proteins are monitored continuously throughout the entire chromatographic elution period. This is relatively inefficient as each peptide is eluted during a brief period within the chromatographic elution period. Conversely, dynamic MRM assays involve scheduling a brief monitoring window that corresponds with the elution time of the peptide of interest. During this monitoring window, data is only acquired for transitions of analytes that elute around this time. This means the retention times of each peptide must be known in advance, and the retention time for each peptide must be stable and reproducible. Any shifts in retention time due to instabilities in chromatographic elution may cause peptides to elute outside of the specific retention time window, which may result in missed quantitation events. Retention time windows of ± 30 seconds relative to the highest point of the chromatographic peak are suitable and can account for slight drifts in retention time over multiple sample injections. As a result, the assay can monitor a greater number of transitions throughout the same experiment, increasing experimental efficiency and decreasing instrument time. However, the multiplexing capacity of any MRM experiment is dependent upon duty cycle and dwell time.

Duty cycle is the amount of time spent monitoring an analyte, while the dwell time is the time spent acquiring data for a transition during each cycle. The user defines the dwell time for each analyte in milliseconds, and the duty cycle is calculated from the dwell time and the number of transitions monitored at that time. The higher the duty cycle, the more cycles are monitored in a defined period, which increases data quality as this ensures a greater number of data points acquired over a chromatographic peak. This increases the quality of the chromatographic peak and therefore increases the accuracy of the peak area measurement. Duty cycle is proportional to dwell time and inversely proportional to the number of concurrent MRM transitions monitored.

Overall, the aim of a dynamic MRM method is to analyse the highest number of peptide analytes during a single injection while maintaining the highest possible duty cycle and dwell time.

This study uses empirical analysis of synthetic peptides to determine the top five transitions for each peptide that produced the greatest signal response. To do this, after selection of tryptic peptides described in Section 3.4.1, selection of suitable transitions was formed by scanning each peptide for all transitions available for MRM assay development. Table 3-VII below shows the transition settings used within the Skyline MS software to filter for the most suitable product ions.

Table 3-VII: Skyline MS transition settings used for the investigation of suitable product ions for MRM-LC-MS analysis of nuclear lamin protein isoforms.

Transition Setting	Parameter
Prediction: Precursor mass and product mass	Monoisotopic
Prediction: Collision energy	Agilent QQQ
Filter: Peptide - Precursor charges	2 ³
Filter: Peptide - Ion charges	1, 2
Filter: Peptide – Ion types	Y
Filter: Product Ion Selection	From ion 3 to last ion
Filter: Special ions	N-terminal to Proline

³ If the 2+ precursor was greater than or equal to 700 *m/z* rounded to one decimal place, then the 3+ precursor was also included.

3.4.3. Comparison of instrument sensitivity using the Agilent 6420 LCTQ vs Agilent Ultivo LCTQ

Initially, a selection of 52 lamin A/C and lamin B1 synthetic tryptic peptides were analysed on both the Agilent Ultivo LCTQ and the Agilent 6420 LCTQ, coupled to an Agilent 1290 Infinity II LC system and an Agilent 1260 Infinity LC system respectively. Both analyses were performed using the same LC column. 2 µL of each synthetic peptide was individually injected and analysed. The LC method and source parameters for each instrument are described in Table 3-II,

Table **3-III** and Table 3-IV. Each synthetic peptide was analysed in a single injection to determine their retention time and to identify the top five transitions that gave the highest peak area. The top five MRM transitions for each peptide were used in subsequent experiments in this chapter. For small length peptides of which there were no more than four product ions that fit the criteria described in Table 3-VII, all four product ions were selected for analysis.

To compare instrument sensitivity, the top four or five MRM transitions determined during the previous experiment were selected for analysis. For this experiment, only transitions from lamin A/C and lamin B1 peptides were chosen (see Table 3-VI for more information). 2 μ L of each synthetic peptide was injected and analysed on each instrument.

Figure **3-4** below shows the resulting chromatograms from the analysis of 52 synthetic peptides analysed on the Agilent 6420 QQQ and Agilent Ultivo QQQ. For peptides where 2⁺ and 3⁺ precursors were analysed, one the precursor ion which resulted in the highest signal intensity was chosen and represented on the chromatogram, as Skyline MS software was unable to plot the signal intensity of both charge states for a single peptide within the same chromatogram.

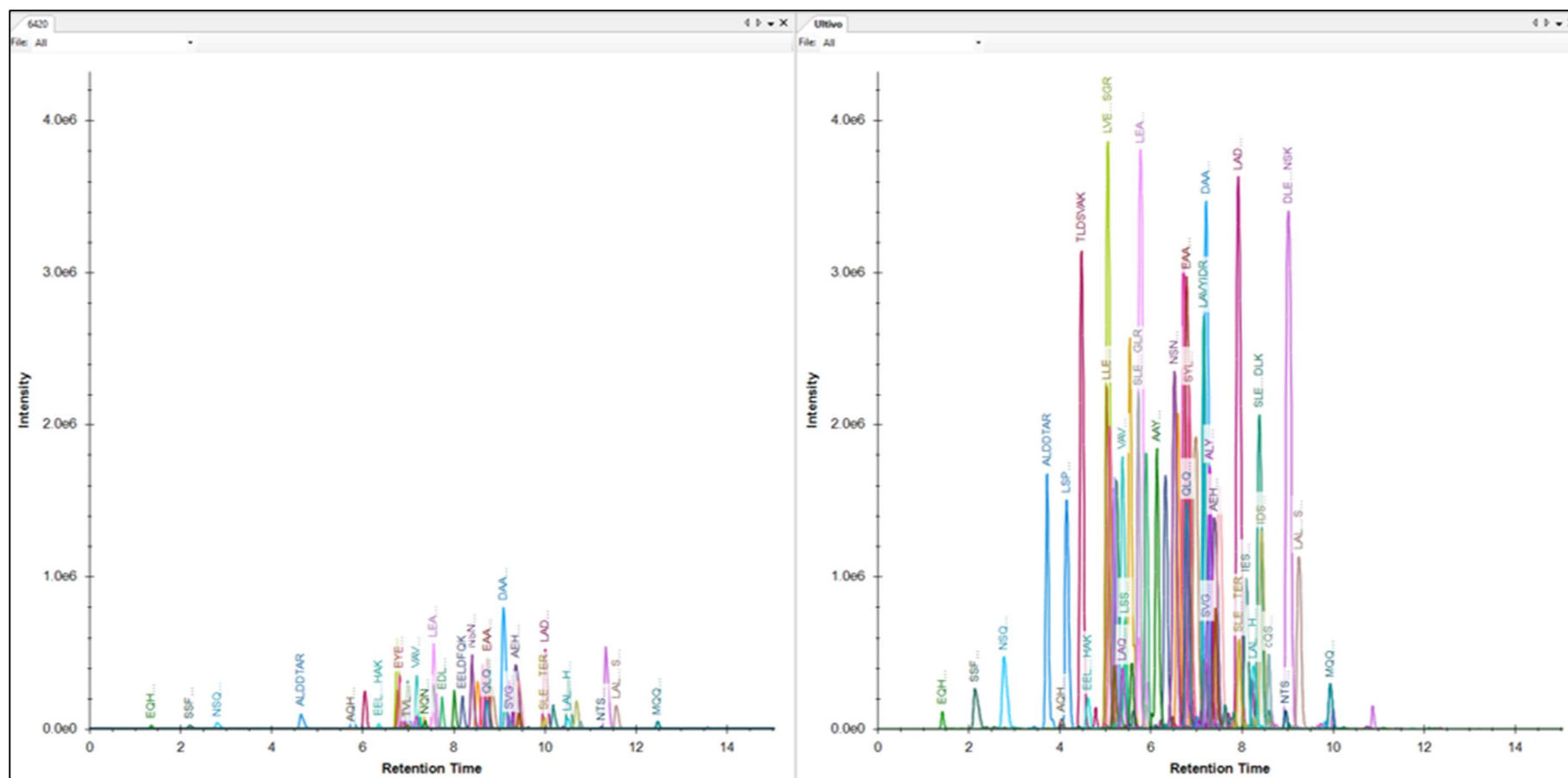


Figure 3-4: Chromatograms of 52 synthetic peptides acquired using the Agilent 6420 (left) and Agilent Ultivo (right), showing the signal intensity and retention time of each peptide (n=1). Data was visualised in Skyline MS software, where the sum of the top 4-5 transitions for each peptide were used to create each chromatographic peak for each peptide precursor.

Based on the results shown in

Figure **3-4**, it was suggested that using the Agilent Ultivo QQQ instrument provided greater sensitivity when detecting nuclear lamin peptides compared to the Agilent 6420 QQQ instrument, based on a greater signal response measured for each peptide. To quantitatively measure the increase in sensitivity when using the Agilent Ultivo compared to the Agilent 6420, statistical analysis via a paired T-test was performed on raw peak area data acquired from both instruments and shows a significant increase in measured peak area for product ions ($n=253$) and peptide precursor ions (that is the sum of all product ion measurements for each peptide precursor) ($n=52$) when analysing samples on the Agilent Ultivo compared to the Agilent 6420 ($p<0.0001$ for both data sets). The fold change of the measured peak area for each transition and each peptide precursor (sum of peak area values for each transition of the associated peptide) was plotted. Fold change was calculated as the measured peak area acquired using the Agilent Ultivo QQQ divided by the measured peak area achieved when using the Agilent 6420 QQQ. These results are shown below in Figure 3-5.

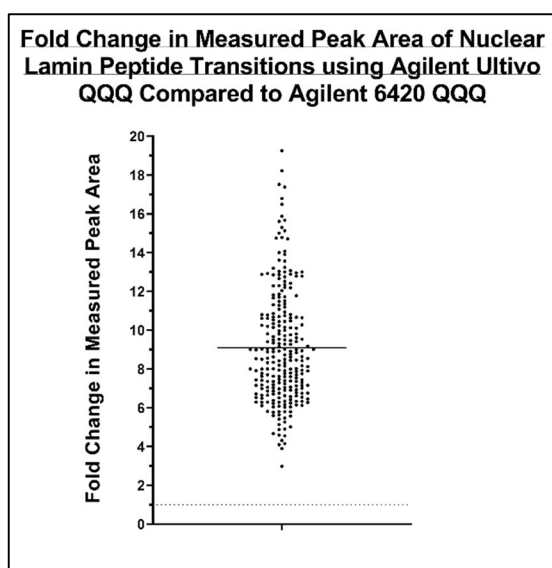
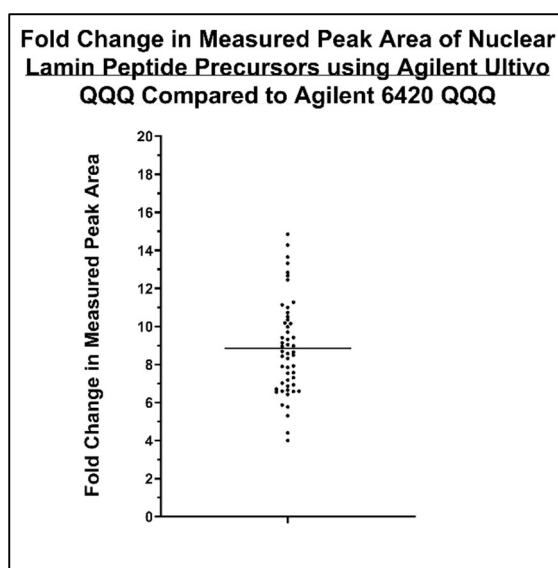
A**B**

Figure 3-5: Fold change in measured peak area of (A) 253 transitions and (B) 52 precursors of selected LMNA/C and LMNB1 tryptic peptides using the Agilent Ultivo LCTQ compared to using the Agilent 6420 LCTQ (n=1). The black line indicates the mean fold change measurement of the fold change values.

The results in Figure 3-5A show a fold change in measured peak area of each transition of between 2.9837 and 19.2508 when analysing synthetic peptide samples using the Agilent Ultivo compared to using the Agilent 6420, with an average fold change of 9.093. No transitions showed a decrease in peak area when using the Agilent Ultivo compared to the Agilent 6420. When examining the fold change of measured precursor ions in Figure 3-5B, the average fold change was 8.8501, with a range of 4.0109 to 14.8569. These results confirm the superior sensitivity of MRM peptide analysis using the Agilent Ultivo LCTQ instrument. Previous research has shown that when analysing 1 pg of reserpine using a conventional Agilent ESI source and an AJS ESI source, an average of 5- to 10-fold improvement in MS and MS/MS sensitivity was achieved (Mordehai & Fjeldsted, 2009). The results in Figure 3-5 support this observation, as the Agilent 6420 uses a conventional Agilent ESI source while the Agilent Ultivo uses an AJS ESI source. The increase in peak area obtained using the Agilent Ultivo can be contributed to improved design of the AJS ESI source compared to the conventional ESI

source used by the Agilent 6420. Further information on the design of the AJS ESI source can be found in Section 3.4.5.

3.4.4. Optimisation of Collision Energy and Fragmentor Voltage

Optimisation of experimental parameters is paramount when attempting to detect low abundant analytes, due to the improvements in assay sensitivity and lowered limits of detection (LOD) and quantitation (LOQ) provided by optimised assays. Two of the most commonly and easily optimised parameters are collision energy (CE) and fragmentor voltage (FV). Both these events are known as collision-induced dissociation (CID) events. CID is a technique to induce fragmentation of selected gas-phase ions. The ions are accelerated by applying an electrical potential to increase their kinetic energy, before colliding with neutral molecules of inert gas. During the collision event, some of the kinetic energy is converted to energy used to break bonds between atoms and fragment the analyte ion into smaller fragment ions.

CE is the kinetic energy is used to collide the analyte ions with inert gas within Q2 and cause fragmentation of the analyte (precursor) ions selected in Q1 into fragment (product) ions, which are then filtered and selected in Q3 and subsequently quantified by the detector. FV is the kinetic energy given to analytes as they exit the heated capillary and are focused into an ion beam before entering the skimmer, which is situated before Q1. These ions are accelerated by the application of a voltage, which promotes collisions with any surrounding species, causing fragmentation. CE and FV are compound-dependent parameters and should be optimised based on each separate analyte ion and transition (Presser, 2009). When optimising FV, fragmentation by CID is unfavourable, as the aim is to give the ions enough energy to enter the skimmer without causing fragmentation. In contrast, CE optimisation aims to fragment the parent ions to create product ions for peptide identification and quantitation, so fragmentation is critical.

Optimisation of CE and FV is commonly performed at the initial stages of method development and optimisation can be performed in a single injection. This results in comparisons between different CEs and FVs that are not influenced by variations between separate sequential injections of the same sample, and do not require extensive instrument time. This section aims to optimise the CE and FV values for the detection of tryptic peptides of nuclear lamins for inclusion in a targeted MRM-based LC-ESI-MS/MS assay. In addition, an investigation into the impact of CE and FV settings on MS signal response was performed.

3.4.4.1. Optimising Collision Energy

CE determines the kinetic energy given to the precursor ions as they exit Q1 and enter Q2 (also known as the collision cell) during QQQ analysis. In the collision cell, precursor analyte ions collide with an inert gas, usually nitrogen to produce product ions. Controlling the fragmentation process within the collision cell by modifying the CE value of each individual precursor ion can result in significant increases in the abundance of a selected product ion, resulting in an increase in signal response and sensitivity. Factors known to influence the optimal CE of precursor ions include the instrument used for MS signal measurement (Révész et al., 2023), precursor ion m/z value (larger m/z molecular ions require more energy) (Memboeuf et al., 2010; Révész et al., 2023) and precursor ion charge state (greater charge state precursor ions require less energy) (Huang et al., 2005; Révész et al., 2023). Multiple instrument vendors provide empirical formulas for calculating the optimal CE based on precursor charge and m/z (Holman et al., 2012). This linear equation is in the form of:

$$CE = a * (\text{precursor } m./z) + b$$

Equation 3-3-1: Linear equation used to predict the optimal collision energy for precursor ion CID fragmentation.

where a and b are the slope and y-intercept respectively. Both a and b differ depending upon the instrument used, and change depending on the charge state of the precursor ion. This calculation has been shown to work best for y-type ions and is considered less effective for b-type ions (Sherwood et al., 2011). In addition, b-type ions tend to require lower CE values than y-type ions for optimal fragmentation, and fragmentation at the amide bonds bound on the amino-terminal side by aspartic or glutamic acid residues tend to require more CE (Sherwood et al., 2011). However, manual determination of optimal CE values is recommended for the production of CE breakdown curves (Mörlein et al., 2020), and previous literature from MacLean and colleagues from the MacCoss Laboratory at the University of Washington has shown that testing various LC-MS/MS systems with 20 doubly charged and 10 triply charged peptides, with four transitions per peptide, vendor-suggested linear equations may not be suitable and suggested recalculation of the linear equations for each charge state resulted in far better performance than previously reported equations (MacLean et al., 2010). Based on this study, the MacCoss laboratory developed instrument-specific and charge state-specific linear equations within their Skyline MS software to predict the optimal CE for peptide precursor ions. For Agilent QQQ instruments, the Skyline MS equation for 2⁺ charged precursor ions is:

$$CE = 0.031 * (\text{precursor } m/z) + 1$$

Equation 3-3-2: Linear equation used by Skyline MS software to predict the optimal collision energy for precursor ion CID fragmentation in doubly charged (2+) precursor ions measured on Agilent QQQ instruments.

For 3⁺ charged precursor ions, the Skyline MS equation is:

$$CE = 0.036 * (\text{precursor } m/z) - 4.8$$

Equation 3-3-3: Linear equation used by Skyline MS software to predict the optimal collision energy for precursor ion CID fragmentation in triply charged (3+) precursor ions measured on Agilent QQQ instruments.

For this experiment, the top five MRM transitions for a selection of tryptic peptides of lamin A/C and lamin B1 that gave the highest signal response in Section 3.4.3 were chosen for analysis. For small length peptides where there were no more than four product ions that fit the criteria described in Table 3-VII, all four product ions were selected for analysis. Furthermore, if the 2⁺ precursor for an analyte ion was greater than 700 *m/z*, the 3⁺ precursor was also measured. Therefore, 253 transitions were measured on the Agilent 6420 (59 precursors, (2⁺ precursors n=52, 3⁺ precursors n=7) and 385 transitions were measured on the Agilent Ultivo (79 precursor ions (2⁺ precursors n=67; 3⁺ precursors n=12)). The difference in the number of precursor ions measured with each instrument is a result of the inclusion of peptides within the first 25 amino acid sequence of lamin A/C and the inclusion of lamin B2 peptides, which were excluded during the initial experimentation on the Agilent 6420.

To investigate the effect of optimising CE on the signal response of nuclear lamin synthetic peptides, a pooled sample of synthetic peptides were analysed by MRM-LC-MS/MS. The signal response of each transition was measured at the Skyline MS-predicted optimal CE for Agilent QQQ instruments for 2⁺ and 3⁺ precursor ions, shown in the formulae above, and at a range of different CE values with a step size of 3 V up to +/- 9 V either side of the Skyline-predicted optimal CE. Optimisation of CE was performed within the same single injection and recorded simultaneously. Due to the multiplexing capabilities of QQQ instruments, 6 – 7 peptides were pooled into each sample and all CE values for those peptides were measured within the same injection. For the LMNC peptide VSGSR, measurements of only two 3 V steps below the software-predicted optimal CE were collected, as the third step gave a voltage below 0 V. All three 3 V steps above the predicted optimal CE were still measured for this peptide.

After measuring the signal response of each transition at varying CE values, the CE value which resulted in the highest signal response was chosen as the optimal CE for that transition. For this experiment, the default FV of 130 V was used for all transitions. Figure 3-6 shows the bar chart of the optimal CE of transitions of synthetic peptides of nuclear lamins with respect to the software-predicted optimal CE (also known as the default CE) analysed on the Agilent 6420 and the Agilent Ultivo.

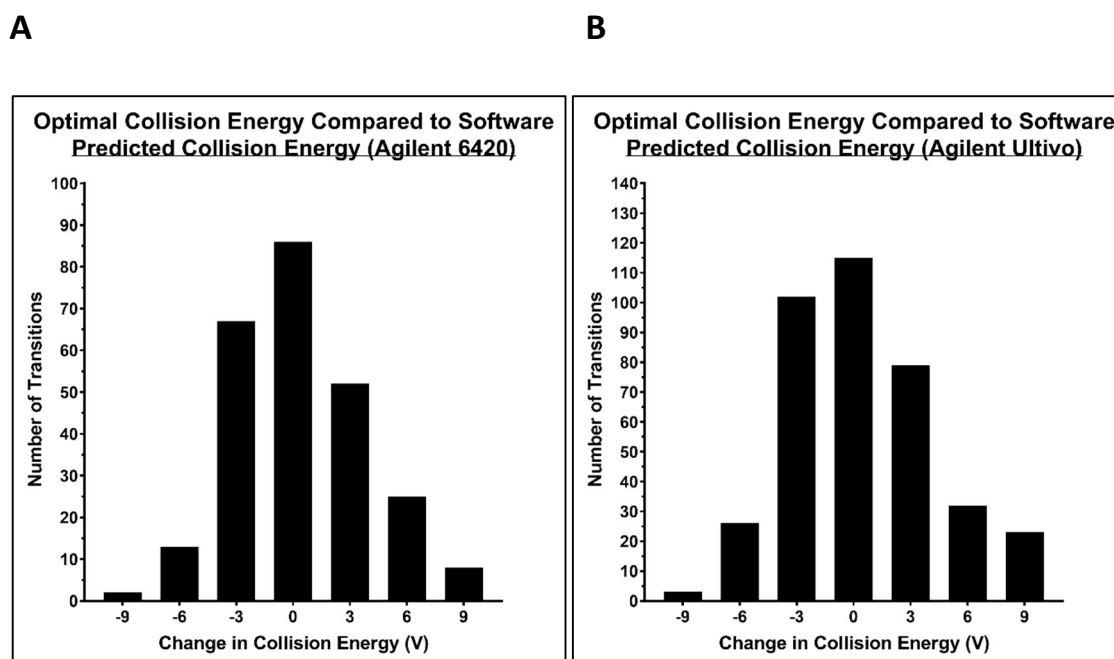


Figure 3-6: Bar charts showing the optimal collision energy of transitions of 253 synthetic peptides analysed on the Agilent 6420 (A) and 380 synthetic peptides analysed on the Agilent Ultivo (B) relative to the Skyline MS predicted (default) CE for MRM analysis on Agilent QQQ instruments (n=1). Default CE is represented as a change in collision energy of 0 V.

When analysing peptide transitions on the Agilent 6420, the results in Figure 3-6A show that for 33.99% (86 of 253 transitions) of transitions, the default CE gave the highest peak area. In 66.01% (167 of 253 transitions) of transitions investigated, a different CE to the default resulted in a higher peak area. When analysing peptide transitions on the Agilent Ultivo, Figure 3-6B shows that 115 of 380 transitions (30.2%)

gave an optimal CE that was the same voltage as the default CE. Furthermore, when performing analysis on the Agilent Ultivo, 265 of 380 transitions (69.7%) had an optimal CE that was different to the default CE. This suggests that, regardless of the instrument used, for over two thirds of the transitions, the default CE provided by the Skyline MS software did not produce the greatest signal response. This highlights the importance of optimizing CE for each transition in targeted MRM experiments.

For transitions in which the optimal CE was different to the default CE (n=167 for the Agilent 6420, n=265 for the Agilent Ultivo), further analysis was performed to show the fold change in measured peak area achieved when the optimal CE was used compared to when the default CE was used. Figure 3-7 shows a scatter plot of the fold change in peak area of each of the transitions where the optimal CE was not the default CE, with peptides analysed on the Agilent 6420 (A) and the Agilent Ultivo (B).

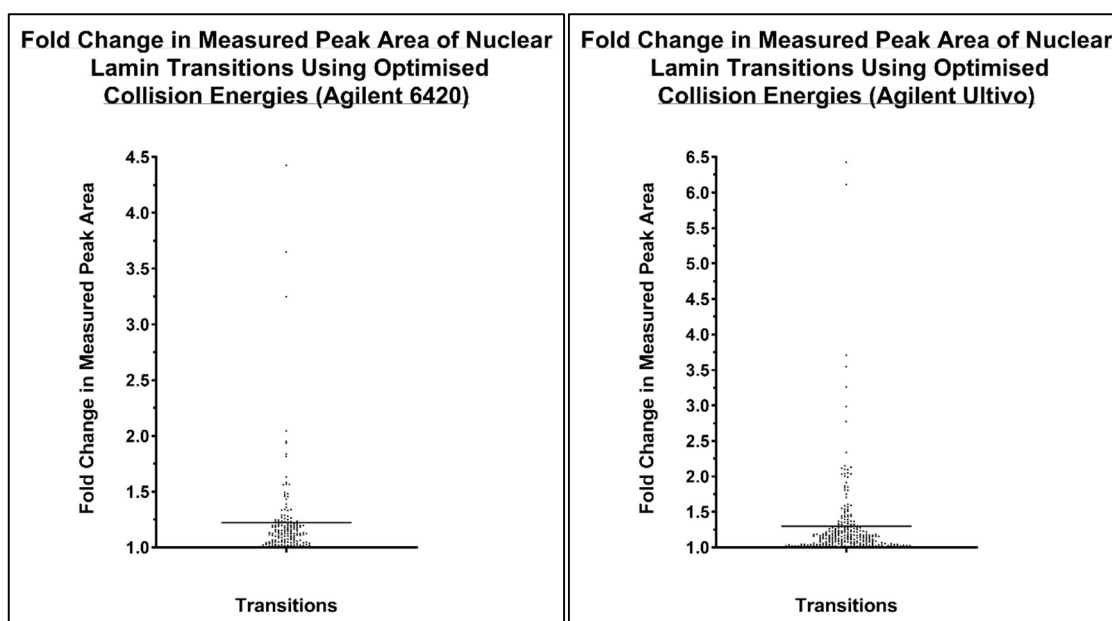
A**B**

Figure 3-7: Fold change in peak area of transitions of nuclear lamin peptides achieved when using the empirically determined optimal CE compared to the default CE, measured on the Agilent 6420 (A, 167 transitions) and the Agilent Ultivo (B, 265 transitions) (n=1). Horizontal line is indicative of the mean.

When using the Agilent 6420, results in Figure 3-7A showed that there was a mean fold change in measured peak area of 1.2224 achieved by optimising the CE (range = 1.0001 - 4.4250). When using the Agilent Ultivo, the results shown in Figure 3-7B indicate that there was a mean fold change in measured peak area of 1.2950 achieved by optimising the CE (range = 1.0004 - 6.4256). This further supports the need for optimisation of CE values when measuring analytes via LC-MRM-MS/MS, as this experiment shows that up to a 4- and 6-fold increase can be achieved in peak area measurement from empirical determination and use of optimal CE values, dependent on the instrument used.

Picotti and colleagues found that a moderate increase in MS signal was achieved through targeted CE optimisation when analysing 96 peptides (Picotti et al., 2010),

while Sherwood and colleagues found that 59% of the transitions they measured gave an increase in MS signal response of greater than 30% when the CE was optimised compared to the default CE provided by the instrument vendor (Sherwood et al., 2009b). Kutzner and colleagues found that CE optimisation led to vast improvements in signal intensity, of between 64% and 94% when designing an MRM-based assay for the detection of fatty acids (Kutzner et al., 2019). Furthermore, MacLean and colleagues found that optimising linear equations for optimal CE prediction for peptide analysis resulted an average of 7.8% increase in peak area measurements when compared to using linear equations recommended by the instrument vendor (MacLean et al., 2010). This further supports the need for empirical optimisation of CE values when measuring analytes via LC-MRM-MS/MS.

Based on previous observations that larger m/z ions require more energy during CID within Q2 (Memboeuf et al., 2010; Révész et al., 2023), the relationship between precursor ion m/z and optimal CE was examined and the results are shown below in Figure 3-8.

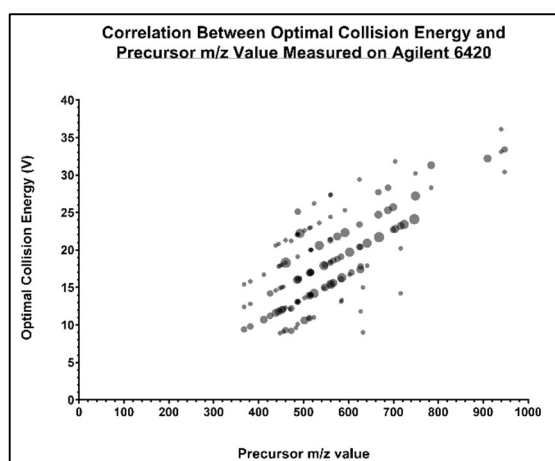
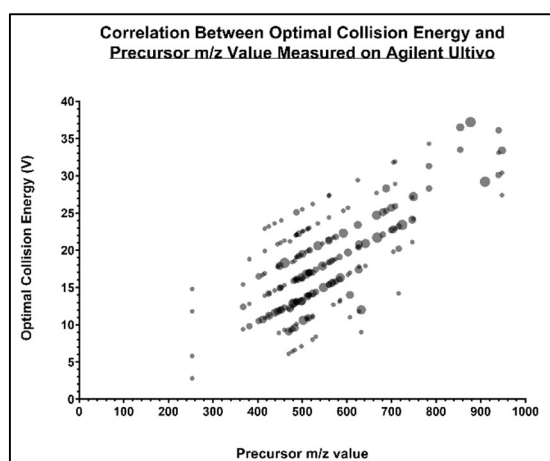
A**B**

Figure 3-8: Relationship between the optimal collision energy for each product ion and precursor ion m/z measured by LC-MRM-MS/MS on the (A) Agilent 6420 QQQ and (B) Agilent Ultivo QQQ. Multiple product ion readings are recorded for each precursor ion as 4-5 transitions were monitored per precursor ion. The size of each bubble is indicative of multiple product ions from the same precursor ion which had the same optimal collision energy. Readings were measured on the A) Agilent 6420 (253 transitions, $R^2 = 0.6034$, $y = 0.003841x - 3.118$) and B) Agilent Ultivo (385 transitions, $R^2 = 0.6094$, $y = 0.03889x - 3.496$).

Both results in Figure 3-8A and Figure 3-8B show a positive correlation between the m/z of the precursor ion and the optimal CE when analysed on the Agilent 6420 QQQ and Agilent Ultivo QQQ respectively. This positive correlation between the m/z of the precursor ion and the optimal CE supports previously published findings.

There are often different linear equations for predicting optimal CE values for precursor ions of different charge states. The linear equations used in Skyline MS software for 2+ and 3+ precursor ions are described in Equation 3-3-2 and Equation 3-3-3 respectively. To examine the effect of charge state on the optimal CE value, the data acquired on each instrument was divided into two groups based on the charge state of the precursor ion. Figure 3-9 shows the relationship between optimal CE and precursor ion

m/z for 2+ charged precursor ions, measured on the Agilent 6420 (A) and the Agilent Ultivo (B).

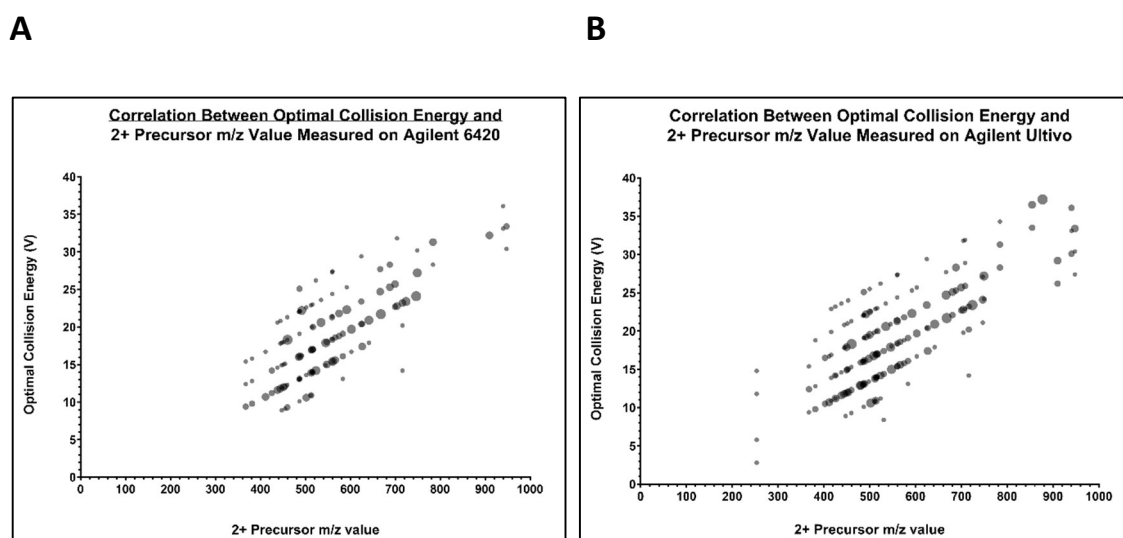


Figure 3-9: Relationship between the optimal collision energy and precursor ion m/z for doubly charged (2+) precursor ions of synthetic nuclear lamin tryptic peptides measured on the A) Agilent 6420 and the B) Agilent Ultivo (n=1). Multiple product ion readings are recorded for each precursor ion as 4-5 transitions were monitored per precursor ion. The size of each bubble is indicative of multiple product ions from the same precursor ion which had the same optimal collision energy. Readings were measured on the A) Agilent 6420 (233 transitions, $R^2 = 0.6464$, $y = 0.03856x - 2.840$) and the B) Agilent Ultivo (325 transitions, $R^2 = 0.6720$, $y = 0.03818x - 2.335$).

The Skyline MS equation for 2⁺ charged precursor ions is shown in Equation 3-3-2. Based on the simple linear regression analysis of the results shown in Figure 3-9, the Skyline MS linear equation for predicting the optimal CE of 2+ charged peptide precursor ions should be adjusted slightly to accurately predict the optimal CE values for precursor ions when using the Agilent 6420 and Agilent Ultivo. It is suggested that the Skyline MS software for predicting the optimal CE should account for differences in instrumentation produced by the same manufacturer and the charge state of the precursor ion. According to the results shown in Figure 3-7, the equation for predicting

the optimal CE of 2+ charged peptide ions when analysed on the Agilent 6420 is shown below as Equation 3-4:

$$CE = 0.03856 * (\text{precursor } m/z) - 2.84$$

Equation 3-4: Empirically determined equation for predicting the optimal CE of 2+ peptides when measured on the Agilent 6420 instrument.

Additionally, the equation for predicting the optimal CE of 2+ charged peptide ions when analysed on the Agilent Ultivo is shown below in Equation 3-5:

$$CE = 0.03818 * (\text{precursor } m/z) - 2.335$$

Equation 3-5: Empirically determined equation for predicting the optimal CE of 2+ peptides when measured on the Agilent Ultivo instrument.

To investigate the accuracy of the Skyline MS linear equation for triply charged (3+) precursor ion collision energy prediction, Figure 3-10 shows the relationship between optimal CE and precursor ion m/z for 3+ charged precursor ions, measured on the Agilent 6420 (A) and the Agilent Ultivo (B) respectively.

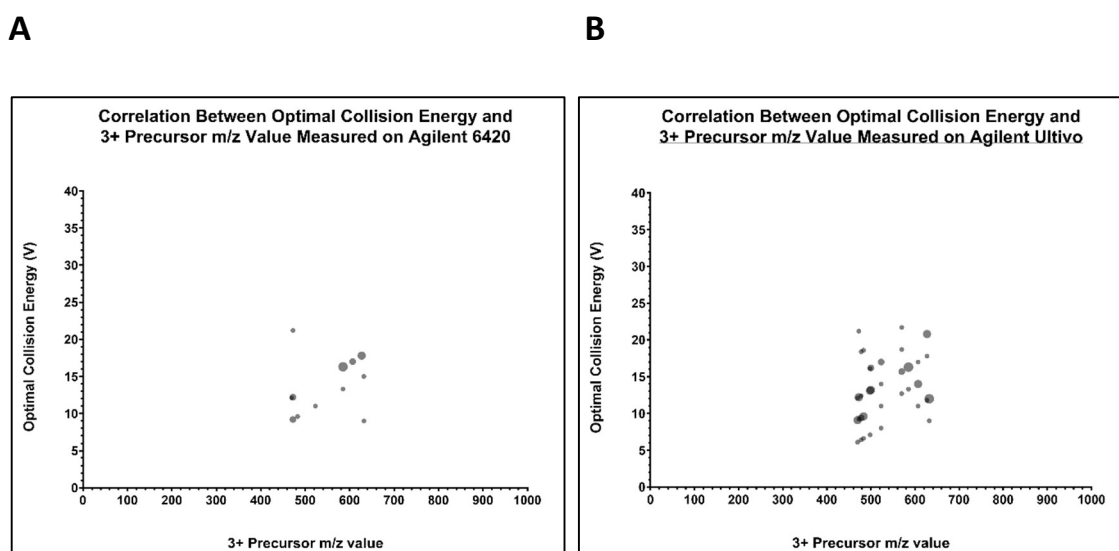


Figure 3-10: Relationship between the optimal collision energy and precursor ion m/z for triply charged (3+) precursor ions of synthetic nuclear lamin tryptic peptides measured on the A) Agilent 6420 and the B) Agilent Ultivo (n=1). Multiple product ion readings are recorded for each precursor ion as 4-5 transitions were monitored per precursor ion. The size of each bubble is indicative of multiple product ions from the same precursor ion which had the same optimal collision energy. Readings were measured on the Agilent 6420 (20 transitions, $R^2 = 0.1472$, $y = 0.02021x + 2.794$) and the Agilent Ultivo (60 transitions, $R^2 = 0.1472$, $y = 0.02486x + 0.08620$).

For 3+ charged precursor ions, the Skyline MS equation for Agilent QQQ instruments is shown in Equation 3-3-3. The results shown in Figure 3-10A and Figure 3-10B suggests that the linear equation for predicting the optimal CE of 3+ charged peptide precursor ions should be adjusted slightly when using the Agilent 6420 and Agilent Ultivo to measure nuclear lamin peptides. Based on the results shown in Figure 3-8, the equation for predicting the optimal CE of 3+ charged peptide ions when analysed on the Agilent 6420 is shown below in Equation 3-6:

$$CE = 0.02021 * (\text{precursor } m/z) + 2.794$$

Equation 3-6: Empirically determined equation for predicting the optimal CE of 3+ peptides when measured on the Agilent 6420 instrument.

Furthermore, the equation for predicting the optimal CE of 3+ charged peptide ions when analysed on the Agilent Ultivo is shown below in Equation 3-7:

$$CE = 0.02486 * (\text{precursor } m/z) + 0.0862$$

Equation 3-7: Empirically determined equation for predicting the optimal CE of 3+ peptides when measured on the Agilent Ultivo instrument.

Continuing the discussion of how charge state affects optimal CE during CID fragmentation within Q2, it has been reported that higher charge state precursor ions require less energy for CID (Huang et al., 2005; Révész et al., 2023). To examine this observation in the current dataset, analysis was performed to compare the optimal CE values observed for 2+ and 3+ peptide precursor ions when measured on the Agilent 6420 QQQ and the Agilent Ultivo QQQ. The results are shown below in Figure 3-11A and Figure 3-11B respectively.

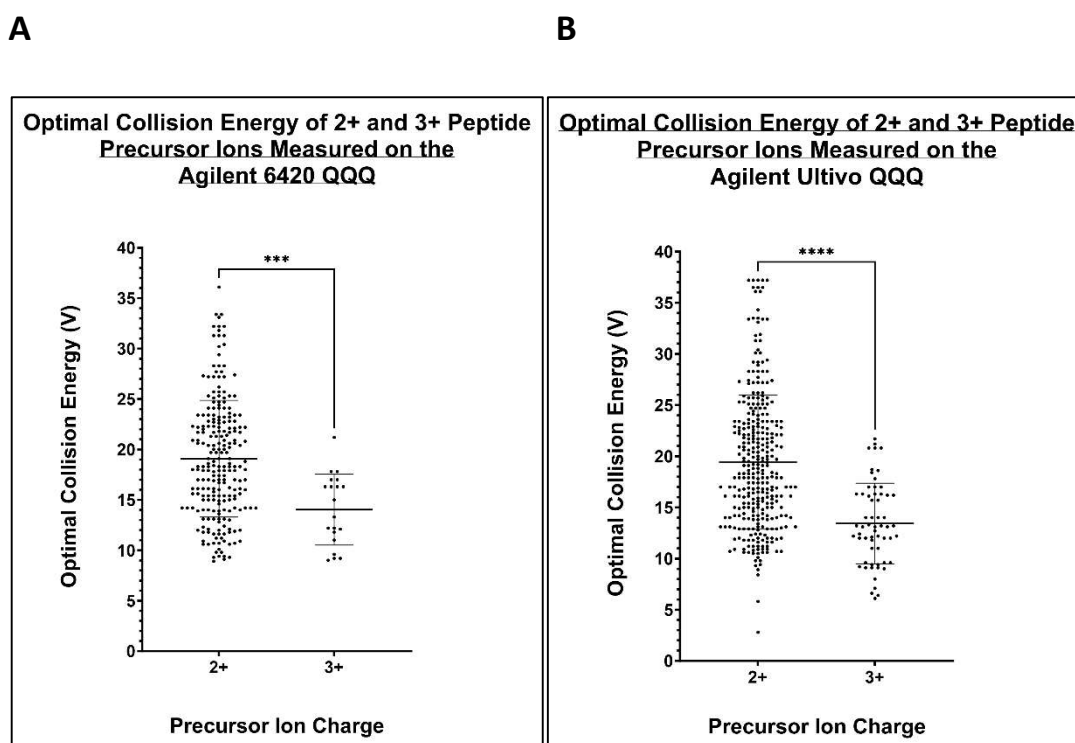


Figure 3-11: Effect of precursor ion charge on the optimal collision energy of 2+ and 3+ peptide precursor ions measured by LC-MRM-MS/MS on the A) Agilent 6420 QQQ and the B) Agilent Ultivo QQQ. The mean is indicated by the horizontal line and error bars represent standard deviation. A) 233 transitions from 2+ precursor ions and 20 transitions from 3+ precursor ions were measured on the Agilent 6420. Unpaired T-test results, $p=0.0002$. B) 325 transitions from 2+ precursor ions and 60 transitions from 3+ precursor ions were measured on the Agilent Ultivo. Unpaired T-test results, $p<0.0001$.

The results in Figure 3-11A and Figure 3-11B show that 3+ precursor ions have a lower optimal CE value than 2+ precursor ions when analysed on both QQQ instruments. Statistical analysis of the optimal CE values for 2+ and 3+ precursor ions via unpaired T-tests showed that 3+ precursor ions have a statistically significantly lower optimal CE when measured on the Agilent 6420 ($p=0.0002$) and Agilent Ultivo ($p<0.0001$). This supports previous findings in this field which suggest that higher charge state precursor ions required less energy to fragment (Huang et al., 2005; Révész et al., 2023).

Overall, the data above shows that CE optimisation can lead to significant fold increases in peak area measurement when analysing peptides via MRM-LC-MS/MS (Figure 3-7). The data in this sections illustrates that both instruments showed similarities in the percentage of peptides that benefit from CE optimisation (Figure 3-6), and the average fold change in peak area when CE was optimised was similar for both instruments (Figure 3-7). Upon CE optimisation, up to a 4-fold increase was seen for peak area measurements of transitions analysed on the Agilent 6420, while this was slightly higher, with peak area measurements reaching up to a 6-fold increase when measured on the Agilent Ultivo. The study compliments previous literature with respect to these findings. Both instruments showed a positive correlation between precursor m/z and optimal CE, which agrees with current findings in this area of research (Figure 3-8, Figure 3-9 and Figure 3-10). Finally, the previous observation that ions with a greater charge require less CE to fragment was confirmed in the current study (Figure 3-11). Published literature suggests that the choice of instrument is a factor affecting the optimal CE used during mass spectrometry analysis (Révész et al., 2023), however the current study found negligible differences in the two instruments used. This may be attributed to both instruments being manufactured by the same vendor.

3.4.4.2. Optimising Fragmentor Voltage

Fragmentor voltage (FV) is the electric potential applied to the end of the atmospheric sampling capillary, which is in a medium pressure environment (ca. 3 torr) and provides the analyte ions with the kinetic energy needed to pass through the CID region from the heated capillary between the electrospray chamber (at atmospheric pressure) and to the skimmer and mass analysers (vacuum pressure, ca 10^{-5} torr). The amount of FV applied helps to focus the ions into a beam and controls the speed at which the ions and transverse the CID region. Since this event occurs before the mass analyser unit, it is often referred to as in-source fragmentation. Figure 3-12 shows the schematic of the ion optics unit where the FV is applied.

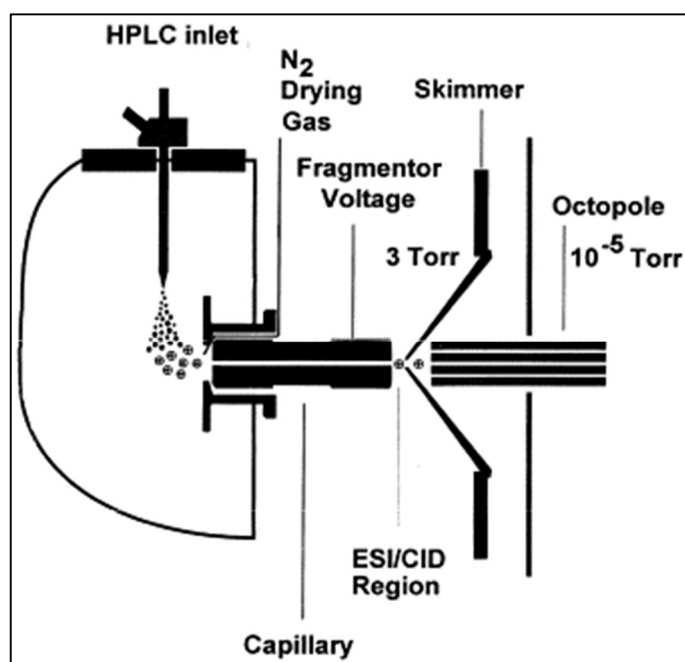


Figure 3-12: Schematic of the ion optics unit of a mass spectrometer, showing the location where the fragmentor voltage is applied. Diagram from (Weinmann et al., 2001).

Although ESI is considered a soft ionisation technique that generates a minimum number of fragments during the ionisation process, analytes may fragment within the ESI source as inappropriate ESI settings are applied, resulting in undesired in-source

fragmentation (He et al., 2020). In-source fragmentation should be avoided in MRM-based assays as it can lead to issues such as reduced sensitivity, and false negative or positive quantitative results (Muehlwald et al., 2018; Sun et al., 2022). In some cases, in-source fragmentation is favourable at this point if the experimental aim was to provide additional structural information about an analyte (Sun et al., 2022), such as when differentiating between D- and L-amino acids (Kong et al., 2014). However, since the aim of the current study is to develop a targeted MRM-based assay, confirmation of the parent ion in Q1 is necessary before fragmentation, therefore an FV value that does not cause in-source fragmentation is required.

The FV is usually set to a default value by the instrument manufacturer. By optimising the FV, the aim is to maximise the analyte ion transmission and minimise CID fragmentation (Presser et al., 2014). FV is a compound-dependent parameter (Presser, 2009) and ions with a larger m/z will require more energy and thus a higher FV at this stage to traverse the space between the heated capillary and the skimmer and Q1. Too high FV values for an analyte ion will result in in-source fragmentation of the ions as they collide with neutral molecules within the CID region. This is unfavourable as the parent ion will be smaller than expected, with a different m/z , resulting in a decrease in assay selectivity as these ions of a different m/z to the target m/z will be filtered out of the mass analyser within Q1 in MRM-type experiments. Conversely, setting the FV too low will result in ions being unable to successfully travel through the CID region, resulting in a low MS signal response. FV has been shown to greatly influence sensitivity and limits of detection and quantitation of analytes (Sun et al., 2022).

In this section, peak area measurements obtained from MRM analysis of peptides at different FV values were compared and evaluated. Since FV is affected by ionisation and the Agilent Ultivo has a technically different ionisation source than the Agilent 6420 due to the use of the AJS ESI, optimisation of all further parameters in this chapter will be conducted on the Agilent Ultivo only.

The same MRM transitions used to optimise CE in Section 3.4.4.1 were used for FV optimisation in this section, resulting in a total of 380 transitions from 79 precursor ions (2^+ precursors $n=67$; 3^+ precursors $n=12$). FV was optimized in a similar way to CE optimisation in Section 3.4.4.1, with the peak area of each transition measured in a single injection, and 6-7 peptides pooled into each sample. FV values between 90 V and 150 V were measured in 10 V steps and the CE used for each transition were determined by the optimisation result in Section 3.4.4.1. Figure 3-13 shows the optimal FV for 380 transitions used to measure synthetic peptides of lamin A, lamin B1, lamin B2 and lamin C using the Agilent Ultivo. After varying the FV of each transition, the FV with the highest MS signal intensity, as determined by the peak area measurement, was chosen as the optimal FV.

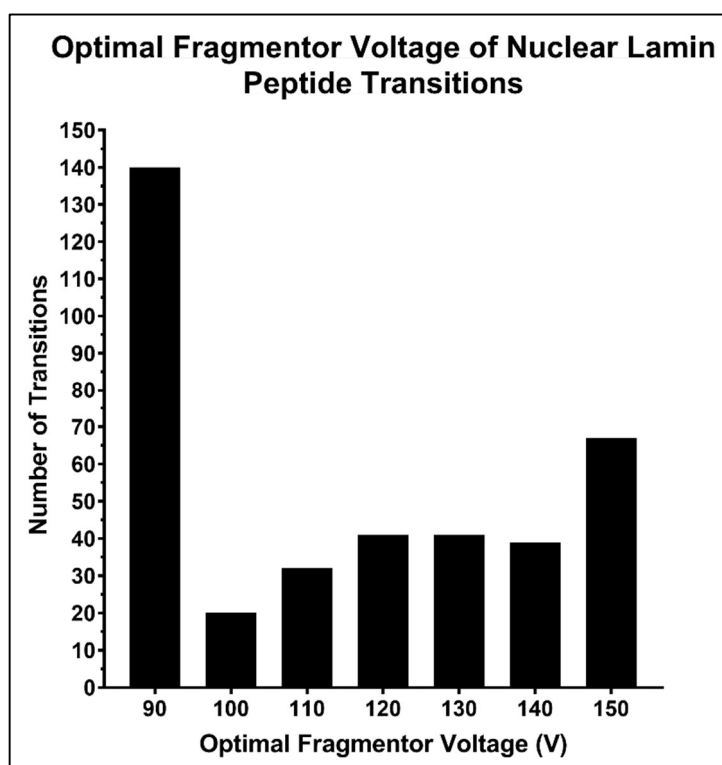


Figure 3-13: Bar chart showing the optimal fragmentor voltage of 380 transitions of synthetic nuclear lamin peptides ($n=1$).

The results in Figure 3-13 show that 339 of 380 transitions (89.21%) had an optimal FV that was different to the default FV of 130 V, and showed that only 41 of 380 transitions (10.7%) had an optimal FV of 130 V. A large proportion of transitions (140 of 380, 36.8%) had an optimal FV of 90 V, which is 40 V less than default FV. These transitions may have benefitted from a further reduction in FV, however that was not investigated. Since almost 90% of peptide transitions had an optimal FV that was different to the default FV, this suggests that the default FV settings on the Agilent Ultivo is too high for most peptide MRM analysis, and FV optimisation for different biomolecules is essential.

For transitions in which the optimal FV was different to the default FV (n=339), further analysis was performed to show the fold change in measured peak area achieved when the optimal FV was used compared to when the default FV was used. Figure 3-14 the fold change in peak area of each of the transitions where the optimal FV was not the default.

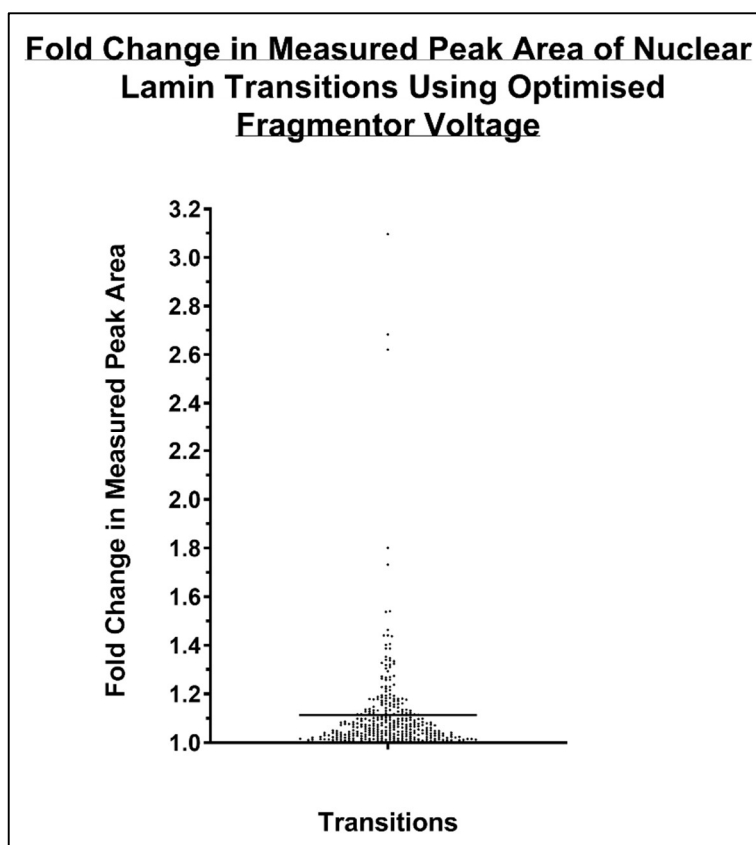


Figure 3-14: Fold change in the measured peak area when using the empirically determined optimal fragmentor voltage compared to default fragmentor voltage when measuring 339 transitions of nuclear lamin peptide precursors by LC-MRM-MS/MS (n=1). Horizontal line indicates the mean.

As shown in Figure 3-14, for 339 transitions where the optimal FV was found not to be the default FV of 130 V, the fold change in measured peak area when using the empirically determined optimal FV ranged from 1.0008 to 3.0963, with a mean fold change of 1.1127. This increase in peak area is not as large as that achieved when optimizing the CE as shown in Figure 3-7 where up to a 6-fold increase in peak area was achieved for some transitions, however Figure 3-14 above shows evidence that up to a 3-fold increase in peak area measurement can be achieved when optimizing the FV for analysis of peptide ions. In addition, Bieber and colleagues found that FV had the greatest influence on signal height of detected compounds, when optimising AJS ESI settings and FV, however they did not optimise CE in their study (Bieber et al., 2019). Therefore, FV optimisation contributes to an increase in assay sensitivity, and the data

above further supports the requirement for FV optimisation for LC-MRM-MS/MS assay development.

As mentioned previously, the consensus in this research area suggests that higher m/z precursor ions require a higher FV. To investigate if the m/z of the precursor ion affects the optimal FV, Figure 3-15 below shows the relationship between m/z value of the precursor ions with their empirically determined optimal FV.

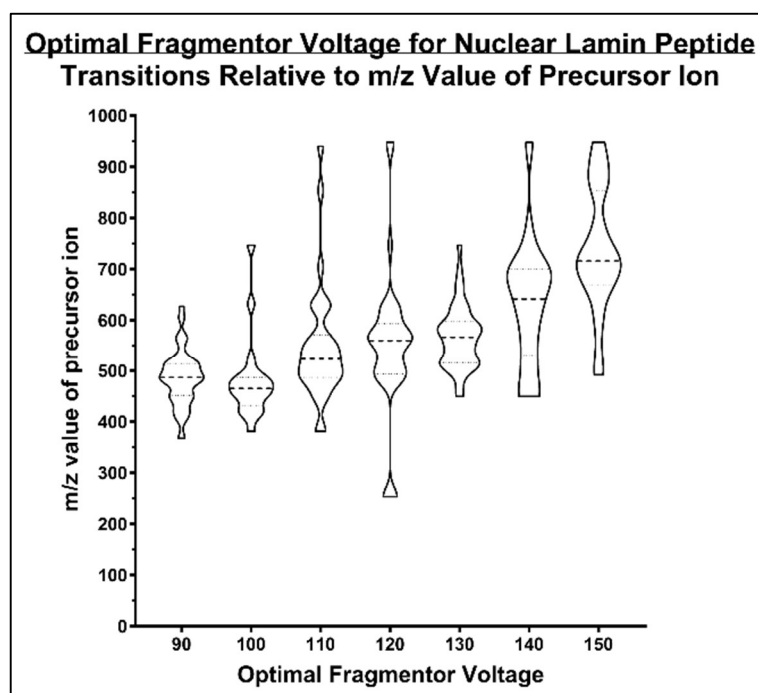


Figure 3-15: Relationship between the m/z of peptide precursor ions and the empirically determined optimal fragmentor voltage for 380 transitions of nuclear lamin peptides ($n=1$). $R^2 = 0.2660$.

Figure 3-15 shows there is a slight trend in that higher m/z precursors have a higher optimal FV ($R^2 = 0.2660$). This result was expected as larger, heavier ions require more energy for them to be able to pass through the skimmer and into the mass analyser.

In summary, the above data shows that FV optimisation can result in significant increases in peak area measurements when analysing peptides via MRM-LC-MS/MS. Almost 90% of peptide ion transitions benefitted from a change in FV which was different from the default FV of 130 V, while over a third of transitions had an optimal FV of 90 V (Figure 3-13). Up to a 3-fold increase in peak area could be achieved when the optimal FV was used for the assay (Figure 3-14). There was also a positive correlation between the precursor m/z and optimal FV (Figure 3-15), which aligns with current knowledge in this area.

3.4.4.3. Summary of CE and FV Optimisation

To conclude, optimizing both CE and FV is important in MRM assay design. Optimisation of the CE for each transition led to a greater increase in fold change in peak area than FV optimisation. This suggests that FV optimisation is less influential on maximizing peak area and analyte detection during MRM analysis compared to CE optimisation. In hindsight, it may have been better to optimise FV before CE, as the FV is applied to the ions before they reach the collision cell, however this does not affect the validity of the results produced in this study.

3.4.5. Optimisation of Agilent Jet Stream Electrospray Ionisation Source Parameters

Commonly described MS optimisation parameters in the literature include CE and FV as discussed previously, however the evaluation of operational mass spectrometer settings is rarer in the scientific literature. A small collection of published literature has aimed to optimise electrospray source parameters for use in assays of several different biological and chemical targets, although very few discuss the effects of each parameter on the measured ion intensity and assay sensitivity. Use of the AJS ESI system has been documented since 2009 (Mordehai & Fjeldsted, 2009), and several groups have used systems with the AJS ESI source for detection and quantitation of a variety of analytes (Bieber et al., 2019; Kukula-Koch, 2017; Moreiras et al., 2018).

The AJS ESI source is made of a number of different hardware elements which aid in the production of an efficient electrospray. Figure 3-16 below shows a diagram of the AJS ESI source and location of several key operational components.

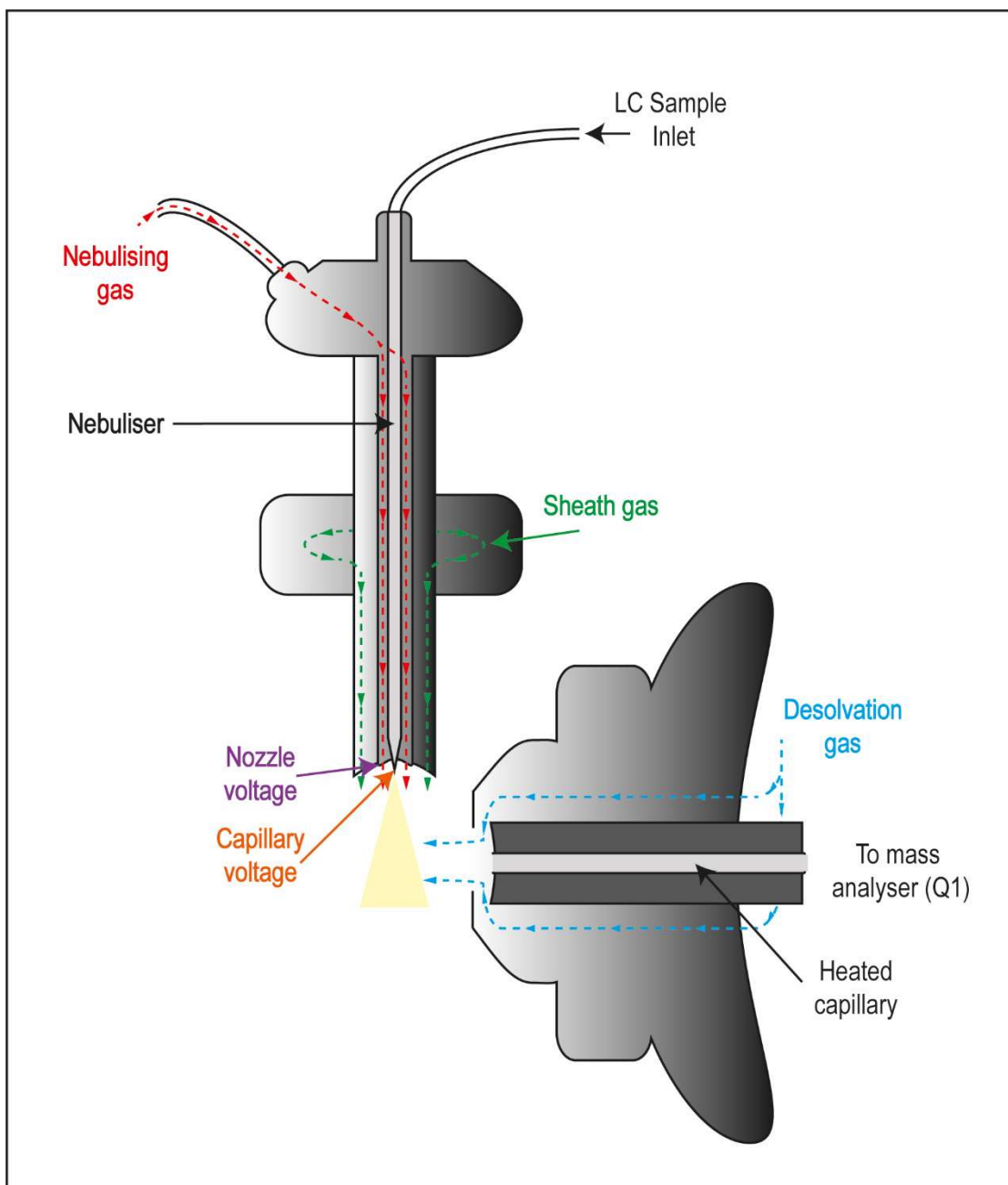


Figure 3-16: Diagram of the Agilent Jet Stream Electrospray Ionisation (AJS ESI) source parameters. Yellow indicates electrospray plume.

Each component in Figure 3-16 will now be discussed, starting with the electrical components. The capillary voltage is the electrical potential applied to the end of the nebuliser (also known as the emitter) to ionise the biomolecules in the solvent and form an electrospray. The nozzle voltage is the voltage applied to a metal ring around the end of the apparatus housing the nebuliser. Adjusting the nozzle voltage allows the

the widening or narrowing of the electrospray plume to incorporate various regions of the plume into the nebulising gas spray for efficient desolvation and entry into the MS inlet.

With respect to gases, the process of ESI is usually pneumatically assisted by nitrogen gas flowing in various directions respective to the sample flow, to aid desolvation and evaporation of the solvent droplets, and help guide the ionised analytes into the mass analyser unit. The first of these gases is the nebulising gas, which flows in the same direction as the sample flow and shears around the eluted sample solution helps guide the sample out of the nebuliser and into the electrospray source. Increasing the pressure of the nebuliser gas may help create smaller solvent droplets which leads to reduced matrix effects. The desolvation gas, also known as the drying gas, is a stream of gas which flows perpendicular to the electrospray plume and also aids solvent droplet desolvation. Both the temperature and flow rate of the desolvation gas can affect the desolvation of sample droplets. Finally, the sheath gas is an additional gas implemented into the AJS ESI system which is not present within convention ESI sources. This superheated nitrogen sheath gas flows in the same direction as the solvent flow and nebuliser gas and confines the electrospray in order to more effectively desolvate the ions and concentrate them into a thermal confinement zone. By columnating the electrospray into this zone, a higher density of ions is presented at the front of the MS inlet which allows more ions to be sampled by the MS, improving sensitivity. According to the instrument vendor, a 5- to 10-fold gain in sensitivity was achieved for flow rates ranging from 0.25 to 2 mL/min using this technology compared to using a convention ESI source (Mordehai & Fjeldsted, 2009). The high temperature of the sheath gas has been shown to result in better desolvation of analyte solvent droplets, however the heat applied may enhance ion suppression since desolvation of the sample too quickly may cause precipitation or degradation of the sample. The flow rate of the sheath gas can also be adjusted to increase solvent desolvation and evaporation and provide increased efficiency of analyte ionisation during the electrospray process.

By manipulating the nebulising gas pressure, and the temperature and flow rates of the desolvation gas and sheath gas, alongside optimisation of the nozzle voltage and capillary voltage, the electrospray can be optimised to produce a stable Taylor cone and the continuous, steady production of droplets of ionised analytes which form gaseous ions able to be measured by the mass spectrometer.

Traditionally, nano-ESI would be used to detect low abundant proteins due to its increased sensitivity compared to conventional flow ESI-MS analysis. This is attributed to the smaller droplet size produced during nano-ESI, which allows for enhanced desolvation and therefore enhanced electrospray efficiency and a greater percentage of ions entering the MS. However, nanoflow comes with technical challenges in operation and chromatography reproducibility and also increases analysis time due to decreased flow rates. Improvements in conventional flow ESI technology can allow for increased sensitivity without compromising robustness and assay time. Use of AJS ESI source can offer reproducible analysis at high throughput without compromising on instrument time and introducing complex hardware. Pergande and colleagues found that conventional flow LC with an AJS ESI source provided a comparable number of protein identifications when analysing HeLa cells compared to nanoflow ESI. AJS ESI provided reproducible results but required 5 times more sample, meaning that if the sample is not limited, use of AJS ESI can be useful in offering more reproducible results compared to nanospray ESI (Pergande et al., 2019a). AJS ESI has been shown to increase ion transfer into the MS by improving desolvation and enhancing the spatial focussing of ions into the MS inlet (Mordehai & Fjeldsted, 2009). Optimising AJS ESI settings for the analyte of interest can further increase electrospray efficiency and thus increase sensitivity for the chosen analyte, if performed suitably.

The way in which optimisation is performed is also of critical importance when considering experiment time, instrument availability, sample availability and budget. Optimization of experimental parameters in such a complex system may be time-consuming or provide insignificant results if not designed efficiently. There are different

strategies for optimisation of systems that contain several variables. The simplest optimisation strategy is known as a “one-variable-at-a-time” (OVAT) (also known as “one-factor-at-a-time” (OFAT)) strategy, in which the effect of a parameter is evaluated by changing a single variable while keeping the other parameters the same. This univariate procedure is very thorough however does not consider potential interactions between variable factors. In addition, the strategy can be time-consuming and may require a high number of experimental runs to be performed but provides a substantial amount of data to help elucidate the influence of each variable within the system being critiqued (Massart et al., 1998).

Another optimization strategy uses a multivariate technique known as design of experiments (DoE). DoE aims to determine significant experimental variables through the screening of variable factors that influence the output or response of the system being optimised. This strategy involves the simultaneous variation of different factors among experimental runs according to a mathematical plan, with the goal of obtaining the maximum amount of data from a minimum number of experimental runs. Mathematical modelling is used to describe the relationship between parameters and all interactions between variables are taken into consideration. For this reason, DoE may be considered more effective than OVAT methods (Dejaegher & Vander Heyden, 2011). Suggested advantages of using DoE over OVAT include the large amount of information from a small number of experiment, and observation of interaction effects between independent parameters (Shokuhfar et al., 2008). In the current study, the OVAT method of optimisation was chosen as the aim was to examine the individual effects of each variable AJS ESI parameter on the peak area response of peptides.

In the previous sections, CE and FV were optimised. These parameters affect the settings within the mass analyser unit of the mass spectrometer. This section will investigate the source parameters within the AJS ESI ion source, which is the first section of the mass spectrometer after LC separation (see Section 1.7.1 for further details). As mentioned previously, the ESI parameter settings are often selected

according to the instrument manufacturer's recommendations or previous user experience, rather than by careful consideration of the specific experimental conditions (such as analyte of interest, solvent system and solvent composition, flow rate, etc). Agilent has published a technical note containing source parameters for the detection of small molecules (Mordehai & Fjeldsted, 2009). The technical note is based on the analysis of pesticides, cosmetics and illicit drugs in biological fluids at standard flow rates, and suggests a 5 to 10-fold increase in sensitivity can be achieved by utilisation of the AJS-ESI source over a traditional ESI source. However, no such technical documentation exists for the analysis of peptides or lipids with the AJS ESI source, which are also commonly analysed using LC-ESI-MS/MS. The analysis of peptides differs from the analysis of small molecules in that peptides are more thermally labile, meaning they are sensitive to high temperatures and have the potential to degrade and fragment in the ionisation source as a result. This is unfavourable to their analysis and may also lead to less efficient use of the advanced thermal gradient focussing technology that is unique to AJS ESI source and has been shown to contribute to the increased sensitivity achieved using the AJS ESI source compared to previous ESI sources. It is also documented that Agilent Jet Stream parameters are very analyte-dependent (Periat et al., 2014; Stahnke et al., 2012).

The aim of this chapter was to thoroughly validate the optimal ESI conditions for the detection of synthetic peptides from nuclear lamin protein isoforms. An additional intention of this section is to discuss the influence of different AJS ESI operating parameters on the measured abundance of synthetic peptides of interest.

For AJS ESI source parameter optimisation, Table 3-VIII shows the range of optimisation for each parameter and the increments used. Parameters were examined in ranges which reflected the operational limits of the Agilent Ultivo LC-QQQ instrument. For sheath gas temperature, desolvation gas temperature, nebuliser pressure and capillary voltage, initial optimisation was performed over a wider range, and then again over a smaller range to study major and minor changes in the operational settings of these parameters. To optimise the ion source for MS analysis of nuclear lamin tryptic

peptides, optimization of MS parameters was conducted according to the OVAT strategy, in which the effect of the tested parameters was assessed by changing the level of a single factor while keeping the other factors at default settings.

Table 3-VIII: Ranges of AJS ESI source parameters used for optimisation of the MRM assay designed to measure nuclear lamin tryptic peptides by LC-ESI-MS/MS.

Source Parameter	Default Value	Range Optimised	Initial increments	Fine-tuning increments
Sheath Gas Temperature (°C)	250	100 - 400	20	10
Sheath Gas Flow Rate (L/min)	11	5-12	1	N/A
Desolvation Gas Temperature (°C)	300	80 - 340	20	10
Desolvation Gas Flow Rate (L/min)	7	6 - 13	1	N/A
Nebuliser Pressure (psi)	15	10 – 60	5	1
Capillary Voltage (V)	4000	500 – 6000	500	100
Nozzle Voltage (V)	1500	0 - 2000	500	N/A

This optimisation study was performed on synthetic tryptic peptides of nuclear lamin isoforms common to both human (*Homo sapiens*) and mouse (*Mus musculus*) protein sequences. 35 µL of each synthetic peptide was pooled into three separate Eppendorf

tubes, with each sample containing synthetic peptides corresponding to lamin A/C, lamin B1 and lamin B2. The samples were lyophilised and resuspended in 140 μ L of 5% ACN with 0.1% formic acid. 1 μ L of each sample was injected per experiment. The starting AJS ESI source parameters were the default method settings of the Agilent Ultivo QQQ instrument. Transitions measured in a dynamic MRM mode during this source optimisation experiment had optimised CE and FV values which were determined in Sections 3.4.4.1 and 3.4.4.2 and are detailed in Appendix IV Table 8-VI. Each worklist for the optimisation of each parameter was carried out in a randomised order. When optimising desolvation gas temperature and sheath gas temperature, a 15-minute wait time was introduced between loading each method and injecting the sample to allow the temperature to stabilise before the analysis begun.

3.4.5.1. Optimisation of Capillary Voltage

Capillary voltage is the electrical potential applied to the end of the nebuliser to ionise the biomolecules in the solvent and form an electrospray (see Section 3.4.5 for more information). Capillary voltage is dependent on the analytes, solvent composition, flow rate and nebuliser diameter, and can have a significant impact on method reproducibility (Kearle & Verkerk, 2010; Marginean et al., 2009; Reschke & Timperman, 2011). Capillary voltage is a compound-dependent parameter (Presser, 2009), and as such, the optimal voltage may be different for various biomolecules of interest (small molecules, lipids, peptides, etc). Presser also states that capillary voltage is somewhat molecular weight-dependent, and therefore peptides of different lengths may also have different optimal capillary voltages.

The earliest studies on electrohydrodynamics, the study of the effects of applied voltages on sprayed liquids, were conducted in the early 1900s by John Zeleny (Zeleny, 1913, 1917). Zeleny's work looked at different shapes of sprays at various spray voltages and documented these using high speed photography. Since then, extensive characterisation of the fluid dynamics for electrospray ionisation at various voltages has been performed (Marginean et al., 2009, 2007). At low voltages, the solvent exiting the emitter wets the outer wall of the emitter rim and does not create an electrospray. With increasing applied voltage, the wetted area decreases. Increasing the voltage further allows for the solvent droplet to anchor to the emitter rim - this is referred to as the "anchoring point". Operation at voltages lower than the anchoring point may result in the deposition of residue on the emitter outer wall, however this has a lesser effect on emitter operation (Marginean et al., 2009). Increasing the voltage past the anchoring point results firstly in a pulsating spray and then increasing the voltage further produces a cone-jet regime. The cone-jet regime is considered an optimal and stable spray for the ionisation and transfer of analytes to the MS inlet. An increase in voltage past the voltage suitable for a cone-jet regime results in a change in wetting properties of the emitter, which means the solvent maintains contact with the outer nebuliser wall and assigns the electrospray to an alternative path with the ESI chamber, producing erratic spray dynamics and an inconsistent electrospray (Marginean et al.,

2009). Electrical discharge can also be seen at high capillary voltages. The use of voltages greater than the discharge point can result in accelerated ageing and even damage to the nebuliser, particularly when using fused silica nanospray emitters (Chen & Cook, 2007; Geromanos et al., 2000).

Marginean and colleagues measured the effect of the LC gradient on the spray current during reversed-phase LC analysis and found that the voltage required to maintain a stable and efficient electrospray decreases significantly as the concentration of organic solvent in the mobile phase increases (Marginean et al., 2007, 2008, 2009). This is due to the lower viscosity of high organic solvents, therefore less energy is required to nebulise the liquid into a spray. This means that when analysing molecules that are eluted in a high composition of aqueous solution, the optimal voltage is higher whereas analytes that are soluble in high organic solutions are ionised, the optimal capillary voltage is lower. As a result, the voltage that ensures optimal operation at the beginning of the analysis may not be appropriate for the solvent mixture when the analyte of interest is eluted from the LC column and enters the nebuliser for ionisation (Marginean et al., 2009). Therefore, a suitable capillary voltage must be chosen that spans the entire LC gradient and does not lead to a detrimental effect on signal intensity of the peptide analytes.

Previous studies have used feedback loops to automatically adjust the applied voltage (Gapeev et al., 2009; Marginean et al., 2008; Valaskovic et al., 2004), however these were only conducted on nanoelectrospray systems and not conventional flow LCMS instrumentation. Furthermore, most LC-MS instrumentation only allows for a constant electrospray voltage to be used throughout the entire analysis, which is set by the user before beginning the experiment. Both mass spectrometry instruments used in this chapter have electrospray ionisation sources for which the capillary voltage is user-defined before starting the analysis and cannot be changed during the experiment. For experiments in which the electrospray voltage remains static throughout the assay, the recommended capillary voltage is one which is above the anchoring point at the

beginning of the analysis (when the solvent composition is highly aqueous) but still below the point in which electrical discharge is seen at the end of the analysis (when the solvent composition is highly organic) (Marginean et al., 2009). This chapter aims to optimise the capillary voltage for individual peptides, which are ionised at different solvent compositions. The aim of this optimisation is to select a voltage that ensures effective electrospray throughout the gradient elution LC period used for the separation of peptides. A consensus voltage in which most peptides give a reasonable ion signal will be chosen as the optimal capillary voltage for the assay.

The capillary voltage was optimized through a range of 500 V to 6000 V, in 500 V increments. Figure 3-17 shows the mean measured peak area acquired for peptide precursors of tryptic peptides of nuclear lamins at different capillary voltages.

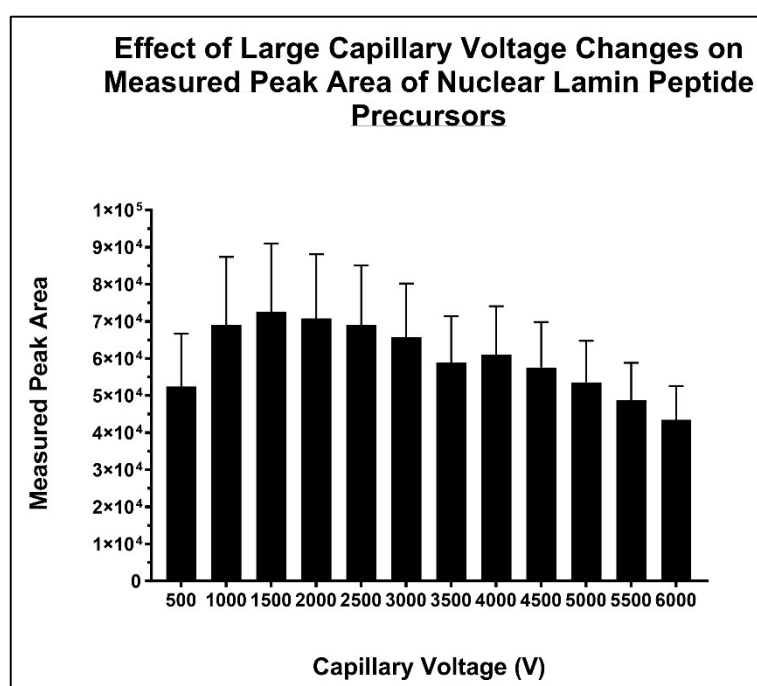


Figure 3-17: Effect of changing the capillary voltage on the mean measured peak area of 77 nuclear lamin peptide precursors, within the range of 500 V to 6000 V (n=1). Error bars indicate standard error of the mean. One-way ANOVA, p=0.9401.

The results of Figure 3-17 show that the average measured peak area was the greatest when the capillary voltage was set to 1500 V. Statistical analysis by use of a one-way ANOVA test showed there was no statistically significant effect of capillary voltage changes on the mean measured peak area of peptide precursors ($p=0.9401$). When examining the peak areas of each individual transition, it was also found that there was no significant difference in the measured peak area over the range of 500 to 6000 V (Appendix IV Figure 8-7).

Changes through finer increments of 100 V were then measured to investigate if small changes to the capillary voltage had a significant effect on measured peak area. Figure 3-18 shows the measured peak area acquired for peptide precursors at different capillary voltages, measured in 100 V increments.

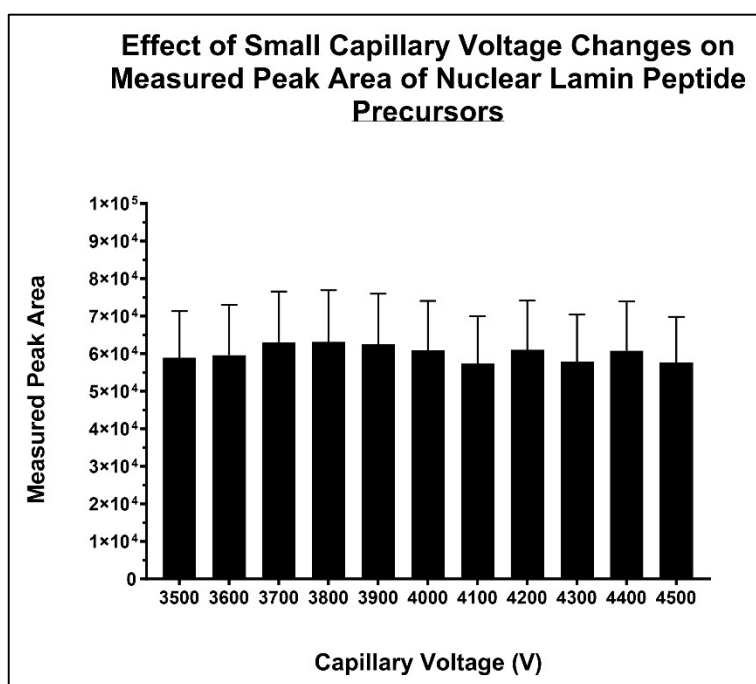


Figure 3-18: Effect of changing the capillary voltage on the mean measured peak area of 77 nuclear lamin peptide precursors, within the range of 3500 V to 4500 V ($n=1$). Error bars indicate standard error of the mean. One-way ANOVA, $p>0.9999$.

Statistical analysis of Figure 3-18 using a one-way ANOVA test shows there was no significant difference in the measured peak area over a range of 1000 V when the capillary voltage was altered in 100 V steps ($p>0.999$). Upon examining each individual transition, it was also found that there was no significant difference in the measured peak area over the range of 3500 to 4500 V (Appendix IV Figure 8-8). This agrees with the previous literature, as Teh and colleagues found that capillary voltage did not significantly affect the peak area of bioactive markers isolated from plants (Teh et al., 2011).

To examine the individual fold change of each precursor when the capillary voltage was changed from the default setting (4000 V) to the empirically determined optimal setting (1500 V), a dot plot was created. Figure 3-19 below shows the fold changes in measured peak area achieved when using the optimal capillary voltage to analyse these peptides compared to the peak area achieved when using the default capillary voltage. In addition, the fold changes of peptides used to measure specific peptides of biological importance including the pan-lamin peptide, pan B-type lamin peptide, and peptides specific to lamin A and C in mice are highlighted separately and shown in the Discriminative peptide group. These results are shown in Figure 3-19.

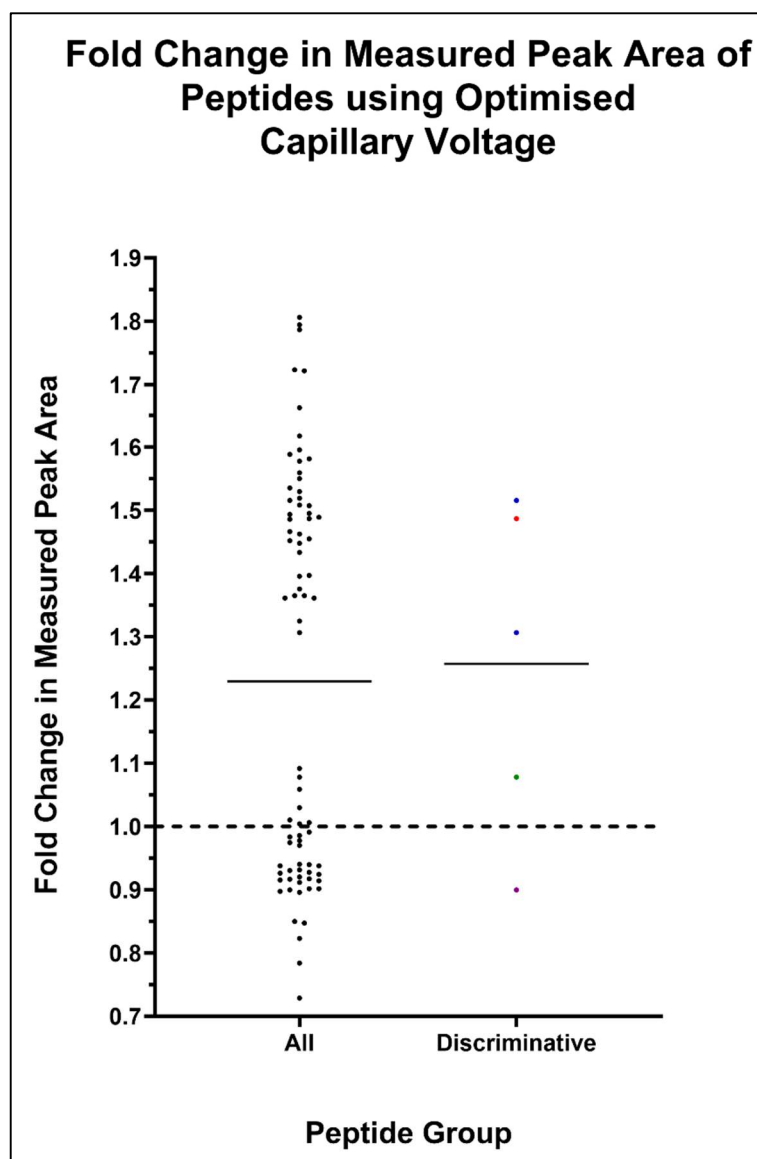


Figure 3-19: Fold change in measured peak area of 77 nuclear lamin peptides achieved upon changing capillary voltage from 4000 V to 1500 V (n=1). Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

When investigating the peptide precursors in Figure 3-19, the mean fold change was 1.2295 with a range of 0.7287 to 1.8062. 31 of 77 (40.26%) of the precursors measured

gave a fold change of less than 1, indicating a detrimental effect on peak area when the capillary voltage was changed from 4000 V to 1500 V. In support of this, when investigating the fold change of each individual transition pertaining to each nuclear lamin peptide upon altering the capillary voltage, 148 of 357 (41.46%) of transitions gave a fold change of less than 1 (Appendix IV Figure 8-9). Since over 40% of precursors measured at 1500 V gave a fold change in measured peak area of less than 1, the capillary voltage was not changed as part of the final analysis. A paired T-test of the raw peak area data suggested that there was no significant difference in the measured peak area achieved for this collection of precursor ions when the optimal capillary voltage of 1500 V was used compared to the default voltage of 4000 V ($p=0.1344$).

Several studies have noted that the optimal capillary voltage is dependent on the solvent flow rate. Studies using AJS ESI sources have used a capillary voltage of 4000 V when measuring metabolites and small molecules at a flow rate of 0.2 - 0.5 mL/min (Amarasinghe et al., 2012; Kozlík et al., 2013; Li et al., 2022; Yang et al., 2022), however 3500 V was used when measuring small molecules in rat plasma with a AJS ESI source at a flow rate of 0.3 mL/min (Zhu et al., 2022) and when measuring sugars at a flow rate of 1 mL/min (Perera et al., 2017). When investigating peptides, Pergande and colleagues have published several papers using 3500 V capillary voltage with the AJS ESI source at a flow rate of 0.2 mL/min (Pergande et al., 2019a, 2019b). In the current study, it was found that changing the capillary voltage to the empirically determined optimal capillary voltage of 1500 V resulted in a decrease in measured peak area for a large number of peptides, and therefore 4000 V was used. Since the solvent flow rate used in this experiment was 0.3 mL/min, this was considered a suitable voltage to use based on previous findings using similar flow rates.

Focussing on the peptides of significant interest, which include the pan-lamin peptide, pan B-type lamin peptide and peptides used to differentiate between lamin A and C in mice, most peptides gave a higher peak area when the capillary voltage was adjusted to 1500 V from 4000 V. Sherwood and colleagues found that optimising the capillary

voltage resulted in 35% of peptides gaining a greater than 30% increase in MS signal response (Sherwood et al., 2009b). This result is similar to the results shown in Figure 3-19, as 39 of 77 peptide ions (50.6%) experienced a greater than 30% increase in peak area measurement when the capillary voltage was adjusted from 4000 V to 1500 V.

Since Presser noted that the optimal capillary voltage is molecular weight-dependent (Presser, 2009), the relationship between m/z and optimal capillary voltage was examined. Figure 3-20 below shows the optimal capillary voltage in relation to the m/z of the precursor ions.

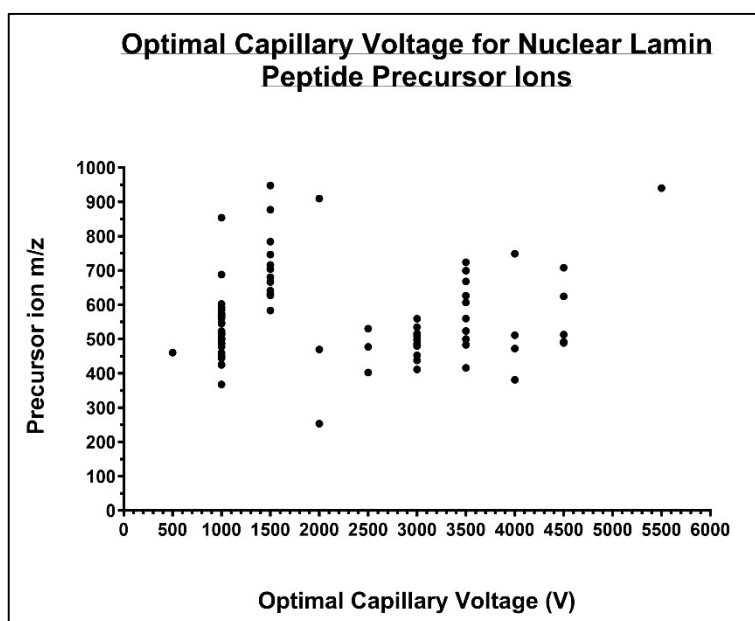


Figure 3-20: Relationship between optimal capillary voltage and m/z value of 77 peptide precursor ions (n=1).

The results of Figure 3-20 suggest no specific trend between precursor m/z and optimal capillary voltage.

There seems to be two clusters of data in Figure 3-19 and Figure 3-20, and further investigation into the group of peptide ions which recorded the highest peak area measurements at 1000 V and 1500 V included all peptides relating to LMNA and LMNC, whereas peptides where the optimal capillary voltage was above 3000 included all LMNB1 and LMNB2 peptides. Since the optimisation of capillary voltage was performed where different isoforms were analysed per injection (LMNA/C, LMNB1 and LMNB2 peptides analysed in separate injections), these results may be a result of erroneous data collection at the point of LMNA/C analysis. This could be due to instrument calibration issues however these would have been identified and rectified prior to analysis during the calibration process. AJS ESI source cleanliness can also affect measurements. Every effort was made to keep the instrument source clean, and calibration was performed after every source clean. Repeat technical measurements would confirm if the results were incorrect, or if LMNA/C peptides do indeed give a greater peak area measurement at lower voltages.

To summarise, the data above shows that there was no significant difference in measured peak area of peptide precursors when the capillary voltage was changed over the range of 500 V to 6000 V (Figure 3-17) and a smaller range of 3500 V to 4500 V (Figure 3-18). Adjusting the capillary voltage to the empirically determined optimal capillary voltage of 1500 V for this set of peptides resulted in two groups of data, one which benefitted from the reduction in capillary voltage and another which experienced a reduction or minor increase in peak area measurements (Figure 3-19). Investigation into the influence of precursor m/z on optimal capillary voltage revealed no significant trend between the two factors (Figure 3-20), although given the technical issue with the injection of lamin A/C peptides, this may mask any potential patterns correlating m/z to optimal capillary voltage in this study.

3.4.5.2. Optimisation of Desolvation Gas Flow Rate

Desolvation gas, also known as drying gas, flows either in the opposite direction or perpendicular to the analytes entering the MS inlet depending on orientation of the specific source used. In an orthogonal electrospray system such as those on the Agilent 6420 QQQ systems, the desolvation gas flows perpendicular to the electrospray and in the opposite direction to the ions entering the mass analyser to effectively desolvate the ions before they enter the mass analyser. Desolvation improves the spatial focussing of the ions and increases sensitivity limiting the entry of undesirable ions into the mass spectrometer, increasing the signal-to-noise ratio (Wang et al., 2007) (see Section 1.7.1.2 for further information on the mechanism of electrospray ionisation).

Desolvation gas flow and temperature have been shown to be critical components in providing effective desolvation of the LC eluent and the subsequent successful production of gas-phase ions during ESI (Lupo, 2018) and should be optimised depending on the solvent composition and solvent flow rate. Drying gas flow rate is considered a flow-dependent parameter (Presser, 2009). Coupled with the nebuliser pressure, both these parameters affect the efficiency of ion formation within the electrospray. Szerkus and colleagues found that source parameters that contributed to most of the variance in MS response were desolvation gas flow, desolvation gas temperature and nebuliser pressure (Szerkus et al., 2016). As the MRM method uses a solvent gradient to elute analyte ions from the LC column, the optimal desolvation gas flow rate may change over the course of the method as the solvent composition changes.

Figure 3-21 shows the mean measured peak area acquired for peptide precursors at different desolvation gas flow rates.

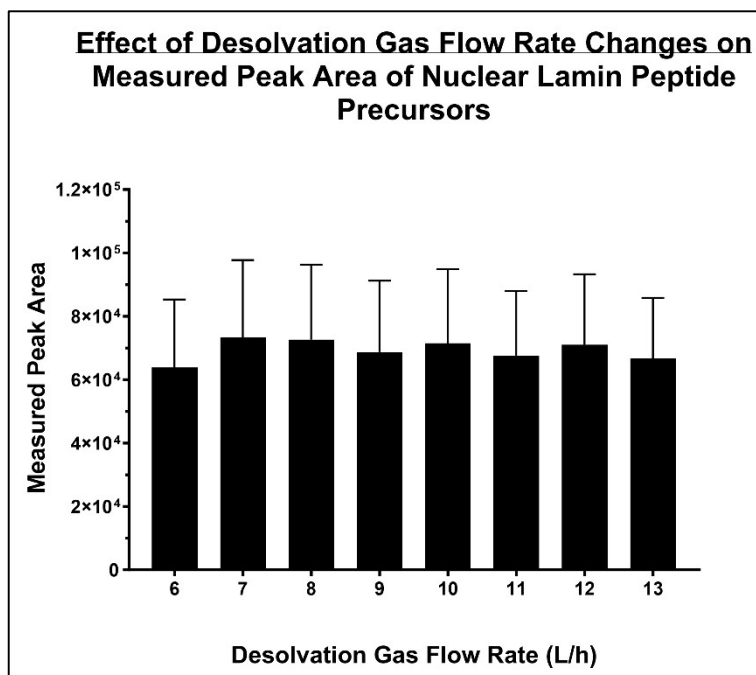


Figure 3-21: Effect of changing the desolvation gas flow rate on the mean measured peak area of 73 nuclear lamin peptide precursors, within the range of 6 L/h to 13 L/h (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p > 0.9999$.

The results of Figure 3-21 show that the measured peak area is greatest when the desolvation gas flow rate is set to 12 L/h. Statistical analysis using a one-way ANOVA test shows there was no significant difference in measured peak area ($p > 0.9999$). Investigations into each individual MRM transition also shows 12 L/h was the optimal desolvation gas flow rate and no significant difference across the range of flow rates examined was observed (Appendix IV Figure 8-10). Two studies by Norton and colleagues found that when analysing zwitterionic and cationic surfactants by capillary electrochromatography coupled with ESI-MS analysis, a decrease in MS signal was observed with an increase in drying gas flow rate (Norton et al., 2006, 2005). In contrast, a later study by the same group measured the MS signal of non-ionic surfactants and found that increasing drying gas flow rate in the range of 2 – 5 L/min increased signal abundance but only slightly, however increasing the drying gas flow rate from 5 L/min to 10 L/min decreased signal abundance (Norton & Shamsi, 2007). In support of this study, Wang and colleagues found that MS response increased as the drying gas flow rate increased from 2 L/min to 6 L/min, then stabilised from 6 L/min to

8 L/min (Wang et al., 2007). It is important to note that these four studies use capillary electrophoresis, which differs from LC and uses a sheath liquid to aid sample ionisation (Stolz et al., 2019). The addition of a sheath liquid may affect the electrospray dynamics and the effect of sheath gas on nebulisation of the sample.

With an LC solvent flow rate of 300 μ L/min and ESI source, Teh and colleagues found that drying gas flow rate did not significantly affect the peak area of bioactive markers isolated from plants when measuring the range of 6 – 12 L/h (Teh et al., 2011). Cuykx and colleagues found that drying gas flow rate and temperature did not affect sensitivity as long as the spray was stable (Cuykx et al., 2017). This publication agrees with the results in Figure 3-21, where changes in desolvation gas flow rate did not significantly affect the measured peak area of peptides.

Figure 3-21 shows the individual fold change of each precursor peak area when the desolvation gas flow rate was changed from the default setting (11 L/h) to the empirically determined optimal setting (10 L/h). As in Figure 3-19, peptides of significant biological interest were highlighted as a separate dataset.

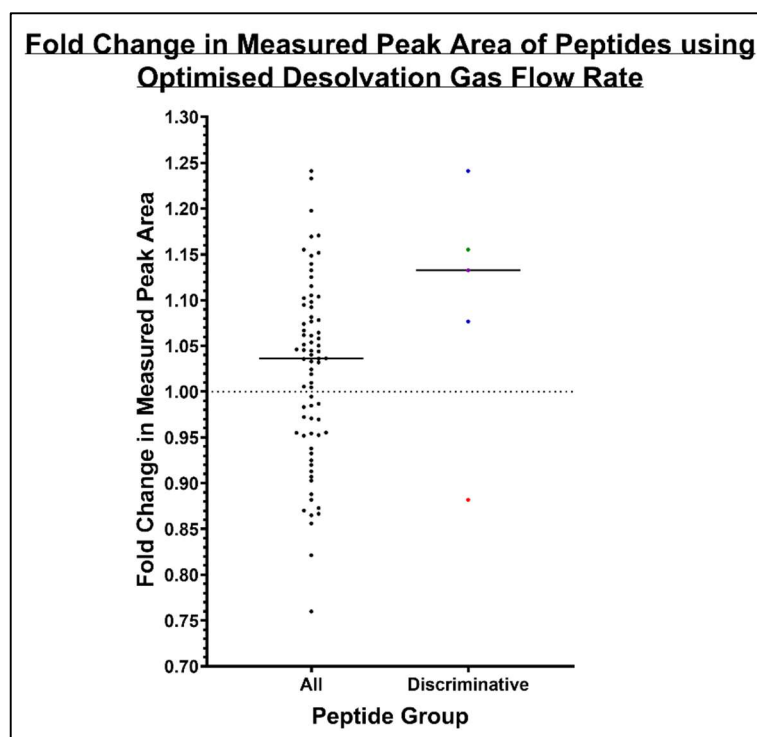


Figure 3-22: Fold change in measured peak area of 73 nuclear lamin precursors achieved upon changing the desolvation gas flow rate from 7 L/h to 10 L/h (n=1).

Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

When investigating precursor peak area in Figure 3-22, the mean fold change was 1.0214 with a range of 0.7600 to 1.2410 when optimising the desolvation gas flow rate. 28 of 73 (38.36%) of the precursors measured gave a fold change of less than 1, indicating a detrimental effect upon changing the flow rate to the empirically determined optimal setting. Biologically significant peptide ions mostly showed a small increase in measured peak area with a desolvation gas flow rate of 12 L/h compared to 7 L/h, with only the pan-lamin peptide LLEGEEER experiencing a decrease in measured peak area. Since 38% of precursors measured at 12 L/h gave a fold change in measured peak area of less than 1, the desolvation gas flow rate was not changed as part of the final analysis. Transition-level analysis also agrees with the findings shown in Figure

3-22 (Appendix IV Figure 8-11), with 150 of 346 transitions resulting in a fold change of less than 1. This confirms that altering the desolvation gas flow rate has a negligible effect on the overall response of nuclear lamin peptides analysed by LC-MRM-MS. A paired T-test of the raw peak area data suggested that there was no significant difference in the measured peak area achieved for these peptide precursor ions when the optimal desolvation gas flow rate of 10 L/h was used compared to the default flow rate of 7 L/h ($p=0.2400$).

A drying gas flow rate of 10 L/h was found to be commonly used in relevant previous publications (Kozlík et al., 2013; Li et al., 2022; Yang et al., 2022). However, one study used a lower desolvation gas flow rate of 7 L/h to detect small molecules in rat plasma at a mobile phase flow rate of 0.3 mL/min with an AJS ESI source (Zhu et al., 2022). A possible reason behind use of a lower drying gas flow rate for the detection of small molecules by ESI-MS may be due to small molecule detection being usually performed with methanol as solvent B and not acetonitrile, which may affect the viscosity and nebulising properties of the ESI spray.

On the other hand, 12 L/h drying gas flow rate was used in the measurement of metabolites using AJS ESI at a mobile phase flow rate of 0.2 mL/min (Amarasinghe et al., 2012). When investigating peptide MS signal, Pergande and colleagues used 14 L/h drying gas flow rate when performing quantitative, label-free proteomic analysis of protein digests of animal tissue samples and HeLa cells (Pergande et al., 2019a, 2019b). Since the AJS ESI source was only able to permit desolvation gas flow rates of up to 13 L/h when the desolvation gas temperature was set to 300°C, desolvation gas flow rates above this could not be tested.

Kutzner and colleagues found that a 6-12% increase in signal intensity could be achieved through optimisation of the desolvation gas flow rate (Kutzner et al., 2019). The data in Figure 3-22 shows that the mean fold change achieved with optimising the desolvation gas flow rate from 7 L/h to 12 L/h was 1.0214, suggesting a negligible

average increase in measured peak area compared to default settings, and changing the gas flow rate to 12 L/h was actually detrimental for a large proportion of peptide analyte ions.

In conclusion, it was found that changes in desolvation gas throughout the range of 6 – 13 L/h did not affect the measured peak area of nuclear lamin peptides (Figure 3-21). In addition, adjusting the desolvation gas flow rate from the default rate of 7 L/h to the empirically optimal flow rate of 12 L/h led to no significant changes in the peak area measurement for nuclear lamin peptides (Figure 3-22).

3.4.5.3. Optimisation of Desolvation Gas Temperature

When analysing thermally labile analytes such as peptides, it is important to exercise caution when using ESI to prevent their degradation in the source (Lupo, 2018). Drying gas temperature is a flow-dependent parameter (Presser, 2009) and is dependent on solvent composition, as water may require a higher drying gas temperature to ensure complete vaporization. Szerkus and colleagues found that desolvation gas temperature and flow rate contributed to most of the variance in MS response (Szerkus et al., 2016).

Figure 3-23 shows the mean measured peak area acquired for peptide precursor ions at different desolvation gas temperatures in the range of 80°C to 340°C.

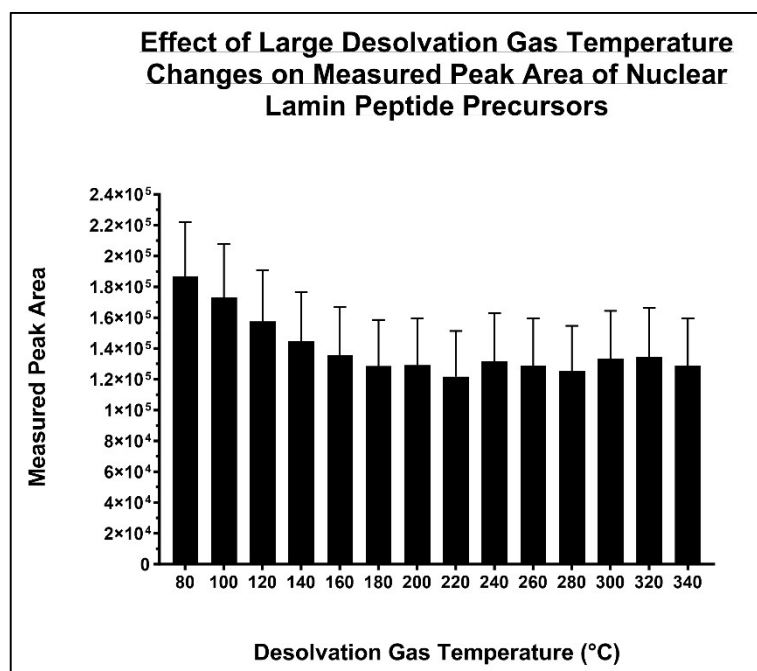


Figure 3-23: Effect of changing the desolvation gas temperature on the mean measured peak area of 71 nuclear lamin peptide precursors, within the range of 80°C to 340°C (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.9779$

The results of Figure 3-23 show that the average measured peak area was the greatest when the desolvation gas temperature is set to 80°C. Statistical analysis using a one-way ANOVA test shows there was no significant difference in the measured peak area ($p=0.9779$). This is also supported by the results observed when each individual transition was measured (Appendix IV Figure 8-12). The solvent composition at the time of peptide elution is highly organic, indicating that a lower desolvation gas temperature may be sufficient to ionise the peptide ions and evaporate solvent droplets without thermal decomposition of the peptides in-source.

Changes through finer increments of 10°C were then measured to investigate if small changes to the desolvation gas temperature had a significant effect on measured peak area. Figure 3-24 below shows the results of this analysis.

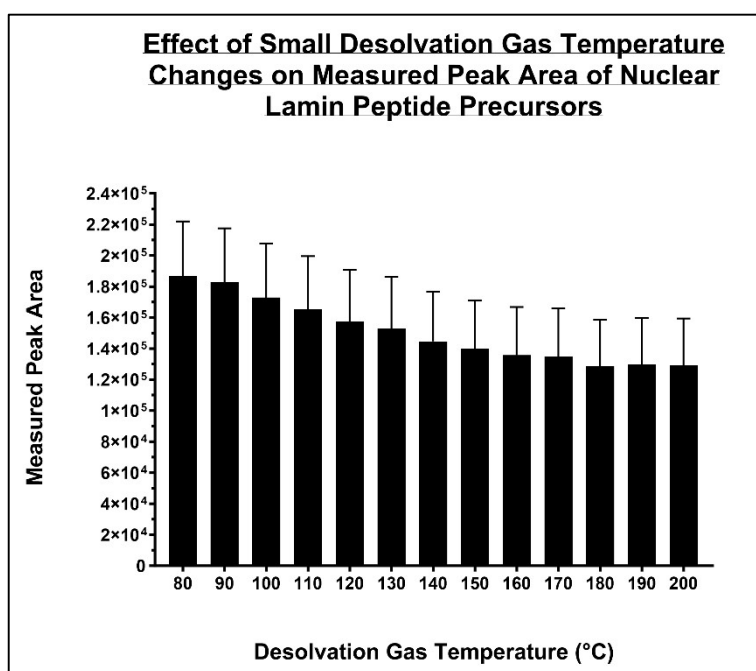


Figure 3-24: Effect of changing the desolvation gas temperature on the mean measured peak area of 71 nuclear lamin peptide precursors, within the range of 80°C to 200°C (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.9621$.

The results of Figure 3-24 show an increase in the average peak area measurement when analysis of nuclear lamin tryptic peptides was performed with a desolvation gas temperature of 80°C compared to results obtained with a temperature of 300°C. Statistical analysis of the results shown in Figure 3-24 using a one-way ANOVA test shows there was no significant difference in measured peak area over a range of 80°C to 200°C and when the desolvation gas temperature was changed in 10°C steps ($p=0.9621$). Additionally, analysis of each individual transition agreed with the results shown in Figure 3-24 (Appendix IV Figure 8-13).

Previously published literature has shown varying results when investigating the effect of drying gas temperature on MS signal response. Cuykx and colleagues noted that drying gas flow rate and temperature did not affect sensitivity as long as the spray was stable (Cuykx et al., 2017). On the other hand, several studies have shown that decreasing the desolvation gas temperature had a negative effect on MS signal. Increasing dry gas temperature from 250°C to 350°C was shown to increase the peak area of bioactive markers isolated from plants (Teh et al., 2011). In addition, Norton and colleagues found that, when analysing cationic surfactants by capillary electrochromatography coupled with ESI-MS analysis, a lower drying gas temperature of 150°C provided lower MS signal compared to 200°C and 250°C (Norton et al., 2006). However, the same group found that increasing drying gas temperature from 100°C to 300°C showed a general decline in signal intensity when measuring zwitterionic surfactants (Norton et al., 2005). The current study supports the finding that increasing the temperature of the desolvation gas leads to a decrease in peak area measurement, and therefore a decrease in sensitivity when measuring nuclear lamin peptides via MRM-LC-ESI-MS/MS, as shown by Figure 3-23 and Figure 3-24. This difference in optimal drying gas temperature between different surfactants shown in Norton's studies gives more evidence to the fact that drying gas is compound-dependent, and therefore the analysis of thermally labile molecules such as peptides and proteins may reasonably require a lower desolvation gas temperature.

To examine the individual fold change of each peptide signal response when the desolvation gas temperature was changed from the default setting (300°C) to the empirically determined optimal setting (80°C) as illustrated in Figure 3-23, a dot plot was created and the results are shown below in Figure 3-25.

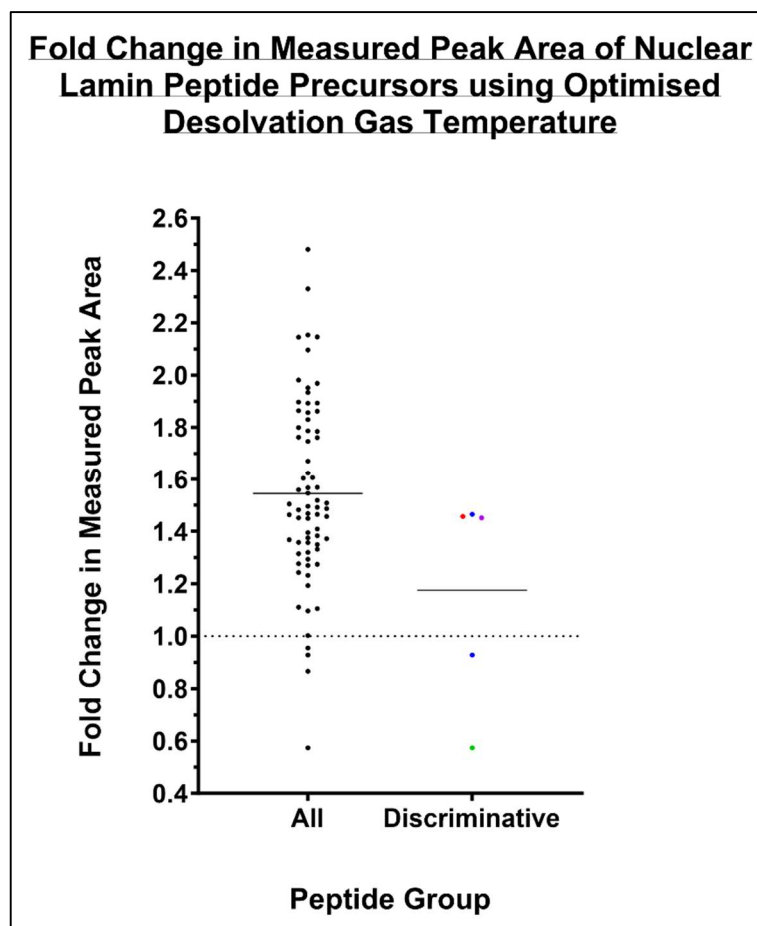


Figure 3-25: Fold change in measured peak area of 71 nuclear lamin precursors acquired upon changing the desolvation gas temperature from 300°C to 80°C (n=1). Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

The mean fold change for precursor ion peak areas (Figure 3-25) was 1.5445 with a range of 0.5740 to 2.4807. Only 4 of 71 (5.63%) of the precursor ions measured gave a fold change of less than 1 when the desolvation gas temperature was changed from 300°C to 80°C for these peptide ions. When examining the isoform-discriminating peptides, two of five peptides showed decreases in measured peak area with a desolvation gas temperature of 80°C. These were the lamin C specific peptide VSGSR and the doubly charged precursor ion of the peptide GSHCSGSGDPAEYNLR, specific to lamin A. Moreover, an inspection of the fold change of each individual transition supports these findings (Appendix IV Figure 8-14). Since both peptides which gave a fold change of less than 1 are crucial in differentiating lamin A and lamin C, adjusting the desolvation gas temperature may not be beneficial for the overall aim of this assay, which was to measure the abundance of different isoforms of nuclear lamins. Conversely, a paired T-test of the raw peak area data suggested that there was a significant difference in the measured peak area achieved for these peptide precursor ions when the optimal desolvation gas temperature of 80°C was used compared to the default temperature of 300°C ($p < 0.0001$).

For peptide analysis, Pergande and colleagues used 250°C drying gas temperature when performing quantitative, label-free proteomic analysis of protein digests at a flow rate of 0.2 mL/min (Pergande, et al., 2019a, 2019b). For the analysis of small molecules, higher temperatures in the range of 300°C to 350°C are preferred (Amarasinghe et al., 2012; Kozlík et al., 2013; Li et al., 2022; Yang et al., 2022; Zhu et al., 2022). As peptides are sensitive to degradation at high temperatures, it is reasonable to think that the desolvation gas temperature should be decreased when analysing molecules that are thermally labile compared to small molecules which may be less affected by high temperatures. The effects of thermal decomposition seem significant on the analysis of peptides in the current study, as a much lower desolvation gas of 80°C was preferable for most peptides analysed, and there was a significant increase in fold change of the peptide precursors when the optimised desolvation temperature was used compared to the default temperature. Overall, the desolvation gas temperature must be set at a value which is a compromise between efficient ion

formation and desolvation of solvent droplets, which requires high temperatures, but also minimise thermal decomposition of the peptide analytes.

In addition, an optimisation of ESI source parameters by design-of-experiment (DoE) protocol found that drying gas temperature and drying gas flow rate showed a significant two-factor interaction (Szerkus et al., 2016). In the current study, this interaction could not be thoroughly investigated due to the use of an OVAT optimisation procedure and therefore, despite the change in desolvation gas temperature from 300°C to 80°C leading to a significant increase in measured peak area for a majority of peptide transitions and precursors, the desolvation gas temperature was not changed as part of the final analysis as the relationship between the temperature and flow rate was not clearly understood at the time.

To summarise, a desolvation gas temperature of 80°C gave the highest mean measured peak area of nuclear lamin peptides (Figure 3-32 and Figure 3-24). When analysing peptide ions in this study, a decreased desolvation gas temperature from 300°C to 80°C resulted in a significant increase in the fold change in peak area measurement for over 90% of peptides analysed (Figure 3-25). Factors which are thought to contribute to a significant increase in peak area at a lower temperature include the biochemical characteristics of peptides and solvent composition at the time of analyte elution.

3.4.5.4. Optimisation of Nebuliser Pressure

As part of the ESI source, a nebulising gas is introduced which flows in the same direction as the sample solvent. The nebulizer pressure controls the speed at which the nebulising gas flows, which together with drying gas temperature and flow rate, play a significant role in the quality and quantity of the ions produced in the source (Herrero et al., 2005). In addition, the use of a nebulising gas helps to eliminate electrical discharge at high capillary voltages (Bruins, 1998) and this is important in cases where high capillary voltages may be required when solvents with high surface tension, such as high aqueous solutions, are used. The nebuliser pressure is both HPLC flow rate- and solvent composition-dependent (Presser, 2009). The use of a nebulising gas aids aerosol formation and reduces transit time to the MS inlet. Miller and colleagues showed that changing the nebuliser pressure affected various factors of the ESI process, including the droplet speed at different nitrogen pressures and the MS signal response (Miller et al., 2020). As mentioned previously, nebuliser pressure was among one of the source parameters that was found to contribute to the most variance in MS signal response in previous studies (Szerkus et al., 2016).

Figure 3-26 below shows the mean measured peak area acquired for peptide precursor ions at different nebuliser pressures in the range of 10 psi to 60 psi, in 5 psi increments. Data obtained with the nebuliser pressure set to 0 psi and 5 psi was not included in the final analysis as very little MS signal response was observed amongst the set of peptides analysed. This may be due to the nebuliser pressure being insufficient to stabilise the electrospray plume and allow analyte ions to enter the MS inlet.

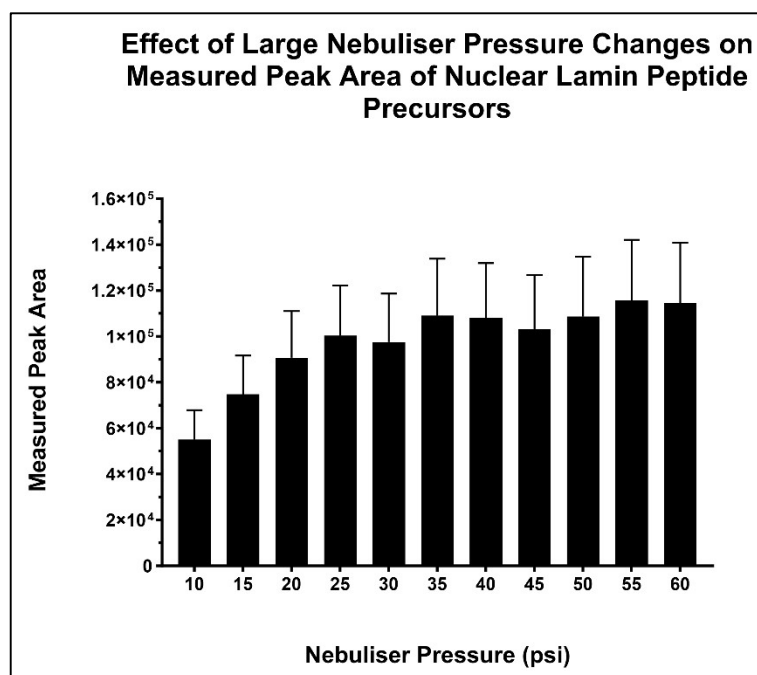


Figure 3-26: Effect of changing the nebuliser pressure on the mean measured peak area of 74 nuclear lamin peptide precursors, within the range of 10 psi to 60 psi (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.7546$

The results in Figure 3-26 show that the average fold change in measured peak area is greatest when the nebuliser pressure is set to 60 psi. Statistical analysis using a one-way ANOVA test shows there was no significant difference in the fold change in measured peak area ($p=0.7546$). Conversely, inspection of the peak area measured for each transition suggests that the mean measured peak area was the highest when the nebuliser pressure was set to 60 psi and was significantly different across the range tested Appendix IV Figure 8-15, $p=0.0111$). Norton and colleagues found that MS signal response decreased with increasing nebuliser pressure (Norton et al., 2006, 2005). Additionally, varying the nebuliser pressure across the range of 30 to 60 psi was shown to decrease peak area when using LC-ESI-MS (Teh et al., 2011). Wang and colleagues noted a decrease in signal intensity above 45 bar of nebuliser pressure but did not speculate the cause. This decrease in signal intensity at higher nebuliser gas pressures was not observed in Figure 3-26. It is important to note that Wang and colleagues were investigating proteins and not peptides, and looking at folded protein ions vs unfolded ions, using ESSI and not ESI (Wang et al., 2011).

Next, changes in peak area when changing the nebuliser pressure in 1 psi steps were measured over a range of 25 to 35 psi. Figure 3-27 shows the mean measured peak area acquired for peptide precursor ions.

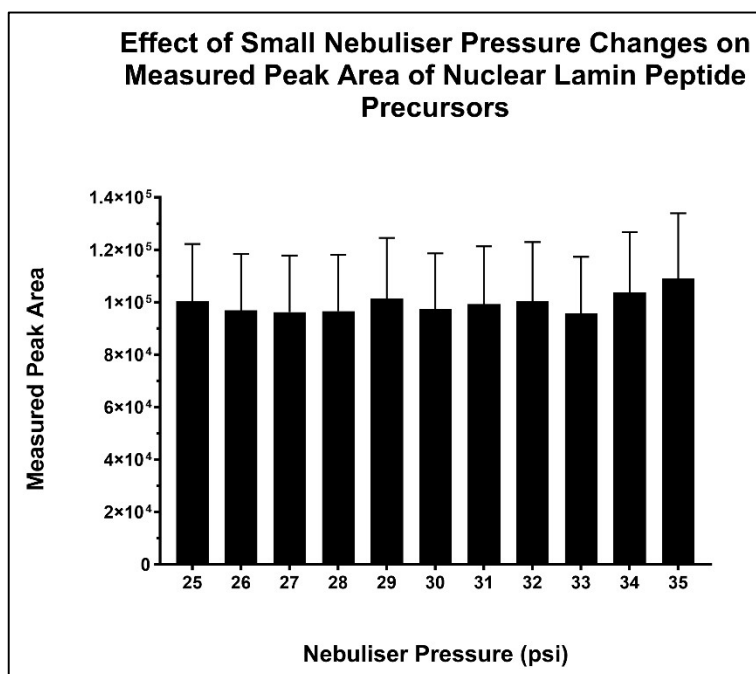


Figure 3-27: Effect of changing the nebuliser pressure on the mean measured peak area of 74 nuclear lamin peptide precursors, within the range of 25 psi to 35 psi (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p>0.9999$

Figure 3-27 suggests an increase in peak areas observed with increasing nebuliser pressure, however statistical analysis shows no significant difference in the measured peak area of nuclear lamin peptides over a range of 10 psi using a one-way ANOVA test ($p>0.9999$). The results shown in Figure 3-27 are supported by data showing the peak area for each of the 346 transitions measured also did not significantly change when the nebuliser pressure was adjusted between the range of 25 – 35 psi (Appendix IV Figure 8-16).

The fold change in peak area observed when measuring peptide precursors by LC-MRM-MS/MS upon adjusting the nebuliser pressure from the default setting (15 psi) to the empirically determined optimal setting (60 psi) is shown below in Figure 3-26.

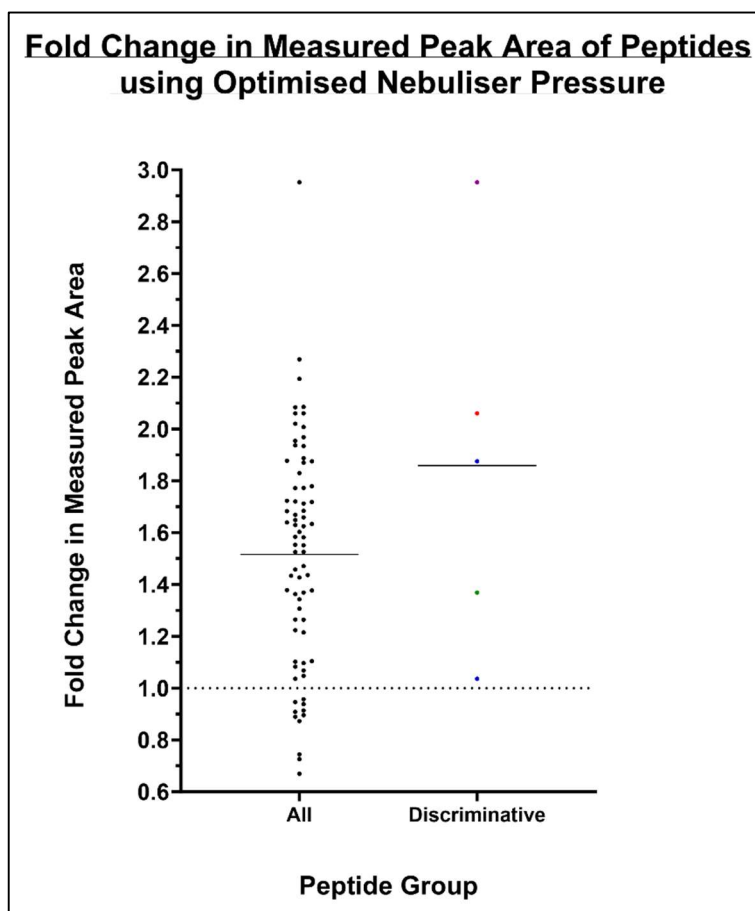


Figure 3-28: Fold change in measured peak area of 74 nuclear lamin precursors upon changing nebuliser pressure from 15 psi to 60 psi (n=1). Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

When investigating precursor ion peak area in Figure 3-28, the mean fold change in measured peak area was 1.5162 with a range of 0.6697 to 2.9524. 11 of 74 (14.86%) of

the precursors measured gave a fold change of less than zero, indicating a detrimental effect on peak area when the nebuliser pressure was changed from 15 psi to 60 psi. Additionally, there was a mean fold change of 1.519 when examining each individual transition, with only 14.74% of transitions (51 of 346 total transitions) resulting in a fold change in peak area of less than 1 (Appendix IV Figure 8-17). In addition, a paired T-test of the raw peak area data suggested that there was a significant difference in the measured peak area achieved for these peptide precursor ions when the optimal desolvation gas temperature of 80°C was used compared to the default temperature of 300°C ($p=0.0004$). Given the significant increase in peak area when the optimised nebuliser pressure was used to measure peptide precursors, and only a small amount of peptides experienced a small reduction in fold change in peak area, and all the isoform-differentiating peptides gave an increase in signal upon increasing the nebuliser pressure from 15 psi to 60 psi, the nebuliser pressure was changed from 25 psi to 60 psi as part of the final optimised method.

When analysing the published literature, it was found that 20 psi of nebuliser pressure was used when detecting small molecules by LC-MS-MS with an AJS ESI source and a sample flow rate of 0.32 mL/min (Yang et al., 2022). In support of this, a 2008 study by Maragou and colleagues found that the lower the nebuliser pressure, the better the signal obtained when their group optimised the pressure within the range of 20 – 60 psi for analysis of estrogenic compounds in beer by LC-ESI-MS (Maragou et al., 2008). AJS ESI has been used in the food industry to measure sugars using a solvent flow rate of 1 mL/min and a nebuliser pressure of 35 psi (Perera et al., 2017). Pergande and colleagues also used 35 psi nebuliser pressure in their quantitative, label-free proteomic analysis of protein digests using AJS ESI at a flow rate of 0.2 mL/min (Pergande et al, 2019a, 2019b). For pharmaceutical analysis, AJS ESI source has been used to detect compounds with a nebuliser pressure of 45 psi at 0.5 mL/min (Kozlík et al., 2013). 50 psi nebuliser pressure was used in the measurement of metabolites using AJS ESI at a flow rate of 0.2 mL/min (Amarasinghe et al., 2012). Therefore, there seems to be little consensus on the optimal nebuliser pressure for a specific sample flow rate and solvent composition.

With respect to higher nebuliser pressures used in biomolecule analysis, Kutzner and colleagues optimised ESI source parameters and found the optimal nebuliser setting to be 60 psi for the detection of fatty acids, gaining a 16-23% increase signal intensity upon optimisation (Kutzner et al., 2019), while Moreiras and colleagues determined that 60 psi was the optimal nebuliser pressure for the detection of small molecules by ESI-LC-MS/MS (Moreiras et al., 2018). These pressure values coincide with the current study, as the results in Figure 3-26 show that the MS signal seems to plateau after a nebuliser pressure of 35 psi is reached, while 60 psi was ultimately chosen as the optimal nebuliser pressure.

In summary, changing the nebuliser pressure over the range of 5 – 60 psi resulted in overall significant changes in the mean measured peak area of nuclear lamin peptides, when each individual transition was analysed, but no significance was observed when this data was translated to a precursor ion measurement (Appendix IV Figure 8-15 and Figure 3-26). Across a smaller range of 25 – 35 psi, no significant change in peak area was observed when analysing signal response (Figure 3-27 and Appendix IV Figure 8-16). Adjusting the nebuliser pressure from 15 psi to 60 psi resulted in an up to 3-fold change in measured peak area, with an average fold change of 1.5 (Figure 3-28) and a statistically significant increase in peak area measurements was observed upon optimisation. These results suggest that optimising the nebuliser pressure is an important consideration when designing sensitive MRM-based assays for peptide analysis and the optimised nebuliser pressure of 60 psi was applied to the finalised LC-MRM-MS/MS method for detection of nuclear lamin isoforms.

3.4.5.5. Optimisation of Nozzle Voltage

The nozzle voltage is the voltage applied to a metal ring around the end of the apparatus where the nebuliser sits, and allows the application of an additional electrical potential as the analytes leave the nebuliser (see Figure 3-16). Adjusting the nozzle voltage allows the widening or narrowing of the electrospray plume to incorporate various regions of the plume into the nebulising gas spray for efficient desolvation and entry into the MS inlet. Altering the nozzle voltage has been shown to be useful in investigating matrix effects. In a technical application note produced by the instrument manufacturer Agilent, the recommended default nozzle voltage used for the analysis of the small molecule reserpine was 600 V (Mordehai & Fjeldsted, 2009), however optimisation was advised for best analyte response as nozzle voltage is considered a compound-dependent parameter (Presser, 2009).

Figure 3-29 shows the mean measured peak area recorded at different nozzle voltages in the range of 0 V to 2000 V when analysing peptides via LC-ESI-MRM-MS/MS.

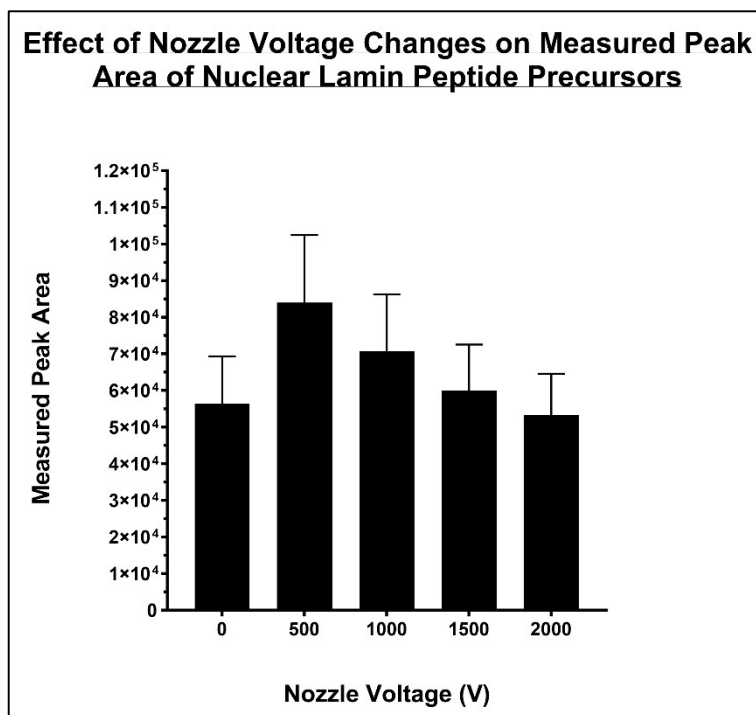


Figure 3-29: Effect of changing the nozzle voltage on the mean measured peak area of 75 nuclear lamin peptide precursors, within the range of 0 V to 2000 V (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.5490$.

The results of Figure 3-29 show that the average measured peak area was greatest when the nozzle voltage was set to 500 V. Statistical analysis using a one-way ANOVA test shows there was no significant difference in the measured peak area across the range of 0 – 2000 V ($p=0.5490$). A similar pattern was observed when examining the peak area of each individual transition, however the results were statistically significant at a transition level ($p=0.0424$, Appendix IV Figure 8-18).

Figure 3-30 below shows the individual fold change of each peptide precursor when the nozzle voltage was changed from the default setting (1500 V) to the empirically determined optimal setting (500 V).

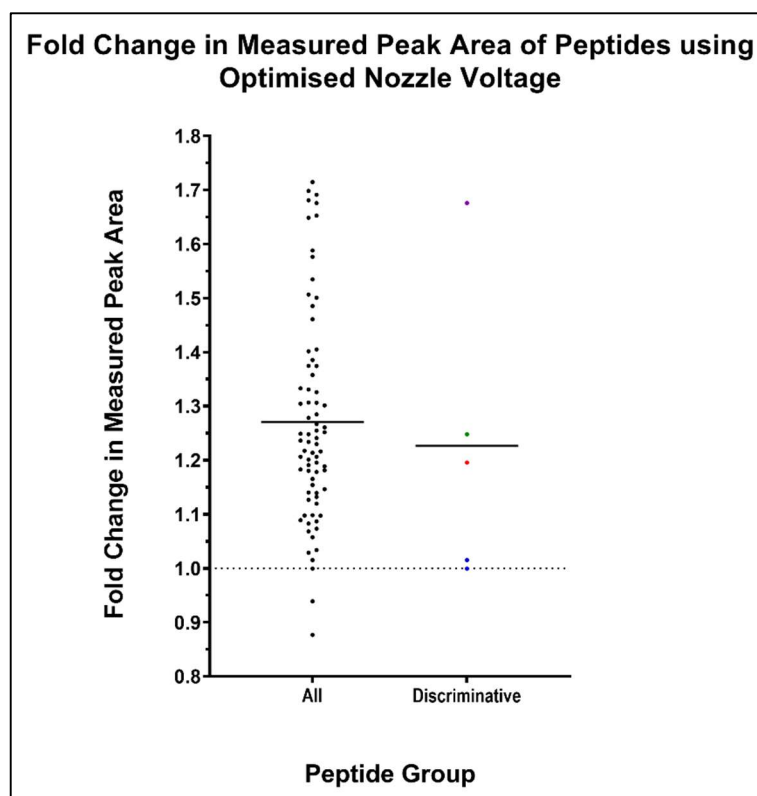


Figure 3-30: Fold change in measured peak area of 75 nuclear lamin precursor ions upon changing nozzle voltage from 1500 V to 500 V (n=1). Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

When investigating precursors in Figure 3-30, the mean fold change was 1.2704 with a range of 0.8767 to 1.7146, while 3 of 75 (4.00%) of the precursors measured gave a fold change of less than 1. Furthermore, four of five isoform-specific peptide precursor ions gave a positive fold change in response to changing the nozzle voltage from 1500 V to 500 V, while the isoform-specific peptide precursor (2+ charged ion of GSHCSGSGDPAEYNLR, the lamin A-specific peptide), gave a fold change of 0.9992. In addition, examination of the data from each individual transition supported these findings that adjusting the nozzle voltage was beneficial to the MS measurement of nuclear lamin peptides (Appendix IV Figure 8-19). These results suggest that adjusting

the nozzle voltage to 500 V had an overall positive effect on MS signal and assay sensitivity. Following this, a paired T-test of the raw peak area data suggested that there was a significant difference in the measured peak area achieved for these peptide precursor ions when the optimal nozzle voltage of 500 V was used compared to the default nozzle voltage of 1500 V ($p=0.0002$). As a result, the nozzle voltage was adjusted from 1500 V to 500 V as part of the final optimised LC-MRM-MS/MS method.

In previous research, 500 V was used as the nozzle voltage when using HILIC coupled with AJS ESI-MS to analyse small molecules at a flow rate of 0.5 mL/min with ammonium acetate and ammonium formate buffer solvents (Kozlík et al., 2013). Furthermore, a nozzle voltage of 750 V was used to measure HeLa protein digest via standard flow LC with AJS ESI source at a sample flow rate of 0.2 mL/min (Pergande et al, 2019a, 2019b). These studies support the findings of this chapter where 500 V was determined as the optimal nozzle voltage for LC-ESI-MRM-MS analysis of peptides (Figure 3-29). In contrast, Moreno-González and colleagues found that increasing the nozzle voltage to 2000 V improved the sensitivity of small molecule detection (Moreno-González et al., 2020). They also noted that use of the nozzle voltage was critical for analysis of neutral compounds, however the nozzle voltage was suggested to have a negligible effect on charged compounds, since the study found that the formation of charged ions was mostly affected by capillary voltage and not nozzle voltage when using the AJS ESI apparatus (Moreno-González et al., 2020). The results in Figure 3-30 show that changing the capillary voltage from 1500 V to 500 V had a significantly positive impact on the measured peak area of peptides. Bieber and colleagues found that, of the AJS ESI sources optimised for their assay, nozzle voltage optimisation contributed the most to ESI ionisation efficiency (Bieber et al., 2019).

To summarise, the data shows that changes in nozzle voltage had a notable effect on the mean measured peak area of peptides throughout the range of 0 to 2000 V (Figure 3-29), which was statistically significant when the data was analysed as individual transition peak areas (Appendix IV Figure 8-18). 500 V was found to be the optimal

nozzle voltage for peptide analysis in this study using an AJS ESI source at a solvent flow rate of 0.3 mL/min. By changing the nozzle voltage from the default value of 1500 V to the empirically optimised voltage of 500 V, an average fold change of 1.27 was achieved with up to 1.7-fold increase in some cases, and analysis of the raw peak area data suggests this change was statistically significant (Figure 3-30). Considering this, the nozzle voltage was adjusted to 500 V as part of the final optimised MRM method for nuclear lamin MS quantitation.

3.4.5.6. Optimisation of Sheath Gas Flow Rate

During ESI, both the sheath gas and nebuliser gas are applied in a coaxial fashion to the sample flow emitted from the nebuliser and affect the efficiency of ion formation within the electrospray by aiding desolvation of highly charged aerosolised electrospray droplets, so that fully desolvated ions can enter the mass analyser. The addition of a sheath gas flowing around the emitter results in better nebulisation and desolvation of molecular ions and also helps direct the spray emerging from the nebuliser tip towards the mass spectrometer by collimating the electrospray into a concentrated area at the front of the MS inlet. The creators of the superheated sheath gas technology, Agilent Technologies Inc, claim that full confinement of the spray by the super-heated nitrogen sheath gas eliminates sample recirculation and reduces peak tailing, which is important when measuring peak area to determine analyte concentration and measurements (Mordehai & Fjeldsted, 2009). Sheath gas flow rate is known to be a flow-dependent parameter (Presser, 2009).

Figure 3-31 shows the results of altering the sheath gas flow rate on the mean measured peak area of peptide precursor ions in range of 5 L/h to 12 L/h.

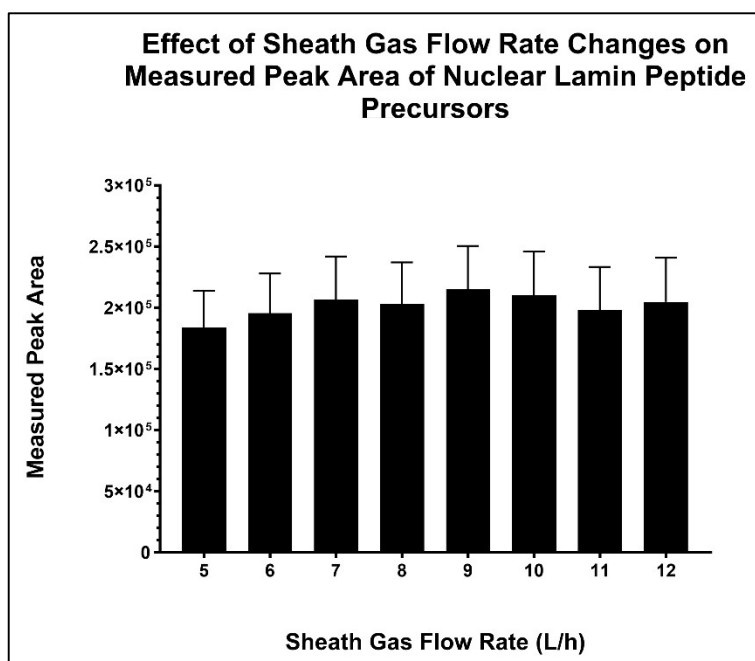


Figure 3-31: Effect of changing the sheath gas flow rate on the mean measured peak area of 76 nuclear lamin peptide precursors, within the range of 5 L/h to 12 L/h (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.9992$.

The results of Figure 3-31 show that the average measured peak area was the greatest when the desolvation gas flow rate was set to 9 L/h. Statistical analysis using a one-way ANOVA test shows there was no significant difference in the fold change in measured peak area across the range of 5 – 12 L/h ($p=0.9992$). Furthermore, when examining each individual transition, the results show a similar pattern to that observed in Figure 3-31 (Appendix IV Figure 8-20).

Following this, Figure 3-31 shows the individual fold change of each peptide precursor when the sheath gas flow rate was changed from the default setting (11 L/h) to the empirically determined optimal setting (9 L/h).

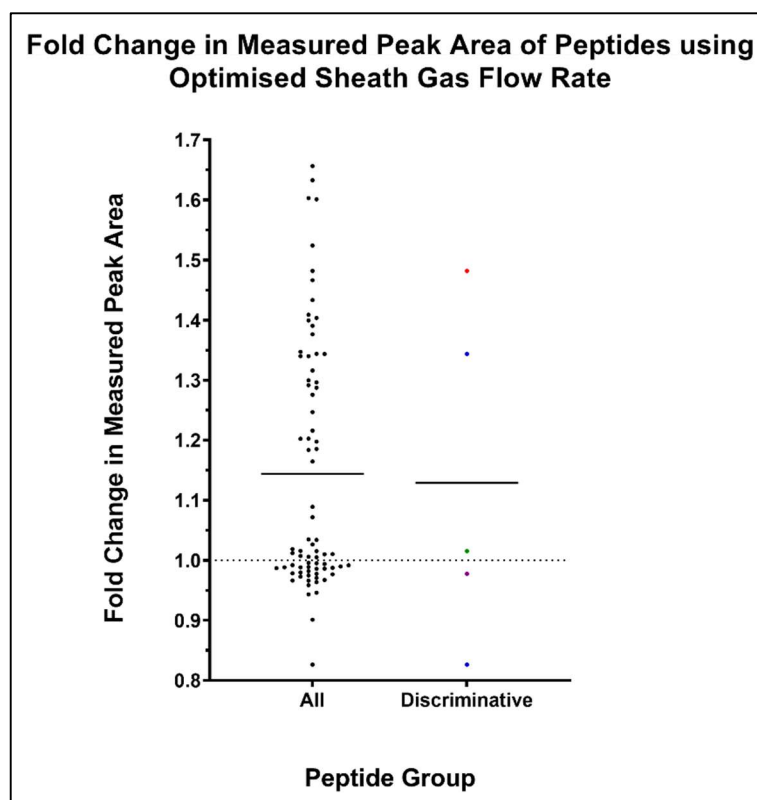


Figure 3-32: Fold change in measured peak area of 76 nuclear lamin precursor ions upon changing sheath gas flow rate from 11 L/h to 9 L/h (n=1). Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

Figure 3-32 above shows the mean fold change in measured peak area upon changing the sheath gas flow rate from 11 L/h to 9 L/h was 1.4139 (range: 0.8264 - 1.6565). 30 of 76 (39.47%) of the precursors measured gave a fold change of less than 1, while a large portion of peptide precursor ions showed a fold change in measured peak area around 1. This pattern was also observed when examining each individual transition (Appendix IV Figure 8-21). Furthermore, Figure 3-32 shows that two of five isoform-specific precursor ions had lower measured fold changes in peak area values at 9 L/h compared to data obtained using a flow rate of 11 L/h (pan B-type lamin peptide LSPSPSSR and the doubly charged precursor ion of the lamin A-specific peptide

GSHCSGSGDPAEYNLR). Considering these factors, the sheath gas flow rate was not changed as part of the final analysis. Conversely, a paired T-test of the raw peak area data suggested that there was a significant difference in the measured peak area when the optimal sheath gas flow rate of 9 L/h was used compared to the default flow rate of 11 L/h ($p=0.0007$).

Most studies used a sheath gas flow rate of between 10 and 12 L/h, which is recommended for a flow rate of 0.2 – 0.7 mL/min (Presser et al., 2014). 10 L/h was used in the analysis of small molecules at a flow rate of 0.5 mL/min (Kozlík et al., 2013) while a flow rate of 10 L/min was used to measure sugars using a solvent flow rate of 1 mL/min (Perera et al., 2017). Studies that used 11 L/h were those involved in the detection of pharma pharmaceutical compounds (Li et al., 2022; Zhu et al., 2022) and protein digests (Pergande et al., 2019a, 2019b). One study used a sheath gas flow rate of 12 L/h using an AJS ESI source to detect small molecules in blood and urine at a solvent flow rate of 0.32 mL/min (Yang et al., 2022). In addition, Riter and colleagues showed that the higher the solvent flow rate and the sheath gas flow rate, the higher the signal intensity (Riter et al., 2005).

To conclude, the data suggests that changing the sheath gas flow rate within the range of 5 – 12 L/h did not have a significant impact on the measured peak area of peptides (Figure 3-31). The empirically determined optimal sheath gas flow rate was shown to be 9 L/h, however, when measuring the fold change in the peak area of individual peptide precursor ions at 9 L/h compared to the default flow rate of 11 L/h, a significant increase in fold change of the measured peak areas was observed with the new gas flow rate (Figure 3-32). Despite this, many peptides did not achieve a substantial increase in fold change and therefore, the sheath gas flow rate was not changed as part of the final optimised LC-MRM-MS, MS method.

3.4.5.7. Optimisation of Sheath Gas Temperature

As mentioned previously, the overall ESI source temperature is important in the analysis of peptides due to their thermal instability. As with the desolvation gas temperature, higher temperatures result in more efficient ion formation but also can result in some thermal decomposition within the electrospray. Mordehai and Fjeldsted have shown that the use of a superheated sheath gas produces a thermal confinement zone, which acted as an insulator for the electric field generated by the capillary voltage and thus aids ionisation efficiency of the analytes exiting the nebuliser. In addition, the electrospray plume produced from the nebuliser is much narrower when using a low sheath gas temperature of 25°C compared to the plume observed at 350°C, which helps to concentrate the gaseous analyte ions in front of the MS inlet (Mordehai & Fjeldsted, 2009). When considering the sample solvent composition, water may require a higher sheath and desolvation gas temperatures to ensure complete desolvation of the aqueous solvent droplets formed during ESI. The sheath gas temperature is known to be a compound-dependent parameter (Presser, 2009).

Figure 3-33 below shows the effect of sheath gas temperature on the MS signal response of peptide precursors, when the sheath gas temperature was adjusted in the range of 100°C to 400°C.

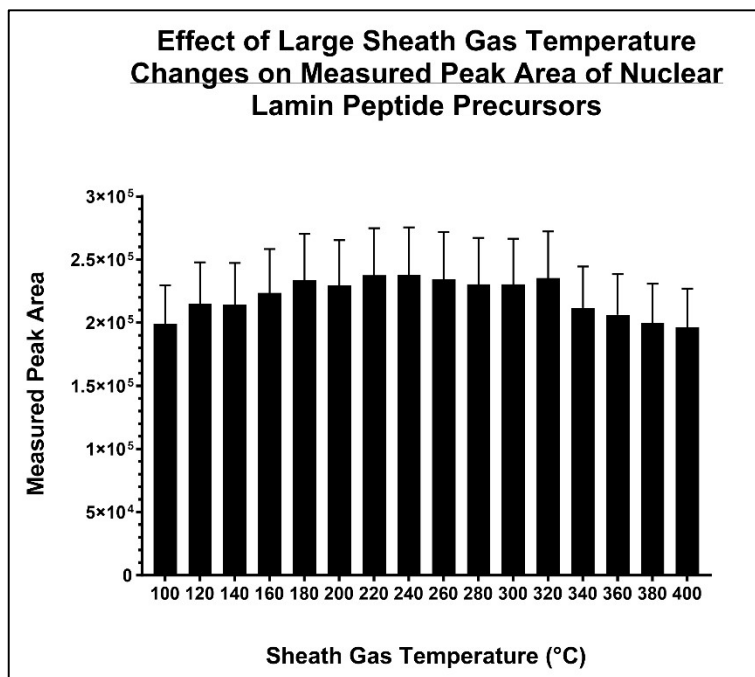


Figure 3-33: Effect of changing the sheath gas temperature on the mean measured peak area of 75 nuclear lamin peptide precursors, within the range of 100°C – 400°C (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.9998$.

Figure 3-33 shows a somewhat Gaussian pattern of peak area measurements with respect to changes in sheath gas temperature, with the highest measured peak area achieved with a sheath gas temperature of 250°C. Statistical analysis of the results shown in Figure 3-33 using a one-way ANOVA test shows there is a significant difference in the fold change in measured peak area ($p=0.9998$), therefore changing the sheath gas temperature was shown to have little effect on the peak area of peptide ions during MS analysis. Additionally, the results of analysing the fold change of each individual transition support the results shown in Figure 3-33 (Appendix IV Figure 8-22).

Changes through finer increments of 10°C were then measured to investigate if small changes to the sheath gas temperature had a significant effect on measured peak area.

Figure 3-34 shows the measured peak area acquired for peptide precursor ions at different sheath gas temperatures.

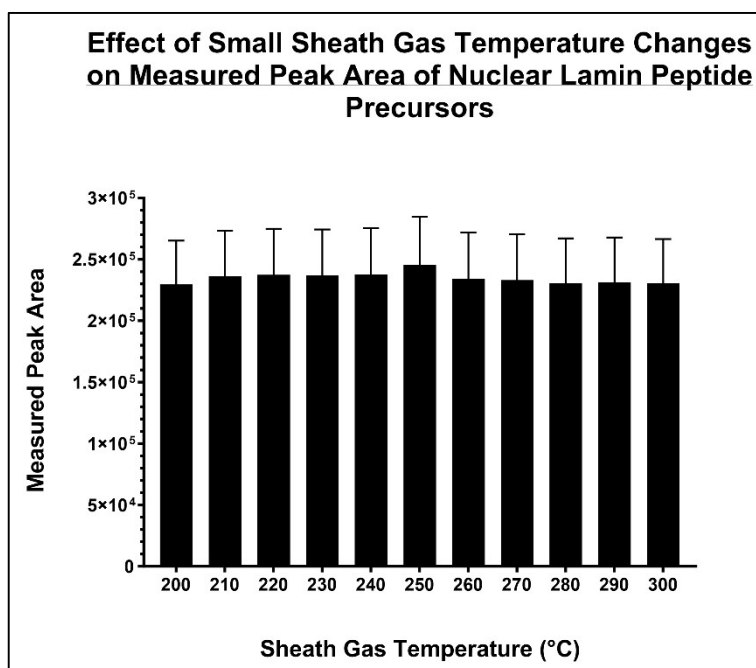


Figure 3-34: Effect of changing the sheath gas temperature on the mean measured peak area of 75 nuclear lamin peptide precursors, within the range of 200°C – 300°C (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p > 0.9999$.

The results of Figure 3-34 suggest that the default sheath gas temperature of 250°C gave the highest average peak area measurement when analysing nuclear lamin peptide precursors by LC-ESI-MRM-MS/MS. Statistical analysis of the results shown in Figure 3-34 using a one-way ANOVA test showed that there was not a significant difference in the fold change in measured peak area ($p > 0.9999$) over a range of 200°C to 300°C and when the sheath gas temperature was changed in 10°C steps. In addition, when analysing the data on a transition level, it was also found that 250°C gave the greatest peak area across this range of sheath gas temperatures and sheath gas temperature had no significant effect on measured peak area over this range of temperatures examined (Appendix IV Figure 8-23).

Most recent publications used a sheath gas temperature between 250°C and 350°C (Perera et al., 2017; Pergande et al., 2019a, 2019b; Yang et al., 2022) with several studies focussing on the analysis of small molecules utilising temperatures of 350°C (Kozlík et al., 2013; Li et al., 2022; Zhu et al., 2022). In contrast, Moreno-González and colleagues found that, when using micellar electrokinetic chromatography (MEKC) with an AJS-ESI source for the analysis of small molecules, analysis of the analytes at low sheath gas temperatures produced the best results (Moreno-González et al., 2020). Since 250°C was the default temperature and also the empirically determined optimal sheath gas temperature shown in Figure 3-34, this temperature was used as part of the final LC-MRM-MS/MS method for analysing nuclear lamins.

To conclude, it was found that the optimal sheath gas temperature for analysis of this group of nuclear lamin peptides was 250°C, which is the same as the default temperature (Figure 3-33). Furthermore, results showed that changes in sheath gas temperature within the ranges of 100–400°C (Figure 3-33) and 200-300°C (Figure 3-34) did not significantly affected the peak area measurements of nuclear lamin peptide transitions measured by LC-ESI-MRM-MS.

As a result of the optimisation of AJS ESI source settings in Section 3.4.5.1 – 3.4.5.7, the final optimised AJS ESI source parameters are detailed below in Table 3-IX.

Table 3-IX: Optimised AJS ESI source parameters for LC-MRM-MS analysis of nuclear lamin peptides using the Agilent Ultivo QQQ MS instrument.

AJS ESI Source Parameter	Optimised Value
Sheath Gas Temperature (°C)	250
Sheath Gas Flow Rate (L/min)	11
Desolvation Gas Temperature (°C)	300
Desolvation Gas Flow Rate (L/min)	7
Nebuliser Pressure (psi)	60
Capillary Voltage (V)	4000
Nozzle Voltage (V)	500

3.4.5.8. Comparison of MS Response using Optimised vs Unoptimised Source Parameters

After optimisation of each AJS ESI source parameter via the OVAT method, the AJS ESI source settings that gave significant increases in peak area when optimised were the desolvation gas temperature, nebuliser pressure, nozzle voltage and sheath gas flow rate. The change in desolvation gas temperature was not included as part of the final optimised source parameters as the relationship between desolvation gas temperature and flow rate was not fully understood at the time of analysis. In addition, many peptide precursor ions showed a fold change of close to 1 when the sheath gas flow rate was changed from 11 L/h to 9 L/h, suggesting no notable difference in the peak area measurements for a large number of analytes. On this basis, the sheath gas flow rate was maintained at the default value. Therefore, the two settings that were altered as a result of the OVAT analysis of AJS ESI source settings in this study were the nebuliser pressure and the nozzle voltage. These optimised values were used as part of the final analysis to produce a fully optimised LC-ESI-MRM-MS/MS method for the semi-quantitative analysis of nuclear lamin protein isoforms in biological samples from humans and mice.

To investigate the differences in measured peak area achieved with fully optimised AJS ESI source settings compared to default source settings, synthetic peptides corresponding to sequences found in lamin A, lamin C, lamin B1 and lamin B2 in were analysed in triplicate injection with empirically optimised AJS source parameters and default source parameters, and when either the optimal nebuliser or optimal nozzle voltage were used. Figure 3-35 shows the measured peak area observed when the optimal nebuliser pressure of 60 psi was used, when the optimal nozzle voltage of 500 V was used, and when both the optimal nebuliser pressure and nozzle voltage was used.

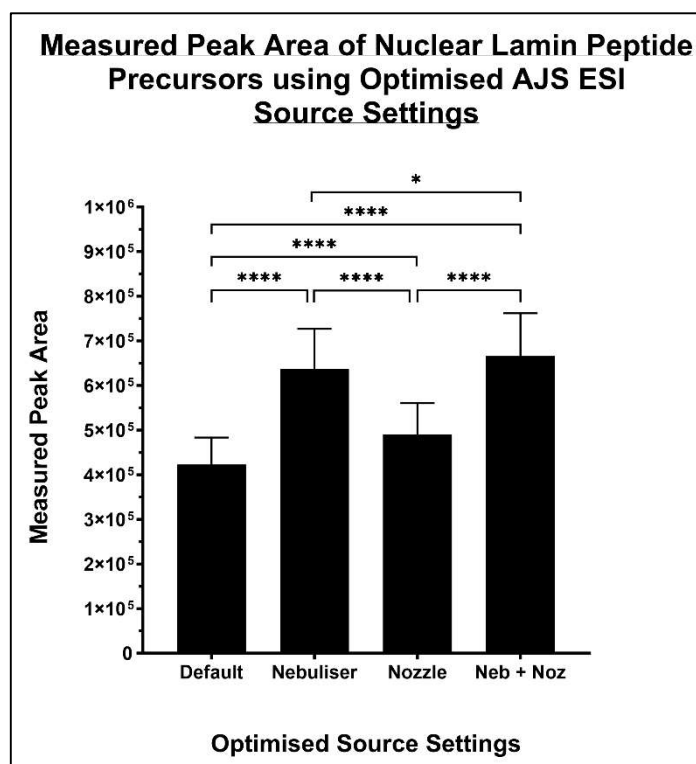


Figure 3-35: Mean measured peak area of 79 nuclear lamin peptide precursors achieved when using optimised AJS ESI source settings (n=3). Error bars indicate standard error of the mean (SEM). “Neb + noz” indicates the measured peak area achieved when both the nebuliser pressure and nozzle voltage were altered to their empirically determined optimal settings. Paired t-test (Default vs Nebuliser, $p < 0.0001$. Default vs Nozzle, $p < 0.0001$. Default vs Neb + Noz, $p < 0.0001$. Nebuliser vs Nozzle, $p < 0.0001$. Nebuliser vs Neb + Noz, $p < 0.0001$. Nozzle vs Neb + Noz, $p = 0.0291$).

Figure 3-35 shows the measured peak area of precursor ions when using default AJS ESI settings, optimised nebuliser settings with all other default AJS ESI parameters, optimised nozzle settings with all other default AJS ESI parameters, and optimised nebuliser and nozzle settings, with all other default AJS ESI parameters. The latter is considered the optimal AJS ESI source settings for analysis of tryptic peptides of nuclear lamin isoforms. The results show that there were significant increases in measured peak area across all conditions. Most notably, a significant increase in peak area was achieved when the default AJS ESI settings were used to measure nuclear lamin peptides compared to when the optimised AJS ESI settings were used.

Furthermore, when analysing the data for each transition, a similar pattern of significant increases when comparing the results observed across these groups (Appendix IV Figure 8-24).

Earlier in this study, Figure 3-28 and Figure 3-30 showed that an up to 3-fold and up to 1.7-fold change in measured peak area were observed when optimising nebuliser pressure and nozzle voltages respectively. The results in Figure 3-35 show that the optimising the nebuliser pressure resulted in a greater change in measured peak area than the change observed when comparing the default peak areas with those measured when the nozzle alone was set at the optimal setting. Only a minor increase peak area measurement was achieved when using the fully optimised AJS ESI settings compared to optimising the nebuliser alone, suggesting that optimising the nozzle settings has the greatest effect on signal response for peptide analysis.

In addition, Figure 3-36 below shows the average fold change achieved for each nuclear lamin peptide precursor when the optimal AJS ESI source settings were used to acquire MRM data compared to the default settings.

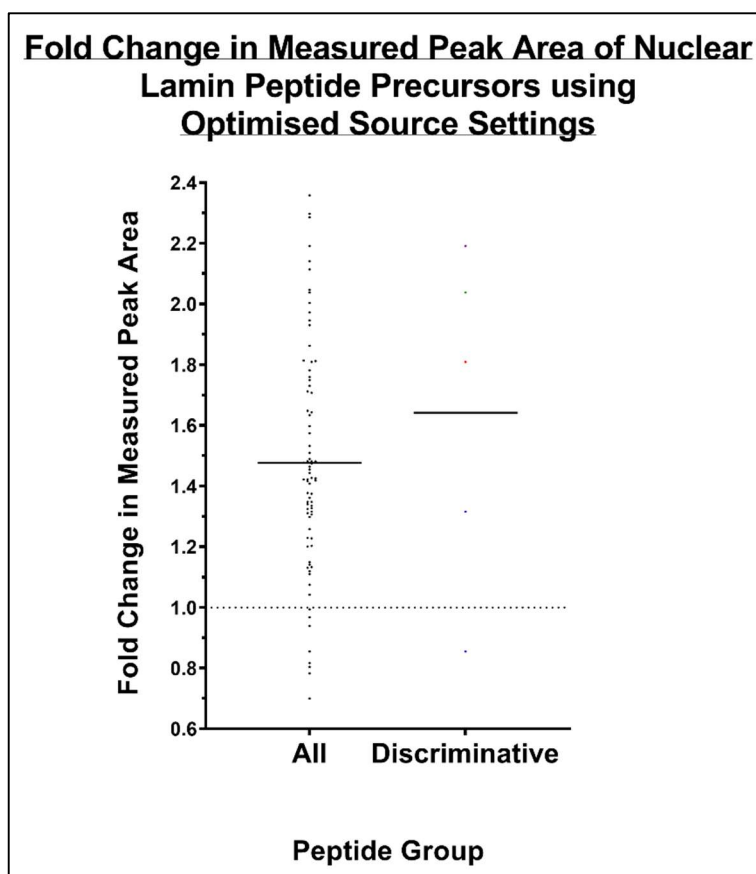


Figure 3-36: Fold change in measured peak area of 79 nuclear lamin precursors achieved upon optimising the AJS ESI source settings (n=3). Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

When investigating the peak area of precursor ions in Figure 3-36, the mean fold change in measured peak area was 1.476 with a range of 0.700 to 2.358. 8 of 79 (10.13%) of the precursors measured gave a fold change of less than 1, indicating a detrimental effect on peak area when the optimal nebuliser pressure and nozzle voltage were applied simultaneously. Most biologically significant peptides gave a higher peak area when the optimised AJS ESI settings were used for nuclear lamin MRM analysis compared to the default AJS ESI settings. The only precursor ion that

gave a decrease in measured peak area was the doubly charged precursor of the mouse lamin-A specific peptide GSHCSGSGDPAEYNLR, which gave a fold change of 0.855. A paired T-test of the raw peak area data obtained from analysis of nuclear lamin peptides at the default settings and the optimised AJS ESI settings found the peak area measurements to be statistically significant between these two groups ($p < 0.0001$). Additionally, when analysing the fold change in peak area of each individual transition, it was found that over 90% of the 382 total transitions used to monitor nuclear lamin isoform expression gave a fold change of greater than 1 when using the empirically determined optimal AJS ESI source settings (Appendix IV Figure 8-25).

Previous studies have found that nebuliser pressure and nozzle voltage are amongst the most influential parameters when it comes to producing the most efficient electrospray (Bieber et al., 2019; Cuykx et al., 2017; Szerkus et al., 2016). As discussed previously in Section 3.4.5.4, the nebuliser pressure is the pressure of the flow of nitrogen nebulising gas which flows in the same direction of the solvent and aims to aid the sample flow out of the nebuliser tip and aid in formation of the electrospray plume. The nozzle voltage controls the widening or narrowing of the electrospray plume by application of an additional electrical potential across the tip of the nebuliser (described in Section 3.4.5.5). Therefore, it may be possible to suggest that these two settings should work complimentary to one another to form an efficiently shaped Taylor cone.

3.4.5.9. Summary of AJS ESI Parameter Optimisation

The results of this study have suggested that optimisation of AJS ESI source parameters can result in significant increases in measured peak area when analysing peptides via MRM-MS/MS, and up to a 1.4-fold increase in peak area measurements can be achieved with optimal AJS ESI settings. Optimisation of ESI source parameters allowed

Furthermore, this study illustrates that the parameters that showed statistically significant increases in measured peak area when adjusting the setting from the default value to the empirically derived optimal setting were: desolvation gas temperature, nebuliser pressure, nozzle voltage and sheath gas temperature.

Published literature corroborate these results, as previous studies found that the nebuliser and nozzle were amongst the factors which had the greatest impact on MS signal response (Cuykx et al., 2017). Szerkus and colleagues found that the source parameters that contributed to most of the variance in MS response were desolvation gas flow, desolvation gas temperature and nebuliser pressure, however nozzle voltage was not measured during their study (Szerkus et al., 2016). Additionally, Bieber and colleagues found that of the AJS ESI sources optimised for their assay, nozzle voltage optimisation contributed the most to ESI ionisation efficiency (Bieber et al., 2019). Other studies found that source temperature and drying gas flow rate were considered the critical variables for ESI (Titato et al, 2007). Meanwhile, in this study, altering the desolvation gas flow rate showed no significant differences in measured peak area over the range tested, while optimisation of source temperature was not investigated.

3.5. Conclusion

The aims of this chapter were to design and optimise a sensitive, selective and robust LC-ESI- MRM-MS/MS method for the measurement of the relative protein abundance of nuclear lamin isoforms found in *Homo sapiens* and *Mus musculus* species. To design the assay, an *in-silico* digestion of four main nuclear lamin protein isoforms was conducted with thorough appreciation and consideration of limiting factors associated with MRM assay development, including but not limited to the peptide length, charge state, peptide modifications and suitable protease. The selection of suitable MRM transitions was performed based on their sensitivity using two different QQQ MS instruments to choose the product ions which gave the greatest signal intensity for protein isoform detection via this technique. A comparison of the sensitivity of two Agilent QQQ instruments was completed with respect to signal response achieved via analysis of appropriate synthetic tryptic peptides corresponding to the nuclear lamin isoforms, and it was found that the Agilent Ultivo QQQ showed greater analyte sensitivity when analysing these molecules (

Figure 3-4 and Figure 3-5).

The optimal collision energy for each synthetic peptide was determined on both instruments and comparisons between the optimal collision energy on each instrument were made. Both MS instruments gave similar optimal collision energies for each synthetic peptide, suggesting that different instrument models from the same vendor do not affect the optimal collision energy (Figure 3-6). Regression analysis was performed, and collision energy linear equations produced from the analysis of these synthetic peptides on each instrument were compared to the Skyline MS software-suggested collision energy linear equations for doubly charged and triply charged peptide precursor ions. It was found that empirical determination of the collision energy linear equation differed slightly from the software-suggested equations for both charge state precursors (Figure 3-8, Figure 3-9 and Figure 3-10). In addition, two thirds of the transitions investigated required collision energies that differed from the software-recommended collision energy to achieve the greatest signal response (Figure

3-6). Optimising the collision energy for transitions of synthetic peptides of nuclear lamins was shown to provide up to a 4-fold and 6-fold increase in MS signal response depending on the instrument used for analysis (Figure 3-7).

Following this, fragmentor voltage optimisation was performed on the synthetic peptides using the Agilent Ultivo QQQ, where it was found that 90% of transitions required adjustments in the fragmentor voltage applied to produce the greatest MS signal response. Furthermore, 37% of peptides analysed required a fragmentor voltage of 90 V for optimal MS signal response which was 40 V less than the default fragmentor voltage for that MS instrument (Figure 3-13). Up to a 3-fold change in measured peak area was achieved using optimised fragmentor voltages for MRM-based MS analysis (Figure 3-14). Optimising collision energy led to a greater average fold change in peak area than optimising fragmentor voltage, which suggests collision energy optimisation is more influential on maximising peak area during MRM analysis of synthetic peptides.

Finally, seven ESI operational parameters were optimised, and their influence analyte ionisation and overall MS signal response were discussed. It was found that optimisation of desolvation gas temperature, nebuliser pressure, nozzle voltage and sheath gas flow rate were among the parameters that gave the most significant increase in measured peak area of peptide precursors when optimised compared to peak area measured using default ESI settings on the Agilent Ultivo QQQ (Figure 3-25, Figure 3-28 and Figure 3-30), which compliments previous findings in this research area. Fold change of up to 2.5, 3.0 and 1.7 were achieved respectively when optimising these ESI source parameters for peptide analysis. Upon optimising ESI parameters, a comparison between the default settings and fully optimised ESI settings was performed which revealed that optimising AJS ESI source parameters can result in significant increases in measured peak area (Figure 3-35 and Figure 3-36). Adjusting both nebuliser pressure and nozzle voltage resulted in only a minor increase in fold change in measured peak area compared to use of optimised nozzle voltage settings

alone, and therefore further investigation into the relationship between these two parameters should be conducted.

The development and extensive optimisation of this MRM-based LC-ESI-MS/MS assay for measurement of the relative expression of nuclear lamin isoforms will be used in future chapters of this thesis to analyse biological samples of human and mouse origin in which the altered protein expression of nuclear lamins has been shown to contribute to various diseases. The optimised source parameters are detailed in Table 3-IX while details of all transitions used to measure nuclear lamin isoforms with optimised CE and FV value can be found in Appendix IV Table 8-VI. These source settings and MRM transition parameters will be used in Chapter 4 and 5 to measure the relative abundance of nuclear lamin isoforms in human cell lines and mouse models.

3.6. Future Work

Ultimately, the aim of this assay was to quantify the expression of different nuclear lamin isoforms in human and mouse biological samples. Since this assay was developed on synthetic peptides suspended in organic solvents, matrix effects cannot be considered and have been shown to have detrimental effects on quantitation of analytes. The experimentally determined optimal settings may not be optimal for more complex samples such as cell lysates containing other cellular materials and sample buffer components.

Some elements of sample matrices may also lead to ion suppression. It has been observed that biological samples give significantly lower MS signal responses compared to pure standard solutions with similar analyte concentrations due to high concentrations of non-volatile compounds from the biological sample, including lipids and salts, accompanying the analyte within the electrospray (Ho et al., 2003). While the use of HPLC columns helps to separate these non-volatile compounds from the analyte (Ho et al., 2003), efforts are still needed to reduce the lipid and salt content within biological samples. These efforts come in the form of extensive sample preparation and purification protocols, where protein extraction aims to remove other organic analytes and the addition of a C18 desalting step aims to remove analytes of small molecular weight via solid phase extraction. These elaborate protocols are time-consuming, expensive and can also lead to poor sample recovery. Mörlein and colleagues suggest analysing samples at a high and low concentration and analysing a neat sample solution and a matrix-based sample that matches the biological samples, to fully appreciate the effects of target analyte concentration and matrix effects that may influence the optimal instrument parameters of a target precursor ion during MRM method development (Mörlein et al., 2020).

To transform this label-free, semi-quantitative assay into an assay capable of use in clinical laboratories, method validation would need to be performed. This includes the determination of assay specificity, linearity, lower limit of quantification, accuracy,

precision, extraction recovery, matrix effects and stability (European Medicines Agency, 2022). In addition, use of LC-MS assays for clinical samples often requires fully quantitative assays. Currently, semi-quantitative isoform measurements are capable of producing ratios of target protein isoforms within the same sample or providing semi-quantitative measurements of the isoforms between different biological samples. To develop a fully quantitative assay from the semi-quantitative assay designed in this study, stable isotope-labelled homologues of nuclear lamin isoforms may be used as internal standards and spiked into the biological samples. These homologues should be identical to the native target tryptic peptides measured in the assay with the exception of deuterated hydrogen atoms which allows the homologue to be differentiated from the native analyte due to their isotope label, as the stable isotope labelled (SIL) internal standard will be heavier than the native analyte of interest and the difference in m/z will be dependent on the number of deuterations (Ho et al., 2003). The production of a standard concentration curve from the deuterated standards of target isoform tryptic peptides would allow for the calculation of the concentration of the peptide within the original sample. These deuterated peptides should be introduced into the sample mixture before the sample preparation protocol begins and allows for a normalisation parameter to be included within the assay. The target protein expression can then be normalised to this reference mixture of peptides. In addition, the use of labelled internal standards compensates for losses during sample preparation and variations in detection sensitivity of the instrument between injections. SILs are used to establish the sensitivity and selectivity of disease biomarkers, and use of SILs in combination with LC-MS/MS provides a high specificity for determining the quantity of a target protein in samples (Ciccimaro & Blair, 2010).

In terms of further optimisation, the effect of modulating the solvent flow rate, LC gradient, source temperature and emitter-MS inlet distance was not conducted in the current study. Previous literature has shown that changing the solvent flow rate affects the optimal source settings, as some of these ESI settings are dependent on solvent flow rate such as nebuliser pressure, capillary voltage, nozzle voltage and desolvation gas flow rate. Considering this, researchers at Agilent Technologies state that

recommended AJS ESI operating parameters for the detection of the small molecule reserpine were consistent across a wide range of flow rates, which reduced the need for optimization at different flow rates (Mordehai & Fjeldsted, 2009). Changing the solvent flow rate may also affect the analyte retention times, and this should be considered when scheduling analyte monitoring windows during dynamic MRM method development. Source temperature was not optimised during this study as this was not included in the list of parameters available for optimisation using the Source Optimiser software and CE and FV Optimiser software, however it may be worthwhile to investigate the effects of source temperature on analyte response when measuring labile analytes such as peptides.

With respect to the experimental design, two main approaches are used for optimisation experiments with multiple variable factors: OFVAT and DoE, as mentioned in Section 3.4.5. OVAT involves changing one factor and keeping the remaining factors constant and observing how this changes a response. Initially, this was considered the most intuitive way to optimise the AJS ESI source parameters due to the vast amount of detail provided in the data obtained from this type of optimisation experiment. OVAT is suitable for simple experiments with few variable factors and no suspected interactions, especially when resources are very limited. In contrast, DOE is more suitable for more complex experiments with multiple factors and suspected interactions, providing a more efficient and thorough experiment at the cost of increased complexity in both experimental design and data analysis. With this in mind, a DoE approach may have been a more suitable method of investigating the optimal AJS ESI source settings in this study, given there are several factors that all contribute to the ESI mechanism. In addition, there are known logistical and technical relationships between some AJS ESI settings, and this is reflective in the user interface of the Agilent Ultivo system. It is known that sheath gas flow rate and temperature are intrinsically linked, as the user interface only allows for flow rates of 2 L/h at a temperature range of 0 – 124°C whereas a high flow rate of 12 L/h only allows for gas temperatures to be in the range of 0 – 400°C. This relationship is not seen, however, with the desolvation gas which allows for temperatures of 80 – 350°C throughout the entire range of 3 – 13

L/h flow rate. It is therefore important to distinguish the relationship between gas temperature and flow rate in future optimisation experiments, particularly with respect to the sheath gas, and to elucidate why these two gases have differences in the relationship between these flow rate and temperature, and ultimately what effect that has on ionisation efficiency. Future work may consider a DoE approach to optimising AJS ESI settings for different biomolecules given the known relationship between some parameters and considering the large number of AJS ESI parameters that affect the electrospray shape, desolvation of droplets and subsequent ionisation of target molecules.

4. Targeted Mass Spectrometry Analysis of Nuclear Lamins in Cell Lines

4.1. Introduction

In Chapter 3 of this thesis, the development of a targeted mass spectrometry method for the relative quantitation of the protein expression of different nuclear lamin isoforms in biological samples of human and mouse origin was reported. Chapter 4 will now describe the use of this LC-MRM-MS/MS method to examine the nuclear lamin isoform abundances in cell lines representative of different B-cell malignancies and in the BL cell line BL-2 with and without the expression of AID.

4.2. Aims and Objectives

The first part of this chapter will investigate the expression of nuclear lamin isoforms in cell lines representative of Burkitt lymphoma (BL-2) and multiple myeloma IgG (OPM-2) and IgE (U266) subtypes. Considering the WB and LC-MSe results in Chapter 2, it was hypothesised that the expression of different nuclear lamin isoforms will vary across different malignant B-cell cell lines. The second part of this chapter will examine nuclear lamin isoform expression in the BL-2 cell line with and without gene inactivation of activation-induced cytidine deaminase ($AID^{+/+}$ and $AID^{-/-}$ respectively). The hypothesis of this study was that the expression of nuclear lamin isoforms will be different in the BL-2 cell line compared to the BL-2 $AID^{-/-}$ cell line. Particularly, the expression of lamin B1 would be upregulated in the BL-2 $AID^{-/-}$ cell line compared to the BL-2 $AID^{+/+}$ cell line. This aim of this chapter was to examine the protein expression of different nuclear lamin isoforms in cell lines using the targeted LC-MRM-MS method optimised in the previous chapter of this thesis. This will be achieved through several objectives:

1. Investigate the protein expression of lamin A/C, lamin B1 and lamin B2 in the BL-2, OPM-2 and U266 cell lines using LC-MRM-MS.
2. Investigate the protein expression of lamin A/C, lamin B1 and lamin B2 in the BL-2 wild-type ($AID^{+/+}$) and BL-2 $AID^{-/-}$ cell lines using LC-MRM-MS.

4.3. Materials and Methods

4.3.1. Reagents

LC-MS grade water and acetonitrile (ACN) were purchased from Fisher Scientific Ltd (Loughborough, UK). >99% formic acid for LCMS was purchased from VWR International Ltd (Leicestershire, UK). Low volume LCMS vials were purchased from Waters Corporation (Wilmslow, UK).

4.3.2. Cell Culture

The culture conditions and details of media components used to culture the BL-2 cell line (also known as BL-2 AID^{+/+}), BL-2 AID^{-/-} cell line, OPM-2 cell line and U266 cell line are detailed in Section 2.3.1 within Chapter 2. After 48-72 hours of continued growth of the BL-2 and BL-2 AID^{-/-} cell lines, a single cell suspension was made, and cells were transferred to a 50 mL tube. Cell suspensions were centrifuged at 1,000 rpm for 3 minutes before resuspension in fresh culture media. Once cells were suspended in fresh culture media, the cell suspension was mixed at a 1:1 v/v ratio with Trypan Blue exclusion dye (Invitrogen by Thermo Fisher Scientific UK Ltd, Loughborough, UK), and the cell viability was determined as outlined in Section 2.3.1 of Chapter 2. For OPM-2 and U266 cell lines, cells were cultured for 72-96 hours, and sub-culturing and counting was performed directly from a single cell suspension without centrifugation. All cell lines were cultured at 37°C, 5% CO₂ with 95% RH.

For downstream analysis, cells were transferred from the cell culture flask to 50 mL tubes for collection. For experiments investigating nuclear lamin expression in BL-2, OPM-2 and U266 cell lines, all cells were greater than 90% viability upon collection, as determined by the Trypan Blue exclusion assay. BL-2 cells were collected by centrifuged at 1000 rpm for 3 minutes, washed twice in PBS and placed on ice, while OPM-2 and U266 cell lines were centrifuged at 800 rpm for 3 minutes with a low deceleration speed before being washed twice in PBS and placed on ice. All cell pellets were then collected by centrifugation at 13,000 rpm for 2 minutes at 4°C and the cell pellet was stored on ice in preparation for immediate subcellular fractionation. Six pellets of 2 x

10⁶ cells each were collected per cell line from the same culture flask for this experiment.

For experiments investigating nuclear lamin expression in BL-2 AID^{+/+} and AID^{-/-} cell lines, all cells were greater than 95% viability upon collection, as determined by the Trypan Blue exclusion assay. Once the number of live cells per mL was calculated, cell pellets were collected by centrifugation at 13,000 rpm for 2 minutes at 4°C and the cell pellet was stored at -80°C. Two pellets of 3 x 10⁶ cells per pellet were collected for each cell line and each biological replicate.

4.3.3. Cell Lysis

4.3.3.1. Subcellular fractionation

Subcellular fractionation was performed on BL-2, OPM-2 and U266 cell lines. Six pellets of 2 x 10⁶ cells from each cell line were fractionated separately and combined at the end of the fractionation protocol. The details of the subcellular fractionation protocol are outlined in Chapter 2 in Section 2.3.2.1. Six nuclear fractions for each cell line were combined into one sample and then sonicated using a probe sonicator for 3 seconds.

4.3.3.2. Whole cell lysis

For experiments investigating nuclear lamin expression in BL-2 AID^{+/+} and AID^{-/-} cell lines, whole cell lysates were prepared from cell pellets following the protocol outlined in Chapter 2 in Section 2.3.2.2 however, a 25-gauge needle was used to lyse cells in this chapter instead of a 26-gauge needle used in the experiments in Chapter 2. Lysates from two cell pellets of each cell line collected at the same time were combined before sonication using a probe sonicator as outlined in Section 2.3.2.2. After sonication, samples were centrifuged at 13,000 rpm for 30 minutes and the supernatant containing the protein lysate was transferred to a fresh Lo-Bind Protein Eppendorf tube.

4.3.4. Protein Quantification

Protein quantification was performed on lysate samples using the Qubit Protein Assay Kit (Thermo Fisher Scientific UK Ltd, Loughborough, UK) using a Qubit 4 fluorometer, as per the manufacturer's instructions.

4.3.5. In-Gel Digestion

For BL-2, OPM-2 and U266 experiment, 120 µg of nuclear lysates were prepared for in-gel digestion while for the BL-2 AID^{+/+} and BL-2 AID^{-/-} experiment, 200 µg of whole cell lysate was prepared for in-gel digestion. Samples were mixed with 4x LDS loading buffer containing 2.5% v/v β-mercaptoethanol. Samples were heated at 95°C for 10 minutes before loading onto a 4-12% Bis-Tris polyacrylamide gel along with an 11-245 kDa protein ladder (New England Biolabs, Herts, UK). For samples in which a total of 120 µg was prepared, 40 µg of lysate was loaded per well. For samples in which a total of 200 µg lysate was prepared, 33.3 µg of lysate was loaded per well. In both experiments, a 1x NuPAGE MES SDS running buffer (ThermoFisher Scientific UK Ltd, Loughborough, UK) was added to the electrophoresis tank and protein separation was conducted at 100 V until the dye front reached the end of the gel.

After electrophoresis, the apparatus was disassembled, and the SDS-PAGE gels were washed in MilliQ 18mΩ ultrapure water three times for 5 minutes per wash. Gels were then stained with a Coomassie-based stain (Imperial Protein Stain) (Thermo Fisher Scientific UK Ltd, Loughborough, UK) for 1 hour at room temperature with gentle rocking. The gels were washed in MilliQ 18mΩ ultrapure water for a total of 1 hour, with water changes every 15 minutes. Gels were stored at 4°C overnight. The next day, the protein bands of interest were excised from the gels using a scalpel. One gel piece was excised from each sample lane which contained proteins ranging from 58 Da to 100 Da in size. Gel pieces for each cell line were pooled transferred to Protein Lo-Bind Eppendorf tubes before destaining by washing twice in de-staining solution (25 mM ammonium bicarbonate (ABC) in 50% ACN) at 37°C for 30 minutes per wash, with shaking at 600 rpm. Samples were reduced through incubation in 3 mM 1,4-dithiotritol (DTT) (Merck Life Sciences UK Limited, Gillingham, UK) at 60°C for 10 minutes. The DTT

solution was removed, and alkylation of proteins was performed by addition of 2.6 μ M iodoacetamide (IAA) (Merck Life Sciences UK Limited, Gillingham, UK). Samples were incubated at room temperature in the dark for 1 hour. The IAA solution was removed, and the samples were then washed twice in de-staining solution. 100% ACN was added to gel pieces and left to incubate for 15 minutes at room temperature before allowing samples to air-dry for 10 minutes.

Samples were trypsinised in 100 μ L of 20 ng/ μ L Sequence Grade Modified Trypsin (Promega, Hampshire, UK) prepared in 100 mM HEPES and incubated at room temperature for 15 minutes. 200 μ L more of the 100 mM HEPES buffer was added to the digestion mixture and samples were left to incubate overnight at 30°C with shaking at 600 rpm. The digested sample solutions were transferred to a separate Lo-Bind Protein Eppendorf tube and 1% trifluoroacetic acid (TFA) was added to the gel pieces. After a 5-minute incubation, any remaining sample mixed in TFA was combined with the digestion sample solution and stored at -80°C. Samples were then desalted using 200 μ L C18 + Carbon TopTips (Glygen, Maryland, USA) as per the manufacturer's instructions and eluted in 70% ACN containing 0.1% TFA. Samples were lyophilised by vacuum centrifugation before resuspension in 25 μ L of 2% ACN with 0.1% formic acid (FA) and transferred to low-volume LC-MS vials (Waters Corporation, Wilmslow, UK).

4.3.6. Liquid Chromatography (LC)

Chromatographic separation was performed using an Agilent 1290 Infinity II LC system. 20 μ L of each sample was injected using an autosampler. Peptide separations were performed by reversed-phase LC separation using an AdvanceBio Peptide Mapping column, 2.7 μ m, 2.1 x 150 mm, 120Å column (PN 653750-902) (Agilent Technologies Ltd, Cheshire, UK). Liquid chromatography was performed with solvent A (100% Milli-Q water with 0.1% FA) and solvent B (100% ACN with 0.1% FA). Peptides were eluted at 0.3 mL/min, with the chromatography method shown below in Table 4-I.

Table 4-I: Liquid chromatography gradient used in the analysis of nuclear lamin synthetic peptides.

Time (mins)	%A	%B	Flow Rate (mL/min)
0	95	5	0.3
15	50	50	0.3
17	10	90	0.3
18	10	90	0.3
20	95	5	0.3
25	95	5	0.3
Post time (5 mins)	95	5	0.3

4.3.7. Multiple Reaction Monitoring-based Mass Spectrometry (MRM-MS)

The MRM-LC-ESI-MS/MS experiments were performed on an Agilent Ultivo QQQ with an Agilent Jet Stream (AJS) ESI source. Nitrogen was used as the collision gas, nebulising gas, desolvation gas and sheath gas. Protein expression was determined by LC-MRM-MS analysis using optimised method described in Chapter 3. The AJS ESI source parameters are detailed in Table 4-II.

Table 4-II: Electrospray ionisation source parameters for LC-MRM-MS analysis of nuclear lamin isoforms in cell lines using the Agilent Ultivo QQQ mass spectrometer.

Source Parameter	Default Value
Sheath Gas Temperature (°C)	250
Sheath Gas Flow Rate (L/min)	11
Desolvation Gas Temperature (°C)	300
Desolvation Gas Flow Rate (L/min)	7
Nebuliser Pressure (psi)	60
Capillary Voltage (V)	4000
Nozzle Voltage (V)	500

A list of MRM transitions and the optimized CE and FV values for this assay are detailed in Appendix IV Table 8-VI. MRM transitions were monitored using unit resolution in both Q1 and Q3 quadrupoles to maximize specificity. Each MRM transition had a minimum dwell time of 20 seconds and a detection window of 1 minute. Scheduled MRM was used to reduce the number of concurrent transitions and to maximize the dwell time for each transition. Data was acquired using Agilent MassHunter Data Acquisition version 1.2 software. MRM data was acquired in the form of .raw files.

4.3.8. Data Analysis

Data in the form of .raw files were uploaded into Skyline MS version 20.0 software. Inspection of each chromatographic peak was conducted to examine peak shape, and peak boundaries were manually adjusted where necessary. Chromatographic peaks that gave a poor shape i.e. were not Gaussian, or contained multiple peaks for one single transition, were not included in the final data set. Information pertaining to chromatographic peak area measurements for each transition during all experiments was exported to Microsoft Excel as .xlsx files. The area under the chromatographic curve (AUC, also known as area) and background signal for each transition was used to calculate the signal-to-noise ratio (S:N). This was determined for each transition measured in each sample by dividing the AUC value by the background signal. The total

peak area of each nuclear lamin isoform was calculated by the addition of the peak area values for all measurable transitions of the isoform-specific tryptic peptides. Peptide ions which gave a signal-to-noise ratio (S:N) of greater than 3:1 were considered above the limit of detection but below the limit of quantitation. For quantitation of each nuclear lamin isoform, the data was filtered to only include transitions which gave an S:N of greater than 10:1. For further verification of suitable transitions, the peak area ratio of each transition was compared between all suitable transitions. Only peptides in which the peak area ratio between transitions gave a coefficient of variation (CV) of less than 20% were chosen for the final analysis. Peptides with two to three quantifiable transitions were used for quantification of the target protein isoform. If the peptide had less than two transitions that were suitable for quantitation, that peptide was not included in the final analysis. If a peptide had more than three quantifiable transitions, the three peptides which gave the greatest peak area were selected for quantitation of that particular peptide. More details on the transitions measured in the LC-MRM-MS/MS assay can be found in Appendix IV Table Table 8-VI. The creation of bar charts and dot plots, and all statistical analysis, was performed using Graphpad Prism version 10 software.

4.4. Results and Discussion

4.4.1. Nuclear lamin expression in B-cell malignancies by LC-MRM-MS

To investigate nuclear lamina dynamics in different types of B-cell malignancies, LC-MRM-MS/MS analysis was conducted to determine the expression of nuclear lamin isoforms in cell lines representative of BL (BL-2), and MM of the IgG and IgE subtypes (OPM-2 and U266 respectively).

To calculate the expression of each nuclear lamin isoform in the B-cell cancer cell lines, the sum of the total peak area of each transition above the limit of quantitation was calculated. Since the same amount of protein in each whole cell lysate was used for sample preparation, the peak area of peptides unique to each nuclear lamin isoform correlates to the amount of protein in the original sample and thus, these can be compared in a semi-quantitative manner across cell lines. The transitions used for quantitation of each nuclear lamin isoform in these samples are described in Appendix V Table 8-VII.

When measuring the protein expression of lamin A/C in malignant B-cell cell lines, only two transitions for lamin A/C peptides were observed above the limit of detection (S:N of greater than 3:1) but not above the limit of quantitation (S:N of greater than 10:1) across all three cell lines, which suggests that lamin A/C is present within all cell lines but not quantifiable in all of the cell lines analysed. When exploring the data, lamin A/C was quantifiable in the OPM-2 cell line, with 12 transitions measured above the limit of detection, suggesting a higher lamin A/C expression in this cell line compared to the BL-2 cell line where only 3 transitions were measured with a S:N of greater than 10:1 and 0 transitions within the U266 cell line sample was measured at quantifiable levels (data not shown). This correlates with previous data shown in Chapter 2 where OPM-2 cells were shown to have a greater lamin A/C expression compared to the BL-2 and U266

cell lines as determined by Western Blot analysis and LC-MS^e analysis (Figure 2-5 and Figure 2-8).

With respect to lamin B1 and lamin B2 expression in these three cell lines, several peptide transitions could be used for their quantitation due to their large peak areas and CV of less than 20%. Figure 4-1 below shows the expression of lamin B1 in BL-2, OPM-2 and U266 cell lines, as determined by the LC-MRM-MS analysis.

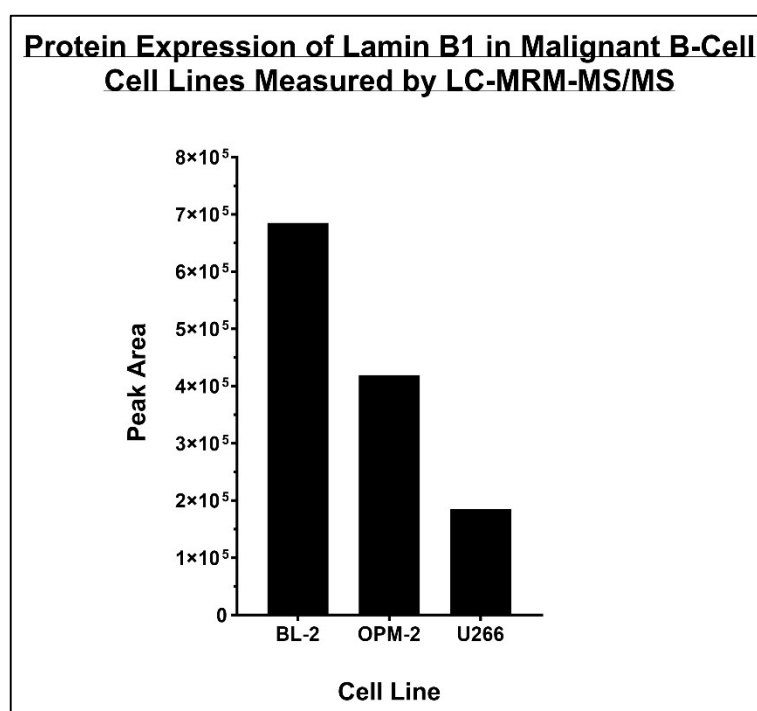


Figure 4-1: Protein expression of lamin B1 in three cell lines of B-cell malignancies, as determined by LC-MRM-MS/MS analysis (n=1).

The data shown in Figure 4-1 suggest that OPM-2 and U266 cells express less lamin B1 protein than BL-2 cells. Lamin B1 expression was the lowest in U266 cells, and the highest in BL-2 cells. This result agrees with the Western Blot data shown in Chapter 2 (Figure 2-6). Lamin B1 expression was greater in OPM-2 cells than in U266 cells, suggesting that this isoform may play a more prominent role in IgG subtype multiple myelomas which is represented by the OPM-2 cell line compared to IgE subtypes such

as that of the U266 cell line, however previous WB data suggested that U266 cells may contain more lamin B1 than OPM-2 cells. LC-MS^e analysis in Chapter 2 seemed to suggest OPM-2 cells contained the most lamin B1 protein, however the accuracy of the LC-MS^e results should be questioned due to the low number of biological replicates analysed and the absence of normalisation of the data to a reference sample or internal standard peptides. In addition, the low number of biological replicates analysed in Figure 4-1 means no conclusive statements on the expression of lamin B1 in B-cell malignancies can be made from these results.

Considering the expression of lamin B2, Figure 4-2 below shows the protein expression in BL-2, OPM-2 and U266 cell lines, as determined by the LC-MRM-MS analysis.

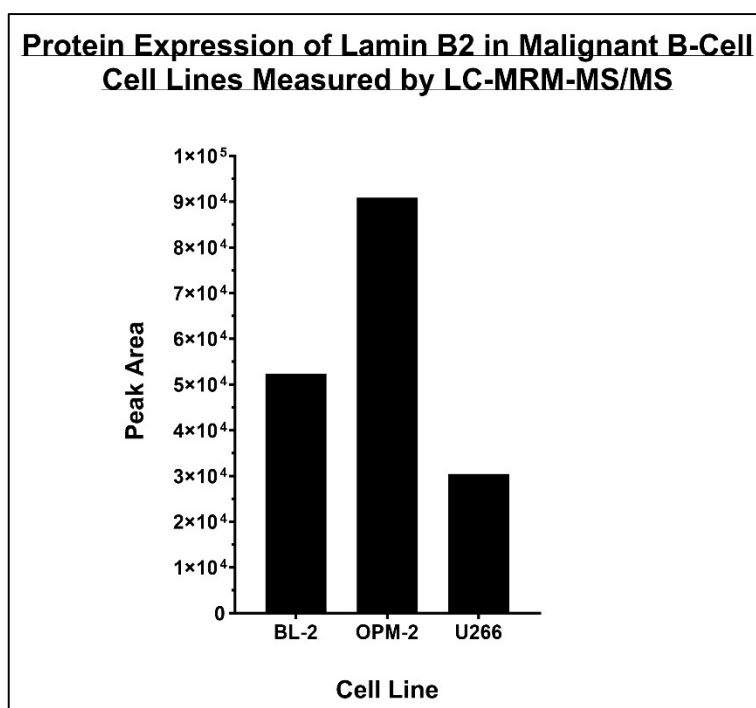


Figure 4-2: Protein expression of lamin B2 in three cell lines of B-cell malignancies, as determined by LC-MRM-MS analysis (n=1).

The data in Figure 4-2 shows that OPM-2 and U266 cells had less lamin B2 protein expression than BL-2 cells. Lamin B1 expression was the lowest in U266 cells, and the

highest in BL-2 cells. This result differs from the WB observations made previously, where U266 cells were suggested to have the highest lamin B2 expression of the three cell lines analysed (Figure 2-7). The discrepancies in these results may be due to biological variations between samples and the normalisation of WB data to housekeeping proteins, while this LC-MRM-MS/MS assay does not contain any normalisation methods.

4.4.1.1. Summary

The data shown in this section suggests that different B-cell malignancies contain different expression patterns of nuclear lamins, which may drive malignant transformation and survival of cancerous cells. OPM-2 seems to have the highest expression of both lamin A/C and lamin B2 across these three cell lines. Correlations between the expression of lamin A/C and lamin B2 have been previously documented with respect to the control of cellular elasticity and nuclear deformability (Vortmeyer-Krause et al., 2020) and the expression of these protein isoforms is dependent on levels of lamin B1 (Shimi et al., 2008) (see Section 1.2 for more information). This may suggest that OPM-2 cells have greater metastatic potential compared to BL-2 and U266 cells.

Decreases in lamin A/C and lamin B2 expression were reported to increase nuclear deformability in previous studies (Vortmeyer-Krause et al., 2020). Since BL-2 cells were shown to have low expression of both lamin A/C and lamin B2 as measured by LC-MRM-MS, it can be speculated that these cells have the greatest ability to modulate the nuclear shape out of the three cell lines analysed, which may aid in migration out of the lymph nodes and subsequent metastasis. Metastasis of BL cells to multiple organs is often seen in BL (Miron et al., 2017; Tang et al., 2016).

Conversely, the higher expression of lamin B2 in the MM cell lines may suggest that nuclear deformability and metastasis is less important to cancer cell survival and

cancer progression in these malignancies, which coincides with the localisation of MM cells being predominantly within the bone marrow (BM), especially in the early stages of disease (Kumar et al., 2017; Ram et al., 2024). In addition, progression to plasma cell leukaemia (PCL) is more common in patients with the IgE MM subtype represented by the U266 cell line, compared to the IgG MM subtype, which OPM-2 represents. One of the defining factors of PCL is the presence of 20% circulating clonal plasma cells (Gundesen et al., 2019; Kyle, 1974). This may explain why U266 cells have less lamin A/C and lamin B2 expression than OPM-2 cells. It would be interesting to explore the nuclear lamin isoform expression in other MM Ig subtypes such as IgD, IgM and IgA, as this may reveal correlations between disease progression to PCL or may lead to insights into patient prognosis, potentially leading to nuclear lamins becoming a prognostic biomarker of disease in MM.

Lamin B1 expression was shown to be the highest in BL-2 cell when comparing the three B-cell cancer cell lines. With respect to MM cell lines, lamin B1 expression was greater in OPM-2 cells than in U266 cells, suggesting that this isoform may play a more prominent role in IgG subtype multiple myelomas compared to IgE subtypes. This also provides further evidence that low expression of lamin B1 is seen throughout B-cell neoplasms, including in transformed plasma cells. A comparison between the expression of lamin B1 in healthy and malignant plasma cells would provide further insights into this topic. Moreover, whether the expression of lamin B1 gradually decreases during the progression of MM to PCL is an interesting topic of exploration and this hypothesis is yet to be explored. The expression of lamin B1 in lymphoma samples compared to MM samples has not been explored previously, so this preliminary data provides novel insights into the role of lamin B1 in transformed B-cells at different stages of maturation upon transformation.

It is important to note that the preliminary results shown above are produced from analysis of one biological replicate of each cell line. As a result, no statistical analysis was performed on these data. Therefore, more replicate analysis is necessary to

confidently assess the protein expression of nuclear lamins and the isoform ratios in these cell lines and confirm the findings shown above.

4.4.2. Nuclear lamin expression in BL-2 AID^{+/+} and BL-2 AID^{-/-} cell lines by LC-MRM-MS

Previous reports have shown that BL-2 cells express AID at both a transcriptional and translational level (Faili et al., 2002a, 2002b; Klymenko et al., 2018). Since lamin B1 is downregulated and AID is upregulated during SHM in BL-2 cells (Klymenko et al., 2018; Woo et al., 2003), and lamin B1 is known to bind to the chromatin at LADs and can affect the expression of genes within these regions (Guelen et al., 2008; Noguchi et al., 2021), it was hypothesized that AID expression may downregulate the expression of lamin B1, leading to the upregulation of genes associated with SHM (see Section 1.3.3 for more information). The aim of this section was to investigate if knockdown of AID in BL-2 cells resulted in the downregulation of lamin B1, and if changes in the expression of other nuclear lamin isoforms were also observed.

To accurately quantify the expression of each nuclear lamin isoform, the peak area data was filtered to only include transitions which gave a S:N of greater than 10:1 and a CV of less than 20% in all samples. The results for lamin A/C expression showed that no transitions for peptides unique to the human lamin A/C protein were measured above the level of quantitation across both cell lines, however two transitions were measured above the level of detection (S:N greater than 3:1). This suggests that the lamin A/C protein isoform is present in these cells but not considered quantifiable by the LC-MRM-MS assay. Conversely, both lamin B1 and lamin B2 were quantifiable by LC-MRM-MS in these cell lines. The transitions used for quantitation of lamin B1 and lamin B2 in these samples are described in Appendix V

Table 8-VIII.

Figure 4-3 below shows the protein expression of lamin B1 in BL-2 AID^{+/+} and BL-2 AID^{-/-} cell lines, as determined by LC-MRM-MS analysis.

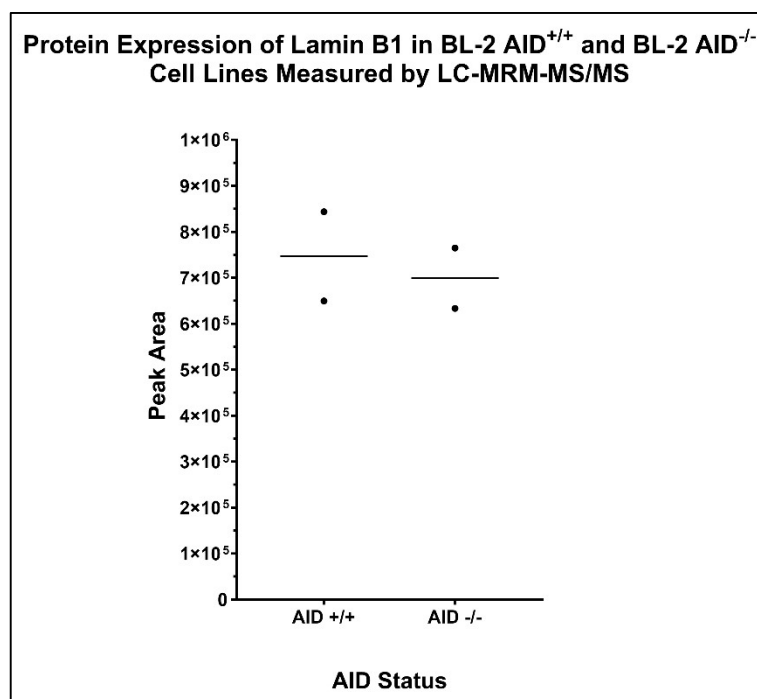


Figure 4-3: Protein expression of lamin B1 in BL-2 AID^{+/+} and BL-2 AID^{-/-} cell lines, as determined by MRM-LC-MS analysis (n=2).

The data shown in Figure 4-3 suggest that lamin B1 protein expression may be similar in BL-2 AID^{-/-} cells compared to BL-2 AID^{+/+} cells, which may suggest no effect of AID knockdown on lamin B1 expression. These data support the LC-MS^e results in Chapter 2, which indicated that AID expression does not affect the protein expression of lamin B1 in BL-2 cells (Figure 2-12).

Finally, lamin B2 expression was quantified using measurements from all peptide transitions with a S:N of greater than 10:1 and a CV of less than 20% between different

sample readings. Figure 4-4 below shows the expression of lamin B2 in BL-2 AID^{+/+} and BL-2 AID^{-/-} cell lines, by LC-MRM-MS analysis.

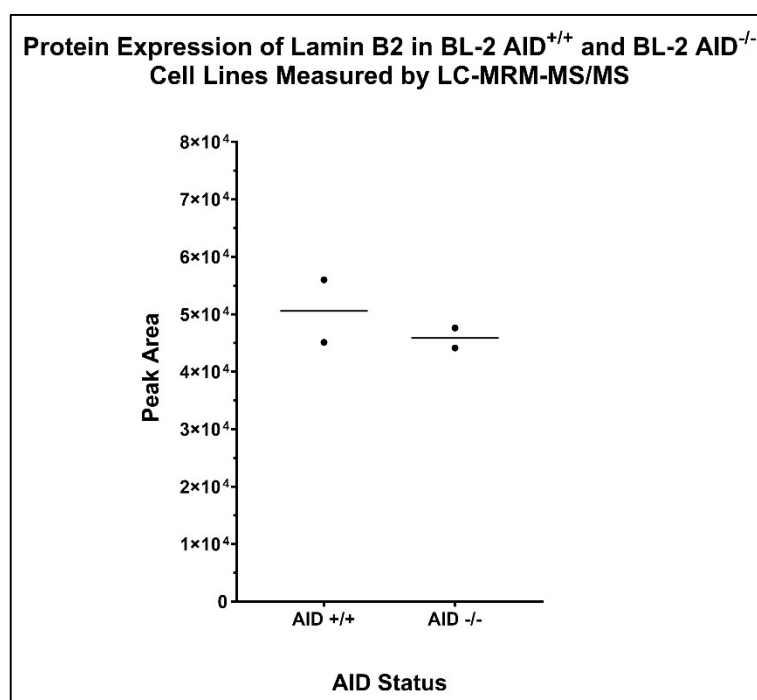


Figure 4-4: Protein expression of lamin B2 in BL-2 AID^{+/+} and BL-2 AID^{-/-} cell lines, as determined by MRM-LC-MS analysis (n=2).

The LC-MRM-MS results shown in Figure 4-4 may suggest that the expression of lamin B2 is slightly lower in BL-2 AID^{-/-} cell line compared to the BL-2 AID^{+/+} cell line.

4.4.2.1. Summary

Analysis of the protein expression of nuclear lamins in BL-2 AID^{+/+} and BL-2 AID^{-/-} cell lines suggest that there was no change in the expression of any B-type nuclear lamin isoforms upon AID knockout. Furthermore, transitions measuring lamin A/C peptides suggest that lamin A/C protein could be detected in both cell lines but not quantified accurately by this mass spectrometry assay. To quantify lamin A/C in these cell lines, more protein lysate could be analysed, and this would be achieved by culturing and

harvesting a greater number of cells per sample, which is an advantage of using cell lines over primary tissues.

With respect to lamin B1, it was hypothesised that since lamin B1 is downregulated and AID is upregulated during SHM, AID expression may negatively affect the expression of nuclear lamins, particularly lamin B1, during B-cell activation. If AID expression was shown to decrease the expression of lamin B1, the results would show the AID^{-/-} cell line contains less lamin B1 than the AID^{+/+} cell line. The data shown in Figure 4-3 suggest that the BL-2 AID^{-/-} cell line contains a similar amount of lamin B1 than the BL-2 AID^{+/+} cell line, which contradicts the hypothesis. LC-MRM-MS/MS analysis of samples from experiments whereby BL-2 cells are induced to undergo SHM and hyper-express AID, i.e. by activation with IgM antibodies or co-culture with activated T-cells as performed in previous studies (Woo et al., 2003) would give further insights into any effects of AID expression on nuclear lamin isoform expression.

Currently there are no studies indicating that lamin B2 may have a role in SHM, BL-2 pathogenesis, or AID activation and the results in Figure 4-4 suggest that lamin B2 expression is not affected by AID expression in the BL-2 cell line.

The preliminary results shown above are produced from analysis of less than three biological replicates of each cell line and as a result, no statistical analysis was performed on this data. Therefore, more replicate analysis is necessary to confidently assess the protein expression of nuclear lamins and the isoform ratios in these cell lines and confirm the findings shown above.

4.5. Conclusion

Overall, this chapter reports experimental work using an optimised LC-MRM-MS assay to quantify the expression of nuclear lamin protein isoforms in a variety of cell lines. This targeted assay was used to investigate the expression of lamin A/C, lamin B1 and lamin B2 across different malignant B-cell cell lines, and in a cell line representative of BL in the presence and absence of AID expression.

The data obtained from the LC-MRM-MS analysis of cell lines representative of B-cell malignancies shows the expression of lamin A/C, lamin B1 and lamin B2 alters between the BL-2, OPM-2 and U266 cell lines. It was suggested that OPM-2 cells expressed the highest protein levels of lamin A/C out of the three cell lines analysed, as multiple transitions were measured above the limit of detection, while BL-2 and U266 cell lines contained few or no transitions measured above the limit of detection. BL-2 cells expressed the greatest amounts of lamin B1 (Figure 4-1) and lamin B2 was also highly expressed in the OPM-2 cell line compared to the BL-2 and U266 cell lines (Figure 4-2). This suggests that nuclear lamins may be of importance as potential biomarkers of disease in neoplastic transformations of B-cells of different maturation levels.

In addition, LC-MRM-MS analysis of nuclear lamin isoforms was conducted in BL-2 cells with and without the expression of the key regulating enzyme involved in antibody diversification, AID. The data obtained suggested that there was no change in the expression of B-type lamins when AID is inactivated in BL-2 cells (Figure 4-3 and Figure 4-4).

Although developing and optimising the MRM-based method in Chapter 2 was performed using synthetic peptides, the analysis of human cell lines via this assay allowed for the identification of the peptides which were the most easily detectable and quantifiable in biological human samples, which would make the method more easily transferrable to a clinical setting. Specific peptides may be modified or degraded

during the sample preparation protocol which cannot be monitored when analysing synthetic tryptic peptides. By using human cell lines, this research is able to better predict which peptides are abundant in human cells and this can aid in the development of absolute quantitation LC-MRM-MS/MS assays. Moreover, the absence of stable isotope labels in this method allowed for comparisons of each nuclear lamin isoform between samples in a semi-quantitative manner in an approach is cost-effective, easy to analyse, and the multiplexing capabilities allow for measurement of all isoforms within the same experiment, saving instrument time and reagent costs.

Overall, this chapter presents use of an optimised LC-MRM-MS assay to quantify the expression of nuclear lamin protein isoforms in a variety of cell lines. This targeted assay was used to semi-quantitatively analyse the expression of lamin B1 and lamin B2 across different B-cell cell lines representative of Burkitt lymphoma and multiple myeloma IgG and IgE subtypes, and in the Burkitt lymphoma cell line absent of AID expression (BL-2 AID^{-/-}).

4.6. Future Work

It is important to note that the preliminary results shown in this chapter are produced from analysis of one or two biological replicates of each cell line. As a result, no statistical analysis was performed on these data. Therefore, future work should focus on repeating this targeted proteomic analysis with more biological replicates of all samples to confidently and accurately report the expression of each nuclear lamin isoform in these cell lines.

In all cell lines examined in this chapter, the number of detectable unique lamin A/C peptides was low, and a S:N of 3:1 was only achievable in very few MRM transitions. According to the European Medicines Agency's IHC Q2(R2) Guideline on validation of analytical procedures, this limitation on the S:N ratio means that the few lamin A/C peptides above the limit of detection cannot be used during quantitative analysis (European Medicines Agency, 2023). This makes the assay currently unsuitable for quantification of lamin A/C. In addition, it is generally accepted that two or more transitions per peptide and at least two transitions per protein should be used for quantitation purposes. The more unique peptides that are detected corresponding to a specific target protein gives greater confidence in the expression of that protein, as there are more contributing transitions used to calculate the overall protein expression. As a result, to provide greater confidence in results of the semi-quantitation of lamin A/C protein expression in these cell lines, at least three transitions measuring unique lamin A/C peptides should be detected with a S:N of greater than 10:1 for accurate LC-MRM-MS quantitation of this isoform in this sample type. This indicates that the sample preparation protocol needs further optimising for low abundant proteins such as lamin A/C or the sensitivity of the assay needs to be increased.

To increase the chance of measuring nuclear lamins in these cell lines by LC-MRM-MS/MS, several approaches can be explored. Firstly, more total protein lysate could be analysed in order to obtain a S:N of greater than 10:1 in the lamin A/C peptide

transitions measured. This could be performed by processing larger cell pellets. Processing larger pellets would require optimisation of the subcellular fractionation, as the relationship between cell number and volume of cell lysis buffer was not linear (data not shown). Alternatively, pooling of several lysates resulting from smaller cell pellets could be used. Secondly, a robust and reproducible method of sample fractionation could be developed and optimised to decrease the sample complexity and increase the concentration of nuclear protein lysate available for analysis. As the samples analysed in this chapter are cell lines, increasing the amount of protein lysate available for each experiment can be done with minimal effort through increasing the number of cultured cells however, multiple cell pellets needed to be fractionated, and several lanes of the same sample were used on an SDS-PAGE in order to load 120 µg of nuclear fractions of cell lysates, which was already a lengthy sample preparation protocol. Future work could focus on MRM analysis of lamin A/C alone instead of in a multiplexing assay along with other nuclear lamin isoforms. This way, the dwell time for lamin A/C peptides could be increased, which may lead to an increase in chromatographic resolution. Additionally, protein purification such as immunoprecipitation protocols could be used to isolate lamin A/C from the complex cell lysates and would increase the concentration of this protein which can be directly injected into the LC-MRM-MS/MS system for analysis.

This study examines the protein expression of lamin A/C in biological samples, however it has been shown that lamin A and lamin C have different roles and their independent expression can vary in different cancers (see Section 1.2 and 1.4 for more information). Therefore, future work could investigate the expression of each A-type lamin isoform individually. Since lamin A and lamin C bands separate well on an SDS-PAGE gel, performing an in-gel digest on each isoform band separately would allow for differentiation of the expression of each individual A-type lamin isoform in the samples. However, as mentioned previously, for cell lines such as BL-2 where lamin A/C expression is barely detectable, the problem of loading enough nuclear lysate to detect these proteins still remains. An immunoprecipitation protocol targeting each specific isoform of lamin A/C may solve this problem.

The expression of nuclear lamin isoforms was compared between cell lines in a semi-quantitative manner using the LC-MRM-MS assay designed in Chapter 2. Normalisation was based on digestion and injection of the same volume of nuclear lysate in each sample. For more accurate normalisation, introduction of internal peptide standards could be used. Incorporating this peptide mixture at the same concentration into each sample at the earliest point of the sample preparation protocol, in this case after bands had been excised from the SDS-PAGE gel for in-gel digestion, would mean any loss of sample during the sample preparation protocol could be accounted for and data could be normalised to the abundance of these internal standard peptides.

5. Targeted Mass Spectrometry Analysis of Nuclear Lamins in Mouse Models

5.1. Introduction

In Chapter 3, a targeted LC-MRM-MS/MS method was developed and optimised to investigate the protein expression of four nuclear lamin isoforms in biological samples from humans and mice. Chapter 5 will now explore the use of this targeted MS method to examine the relative abundance of nuclear lamins in the GCs of a selection of mouse models which have been immunised to illicit an immune response. The mouse models investigated include a model of the lamin B1 overexpressing disease ADLD, a model of *ATG7* conditional knock-down and a model of *LMNB1* conditional knock-down.

5.1.1. B-cell activation

The expression of nuclear lamins in naïve and activated B-cells has been explored briefly, and studies suggest that lamin A/C is undetectable in naïve B cells but increases upon activation (Caruso et al., 2022; Contu et al., 2018; Thiam et al., 2016) while the expression of lamin B1 has been shown to decrease in B-cells upon activation and was theorised to correlate with the initiation of SHM (Klymenko et al., 2018). The expression of lamin B2 as a single isoform has not yet been examined in B-cells. This study aims to investigate the expression of lamin A/C, lamin B1 and lamin B2 in splenocytes of immunised mice to examine the effects of immune activation on nuclear lamin protein expression in activated B-cells and other cells within the spleen.

5.1.2. Autosomal Dominant Leukodystrophy (ADLD)

The neurodegenerative disease adult-onset autosomal dominant leukodystrophy (ADLD) is the only genetic disorder associated with lamin B1 **that** has been shown to result in cell type-specific effects (Padiath & Fu, 2010; Yang et al., 2011a; Yue et al.,

2019). Lamin B1 overexpression in different cell types using mouse models and primary cells has been thoroughly investigated and reviewed in the literature (Neri et al., 2023), however the influence of increased lamin B1 levels on the immune cells has not been examined. This thesis will explore the expression of nuclear lamin isoforms in wild-type (WT) and ADLD mouse models by MRM-based LC-ESI-MS/MS analysis. This thesis utilises a previously described transgenic mouse model of ADLD (MG1 ref. J:197168, Tg(Lmnb1)^{Yfu}) on a C57BL/6 background that expressed four times the normal levels of full-length mouse *LMNB1* under the control of an endogenous promoter (Heng et al., 2013). B-cell activation was induced by immunisation with a mixture of ovalbumin (OVA) and lipopolysaccharide (LPS) and the immune response developed over 4 days. Splenocytes were separated using magnetic activated cell sorting (MACS) into PNA⁺ and PNA⁻ fractions, with the PNA⁺ fraction corresponding to GC B-cells. This technique is a method of separation of various cell populations depending on their surface antigens (CD molecules) and uses magnetic nanoparticles conjugated to antibodies to target specific cell types by capturing them inside a column, which is placed between permanent magnets. Peanut agglutinin (PNA) was the surface antigen used to separate GC B-cells from other cells within the spleen. PNA is a plant-derived glycan-binding protein (lectin) which binds with high affinity to activated B-cells within GCs (Rose et al., 1980, 1981). PNA has been shown to selectively bind to GC cells in peripheral lymphoid organs (Rose et al., 1980). A study by Giovannone and colleagues assessed PNA binding affinity in several B-cell subsets, including in naïve, GC, memory and plasmablast B-cells. The results showed that PNA bound exceedingly strongly to GC B-cells, and PNA binding was greater than 10-fold higher in GC B-cells compared to naïve or memory B-cells. In addition, it was shown that plasmablasts also display strong binding to PNA that equalled that of GC B-cells, suggesting that PNA reactivity may more accurately reflect B-cell activation rather than differentiate cells specifically within the GCs (Giovannone et al., 2018). This suggests that the PNA⁺ fraction contains activated B-cells, including antibody-secreting plasmablasts.

Both PNA⁻ and PNA⁺ cell fractions derived from WT and ADLD samples were analysed to compare the protein expression of each nuclear lamin isoform. Using the cell sorting

model described above, the impact of lamin B1 overexpression on B-cell activation could be assessed *in vivo*. By directly comparing OVA-immunised ADLD animals with WT counterparts, the expression of lamin B1 and other nuclear lamin isoforms could be examined in immune cells through targeted MRM-based mass spectrometry. The hypothesis of this study was that ADLD mouse GC cells contain more lamin B1 protein expression than their WT counterparts in both the PNA+ and PNA- fractions. In addition, comparisons of the expression of lamin B1 in PNA+ cells compared to PNA- cells was performed in ADLD animals and WT animals to examine if ADLD B-cells react similarly to B-cells isolated from wild-type animals upon immune activation.

5.1.3. Autophagy

Since decreases in lamin B1 expression have been previously associated with B-cell activation (Klymenko et al., 2018) and autophagy has been shown to be a mechanism of lamin B1 nuclear disassembly (Dou et al., 2015a, 2015b; Lenain et al., 2015), it was theorised that autophagy may be involved in the removal of lamin B1 during B-cell activation. As Atg7 is a critical protein in the autophagy process, a *Cy1-cre-ATG7* gene knock-out mouse model was used to investigate the effect of *ATG7* expression on the protein expression of nuclear lamins during B-cell activation. This was investigated by blocking the autophagy process by conditional knock-out of *ATG7* in mouse models and measuring the protein expression of lamin B1 and other nuclear lamins in PNA+ and PNA- cells by LC-MRM-MS/MS.

5.1.4. Lamin B1 knock-out

Studies using knockout *LMNB1* mouse models have shown that B-type lamins are crucial for development and differentiation, with fatalities occurring soon after birth due to developmental defects within the CNS, and cardiac and skeletal muscles (Coffinier et al., 2011; Sullivan et al., 1999; Vergnes et al., 2004). With respect to the immune system, lamin B1 has been associated with thymus development and proper functioning (Yue et al., 2019). In consideration of the decreased expression of lamin B1 upon B-cell activation (Klymenko et al., 2018), this chapter aimed to investigate the

relative abundance of each nuclear lamin isoform in *LMNB1*-knockdown (KD) mouse models compared to wild-type mice. Using a conditional knock-out model of lamin B1, the expression of nuclear lamin isoforms can be examined through the targeted MRM-based mass spectrometry assay developed in Chapter 3. The hypothesis was that GC cells from *LMNB1*-KD animals contain less, if any, lamin B1 protein than their WT counterparts, in the PNA+ fractions. In addition, an investigation into the expression of other nuclear lamins in PNA+ B-cells upon *LMNB1*-KD will be explored and compared to the expression of nuclear lamins in WT animals. It was hypothesised that the expression of lamin B1 in *LMNB1*-KD PNA+ B-cells is lower than that of WT PNA+ B-cells. It was also hypothesised that there was a greater expression of lamin B1 in PNA-cells compared to PNA+ cells in both WT and *LMNB1*-KD mouse models.

5.1.5. Mice Models Used in This Study

The strain of mice used in this study are C57BL/6 which is a commonly used strain of genetically modified mice for use as models of human disease. C57BL/6 wild-type mice were used as a control model and were compared to three other mouse models, also on a C57BL/6 background. The first mouse model used in this study was a transgenic model of ADLD on a C57BL/6 background that expresses four times the normal levels of full-length mouse *LMNB1* (*LMNB1*-tg) under the control of an endogenous promoter (Heng et al., 2013). This mouse model was used to model ADLD, a very rare leukodystrophy and leukoencephalopathy associated with the overexpression of *LMNB1* due to gene duplication (Brussino et al., 2009; Padiath, 2019; Padiath et al., 2006) (see Section 1.6.2 for more information).

To study the function of *ATG7* and *LMNB1* genes specifically in GC B-cells, *Cy1*-cre gene knock-out mouse models were used (C57BL/6 *Cy1*-cre-*ATG7* and C57BL/6 *Cy1*-cre-*LMNB1* respectively). In these models, the expression of the Cre recombinase enzyme is induced by transcription of the Ig γ 1 constant region gene segment (*Cy1*) (Casola et al., 2006a). Cre recombinase is an enzyme that is used to carry out modifications within the DNA at specific 34-bp sites known as loxP sites, such as deletions, insertions,

translocations and inversions. This allows the DNA modification to be targeted to a specific cell type or be triggered by specific external stimuli. In this study, ATG7 and LMNB1 genes are repressed in two separate mouse models specifically, by flanking of each gene by two loxP sites in their respective mouse models. Upon activation of Cy1 specifically in GC B-cells, Cre recombinase is expressed and causes DNA modifications at the two loxP sites that flank either the ATG7 gene or the LMNB1 gene depending on the mouse model of interest. By using Cy1-cre-based mouse models, this allows for the timed activation of Cre recombinase in a small fraction of germinal centre B-cells.

Cre-mediated recombination in Cy1-cre-based mouse models has been shown to occur in GC B-cells as early as 4 days after immunisation and involve greater than 85% of GC B-cells (Casola et al., 2006a). In addition, GC B-cells have been detected 4 to 26 days post-immunisation (Shinall et al., 2000). Therefore, for ATG7 and LMNB1 knock-out mouse models, animals were culled 8 days post-immunisation to both allow for the Cy1-cre-based gene knockout mechanism to be activated and for GCs to form. Conversely, the ADLD mouse model and wild-type animals, which do not contain the Cy1-cre mechanism, were culled at 4 days post-immunisation to evaluate the change in lamin B1 protein expression at the earliest timepoint available. Wild-type animals culled at 4 days post-immunisation were used for comparisons in all experiments as GC B-cells are present in high abundance at both time points (Shinall et al., 2000). This also reduced the number of animals needed in these experiments and reduce the number of experiments needed for this type of investigation into nuclear lamins in animal models. After culling of the animals, GC cells were separated from the mouse lymph nodes using MACS separation, as described in Section 5.1.2, and both the PNA- and PNA+ cell fractions were analysed by LC-MRM-MS to investigate the protein expression of different nuclear lamin isoforms.

5.2. Aims and Objectives

This aim of this chapter is to investigate the protein expression of lamin A/C, lamin B1 and lamin B2 in PNA- and PNA+ cells isolated from mouse models, including wild type mice, a mouse model of ADLD, a mouse model of *ATG7* conditional knock-out and a mouse model of *LMNB1* conditional knock-out. Investigation into nuclear lamin protein expression will be achieved by using the targeted LC-MRM-MS method that was developed and optimised in Chapter 3.

The chapter aims will be achieved through the following objectives:

1. Investigate the expression of lamin A/C, lamin B1 and lamin B2 in PNA- and PNA+ splenocytes isolated from wild-type mice using the targeted LC-MRM-MS method.
2. Investigate the expression of lamin A/C, lamin B1 and lamin B2 in PNA- and PNA+ splenocytes isolated from a mouse model of *ATG7* conditional knock-out via LC-MRM-MS analysis and compare these results to the expression of nuclear lamins in the wild-type animals.
3. Investigate the expression of lamin A/C, lamin B1 and lamin B2 in PNA- and PNA+ splenocytes isolated from a mouse model of *LMNB1* conditional knock-out via LC-MRM-MS analysis and compare these results to the expression of nuclear lamins in wild-type animals.
4. Investigate the expression of lamin A/C, lamin B1 and lamin B2 in PNA- and PNA+ splenocytes isolated from an ADLD mouse model via LC-MRM-MS analysis and compare these results to the expression of nuclear lamins in the wild-type animals.

5.3. Materials and Methods

5.3.1. Reagents

Details of the reagents used are outlined in Chapter 4 in Section 4.3.1.

5.3.2. Sample Preparation

Mouse samples were prepared at Barts Cancer Institute, Queen Mary University of London by Dr Marta Crespi-Sallan and Dr Filip Filipisky and were kindly donated for this study. C57BL/6 wild type (WT) mice and ADLD mice described previously (Heng et al., 2013) were used for this study. In brief, B-cell activation was induced in the mice by a single intraperitoneal (IP) injection with 0.5 mg ovalbumin (OVA) and 10 ng lipopolysaccharide (LPS) and monitored for 4 days before euthanasia. In C57BL/6 Cy1-cre-*ATG7* mice (*ATG7*-KD) and C57BL/6 Cy1-cre-*LMNB1* (*LMNB1*-KD) mouse models, B-cell activation was induced by a single IP injection with 100 µg of 4-Hydroxy-3-nitrophenyl)acetyl-conjugated chicken gamma globulin (NP-CGG(16)) with Imject Alum adjuvant (Thermo Fisher Scientific) and monitored for 8 days before euthanasia. At the experimental end date, mice were culled, and spleens were collected from each animal. Each spleen was cut in half, and one half was disaggregated into a single cell suspension. Cells were counted to determine the total cell count. Where possible, MACS was performed to determine the number of PNA⁺ and PNA⁻ cells in each sample and further live-dead staining was performed on MACS-separated cells to determine cell viability in each sample by either via propidium iodide (PI) or 7-aminoactinomycin D (7-AAD) staining. Cells were pelleted and transferred from the Barts Cancer Institute to Sheffield Hallam University on dry ice and stored at -80°C. Information on each animal can be found in Appendix VI Table 8-XI.

5.3.3. Cell Lysis

Whole cell lysates were prepared by resuspension of the cellular pellet in lysis buffer (0.5 M triethylammonium bicarbonate (TEAB), 0.05% sodium dodecyl sulphate (SDS), 1x protease inhibitor cocktail (PIC)) with manual shearing through a 25 G needle ten

times. Samples were sonicated by use of a probe sonicator at 40% power for a total of 30 seconds, with a 15-second incubation on ice after the first 15 seconds of sonication, or by use of a sonicating water bath where samples were sonicated for 30 seconds and then incubated on ice for 30 seconds, and this process was repeated ten times. Samples were centrifuged at 13,000 rpm and the supernatant containing the protein lysate was transferred to a fresh Eppendorf tube.

5.3.4. Protein Quantification

Protein quantification was performed by using the Qubit Protein Assay Kit (Thermo Fisher Scientific UK Ltd, Loughborough, UK) using a Qubit 4 fluorometer, as per the manufacturer's instructions.

5.3.5. In-Gel Digestion

The amount of whole cell lysate loaded onto each SDS-PAGE gel was normalised for PNA+ and PNA- cell fractions from the same mouse, but this amount varied between animals. The amount loaded for each animal was based on loading the entire whole cell lysate for the PNA+ cell sample and loading the same amount of protein lysate from the PNA- cell sample.

Protein lysates were prepared by mixing with 4x LDS loading buffer and 2.5% β -mercaptoethanol. Samples were heated at 95°C for 10 minutes before loading onto a 4-12% Bis-Tris polyacrylamide gel along with a 11-245 kDa protein ladder (New England Biolabs, Herts, UK). A NuPAGE 1x MES SDS running buffer (Thermo Fisher Scientific UK Ltd, Loughborough, UK) was added to the electrophoresis tank and protein separation was conducted at 100 V until the dye front reached the end of the gel. After electrophoresis, the apparatus was disassembled, and the SDS-PAGE gels were washed in MilliQ 18m Ω ultrapure water three times for 5 minutes per wash. Gels were then stained with a Coomassie-based stain (Imperial Protein Stain) (Thermo Fisher Scientific UK Ltd, Loughborough, UK) for 1 hour at room temperature with gentle rocking. The gels were washed in MilliQ 18m Ω ultrapure water for a total of 1 hour, with water

changes every 15 minutes. Gels were stored at 4°C overnight. The next day, the protein bands of interest were excised from the gels in one gel piece using a scalpel. Gel pieces were transferred to Protein Lo-Bind Eppendorf tubes and de-stained by washing twice in de-staining solution (25 mM ammonium bicarbonate (ABC) in 50% ACN) at 37°C for 30 minutes per wash, with shaking at 600 rpm. Samples were reduced through incubation in 3 mM 1,4-dithiotritol (DTT) (Merck Life Sciences UK Limited, Gillingham, UK) at 60°C for 10 minutes. The DTT solution was removed, and alkylation of proteins was performed by addition of 2.6 µM iodoacetamide (IAA) (Merck Life Sciences UK Limited, Gillingham, UK). Samples were incubated at room temperature in the dark for 1 hour. The IAA solution was removed, and the samples were then washed twice in de-staining solution. 100% ACN was added to gel pieces and left to incubate for 15 minutes at room temperature before allowing samples to air-dry for 10 minutes.

Samples were trypsinised in 100 µL of 20 ng/µL Sequence Grade Modified Trypsin (Promega, Hampshire, UK) prepared in 100 mM HEPES and incubated at room temperature for 15 minutes. 200 µL more of the 100 mM HEPES buffer was added to the digestion mixture and samples were left to incubate overnight at 30°C with shaking at 600 rpm. The digested sample solutions were transferred to a separate Lo-Bind Protein Eppendorf and 1% trifluoroacetic acid (TFA) was added to the gel pieces. After a 5-minute incubation, any remaining sample mixed in TFA was combined with the digestion sample solution and stored at -80°C before desalting. Samples were desalted using 200 µL C18 + Carbon TopTips (Glygen, Maryland, USA) as per the manufacturer's instructions and eluted in 70% ACN containing 0.1% TFA. Samples were lyophilised by vacuum centrifugation before resuspension in 25 µL of 2% ACN with 0.1% formic acid (FA) and transferred to low-volume LC-MS vials (Waters Corporation, Wilmslow, UK).

5.3.6. Liquid Chromatography (LC)

Chromatographic separation was performed as detailed in Chapter 4 in Section 4.3.6.

5.3.7. Multiple Reaction Monitoring-based Mass Spectrometry (MRM-MS/MS)

MRM-MS/MS was performed as outlined in Chapter 4 in Section 4.3.7.

5.3.8. Data Analysis

Data analysis was performed as outlined in Chapter 4 in Section 4.3.8. Bar charts were created using Graphpad Prism version 9.2 or version 10.0 software.

5.4. Results and Discussion

5.4.1. Nuclear lamin Expression in Wild-Type Animals

Although samples were received from three wild-type mice, analysis of both PNA+ and PNA- cell fractions in all three animals (A121, FL06 and FL08) showed that there were limited chromatographic results with a S:N of greater than 10:1 for transitions measuring mouse lamin A/C, lamin B1 or lamin B2 peptide sequences. When analysing this dataset of six samples (PNA- and PNA+ samples from three animals), a S:N of greater than 3:1 was detected for only two transitions of peptides unique to lamin A/C (data not shown). When analysing PNA- and PNA+ samples derived from two wild-type animals (A121 and FL08) as a dataset, three transitions used to measure lamin B1 peptide expression achieved an S:N of greater than 10:1 in the PNA+ and PNA- samples for both animals. Despite this, the results differed for each animal within the same group indicating variances in expression levels. Examining the expression of lamin B1 in one animal showed a greater expression of lamin B1 in PNA- cells compared to the PNA+ fraction (A121), whereas samples from the second mouse in the same sample group showed opposing results, with a greater expression of lamin B1 in PNA+ cells compared to PNA- cells (FL08). Together this data indicated that the protein expression of nuclear lamin isoforms in PNA- and PNA+ samples from wild-type animals displayed biological variance, and some samples displayed expression levels below the level of quantification for this type of assay.

When analysing PNA+ and PNA- fractions from a single wild-type mouse with the greatest overall signal intensities for the measurement of transitions of nuclear lamin peptides (A121), only one transition measuring one unique lamin A/C peptide gave a S:N of greater than 10:1 in both fractions. This meant that lamin A/C was not quantifiable in the samples as two or more transitions are necessary to assess the CV% of peptides and confirm the presence of this protein isoform. In addition, no transitions of peptides unique to lamin B2 gave a S:N of greater than 10:1 in PNA- and PNA+ fractions from this animal, therefore lamin B2 was not quantifiable in these samples. However, lamin B2 was measured above the limit of detection, as three transitions

used to measure unique lamin B2 peptides gave a S:N of greater than 3:1 and a CV of less than 20% when comparing peak area ratios between transitions (data not shown). The results suggested that lamin B2 is present in both PNA- and PNA+ fractions in wild-type mice but not within a detectable range for this assay.

With respect to lamin B1 expression, several transitions for lamin B1 peptides gave an S:N of greater than 10:1 in the A121 PNA+ and PNA- samples and a CV of less than 20%, indicating that this protein could be quantified confidently in this animal. Figure 5-1 below shows the expression of lamin B1 protein in PNA+ and PNA- cells from the A121 wild-type mouse.

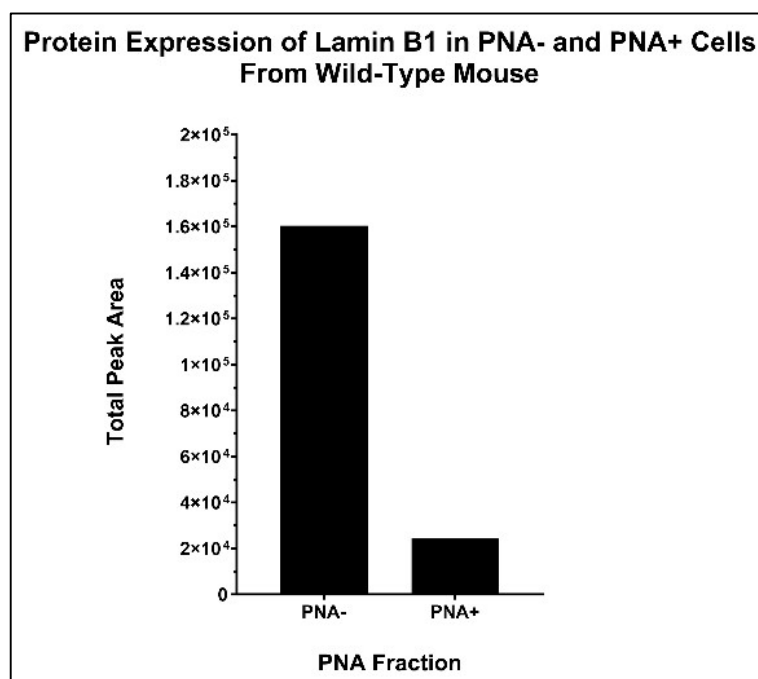


Figure 5-1: Expression of lamin B1 protein in PNA- and PNA+ cells isolated from wild-type mouse (n=1). Expression of lamin B1 calculated by using the sum of peak area measurements of lamin B1 peptide transitions resulting from LC-MRM-MS.

The results shown in Figure 5-1 suggest that the expression of lamin B1 is downregulated in PNA+ cells compared to PNA- cells in the wild-type animal. This

agrees with previous literature in this field showing that lamin B1 is downregulated at a transcriptional and translational level in activated B-cells compared to inactive B-cells (Klymenko et al., 2018). No statistical analysis was performed on this data due to a low number of biological replicates analysed.

5.4.1.1. Summary

To summarise, the preliminary data shown in Figure 5-1 suggests that lamin B1 is downregulated upon B-cell activation. This finding correlates with previously published data in this field (Klymenko et al., 2018). Klymenko and colleagues suggest that lamin B1 downregulation correlates with the initiation of SHM in B-cells, and that this decrease in lamin B1 is transiently seen in highly proliferative B-cells undergoing affinity maturation. The group commented on potential associations between low lamin B1 levels and activation of genes involved in SHM and suggested that decreases in lamin B1 may lead to transcriptional activation of genes involved in SHM which may be present within specific lamina-associated domains. By decreasing lamin B1 expression during B cell activation, genes within LADs, which are usually repressed due to binding of lamin B1 to the genome, are transcriptionally active when lamin B1 is downregulated, leading to the expression of these genes. Considering this, it has not yet been confirmed if genes relating to SHM are found within these domains.

Although no results could be obtained regarding the expression of lamin A/C in PNA+ and PNA- cells from wild-type animals, studies report that lamin A/C levels have been shown to finely regulate the immune system, with macrophages being shown to increase their lamin A/C content upon differentiation and activation while neutrophils downregulate their expression of lamin A/C to enable them to pass through narrow spaces (Saez et al., 2020). In the previous literature, it was reported that lamin A/C expression was upregulated in activated B-cells (Caruso et al., 2022; Contu et al., 2018). The reason for this may be due to lamin A/C expression increasing during differentiation, as noted in several different cell types (Constantinescu et al., 2006). As mentioned in Section 1.4.1, a high lamin A/C expression may be detrimental to cells

which require a higher migration capacity and long-term survival, such as activated B-cells, memory B-cells and plasmablasts, as high lamin A/C expression is suggested to increase nuclear stiffness and slow cellular migration (Harada et al., 2014; Shin et al., 2013; Swift et al., 2013). Thiam and colleagues suggest a mechanism by which dendritic cells possess both high migration capacity and long-term survival capabilities by generating a dense and dynamic perinuclear actin network, and changes in actin nucleation allowed for nuclear deformation and cell migration without decreasing the expression of lamin A/C (Thiam et al., 2016). These researchers suggest that this mechanism may also extend to other immune cells, and we cannot deny this is a possibility in B-cells. In fact, recent studies have suggested that the high lamin A/C expression seen in long-lived macrophages and dendritic cells may protect migrating cells from mechanical stress (Harada et al., 2014).

Conversely, it would be reasonable to suggest that activated B-cells may downregulate their expression of lamin A/C to allow them to migrate between the dark and light zones of the GC during differentiation and affinity maturation (Section 1.3.1) and decreasing the expression of lamin A/C may be beneficial in their movement throughout the GCs. Furthermore, plasma cells and memory B-cells exit the GCs after achieving terminal differentiation and therefore a low lamin A/C expression may be useful in migration out of the GCs.

Regarding lamin B2, studies have shown that *LMNB2* expression decreases in mice after birth (Han et al., 2020) and therefore may be related to cell differentiation. Shin and colleagues showed that lamin B2 was undetectable in most haematopoietic cell types via Western Blot analysis (Shin et al., 2013). In addition, previous mass spectrometry analysis has shown that lamin B2 is expressed at much lower levels than lamin B1 in haematopoietic cells (Shin et al., 2013) and therefore the results suggesting that lamin B2 is present but not quantifiable supports this finding. It has been suggested that lamin B2 is required for cell cycle progression and proliferation (Han et al., 2020) and ensuring proper spindle assembly during mitosis (Kuga et al., 2014).

Therefore, lamin B2 may be important during the highly proliferative stages of B-cell maturation after successful affinity maturation and during differentiation into plasma cells or memory B-cells. As this study could not provide conclusive results on the expression of lamin B2 in PNA- and PNA+ cells, the question of changes in lamin B2 expression upon B-cell activation remain unanswered.

5.4.2. Nuclear lamin expression in an ATG7-KD mouse model

With respect to the relationship between autophagy and nuclear lamin expression, lamin B1 expression was shown to be downregulated in B-cells upon activation (Klymenko et al., 2018) and since autophagy is a mechanism of lamin B1 nuclear disassembly (Dou et al., 2015a, 2015b; Lenain et al., 2015), this section aimed to assess if autophagy is partially responsible as a mechanism for lamin B1 downregulation in activated B-cells. For this analysis, one *ATG7* knock-down animal and one wild-type animal was used (A121). Due to the limited availability of samples, these two animals were immunised for different time periods however, it was assumed that this difference was negligible based on previous literature showing GC B-cells are present from 4 to 26 days post-immunisation (Shinall et al., 2000). No statistical analysis was performed, and no conclusive results can be reported due to the low number animals available.

To examine the expression of nuclear lamin isoforms in *ATG7* knock-down mouse models upon B-cell activation, PNA+ and PNA- cells were analysed by LC-MRM-MS. Only one transition for one peptide unique to lamin A/C was above the limit of quantification (S:N of greater than 10:1). Using only one transition to determine the relative quantitation of a protein isoform is unsuitable, and using two or more transitions enhances the specificity and selectivity of the target peptide and is considered more reliable for quantitation via MRM.

With respect to lamin B1, the previous literature and the results shown in Figure 5-1 suggest that lamin B1 is downregulated in PNA+ GC cells compared to PNA- cells in wild-type animals. If lamin B1 protein expression is not affected by the expression of *ATG7*, lamin B1 would also be downregulated in *ATG7*-knock down PNA GC + cells compared to PNA- cells. Multiple transitions of lamin B1 peptides were quantifiable within the PNA- fraction, whereas these were not even within detectable range in the PNA+ fraction. This suggests that, since the same amount of total protein was loaded in each fraction, there was more lamin B1 in the PNA- fraction than in the PNA+ fraction.

Therefore, it may be speculated that lamin B1 is downregulated in PNA+ GC cells compared to PNA- cells in *ATG7* knock-down mouse models. Considering this, for the expression of lamin B1 to be examined accurately, transitions need to be measured at quantifiable levels in both fractions, which was not possible in this experiment.

Using an S:N of greater than 3:1 to determine if each nuclear lamin isoform can be detected in each sample, four lamin A/C and four lamin B1 transitions monitored gave an S:N of greater than 3:1. The data suggests that both lamin A/C and lamin B1 are present in both PNA+ and PNA- cells in *ATG7*-KD mouse models, however lamin B2 peptide transitions were not observed at detectable levels in these samples using this MRM-based LC-MS/MS assay. This suggests that this assay may not be sensitive enough to assess the protein expression of nuclear lamin isoforms in PNA-separated GC samples. Furthermore, increasing the amount of protein analysed for each sample may be necessary to identify all nuclear lamin isoforms at detectable and measurable levels using this method. This is difficult due to the small size of the mice spleens used. Alternatively, the absence of lamin B2 transitions detected by LC-MRM-MS/MS may also suggest that lamin B2 is not expressed in these cells. Further experimentation via more sensitive methods may be able to determine if lamin B2 is present or absent in these sample types.

Next, the PNA- fractions from one wild-type and one *ATG7* knock-down mouse were analysed using the optimised LC-MRM-MS/MS method. Figure 5-2 below shows the results of this analysis.

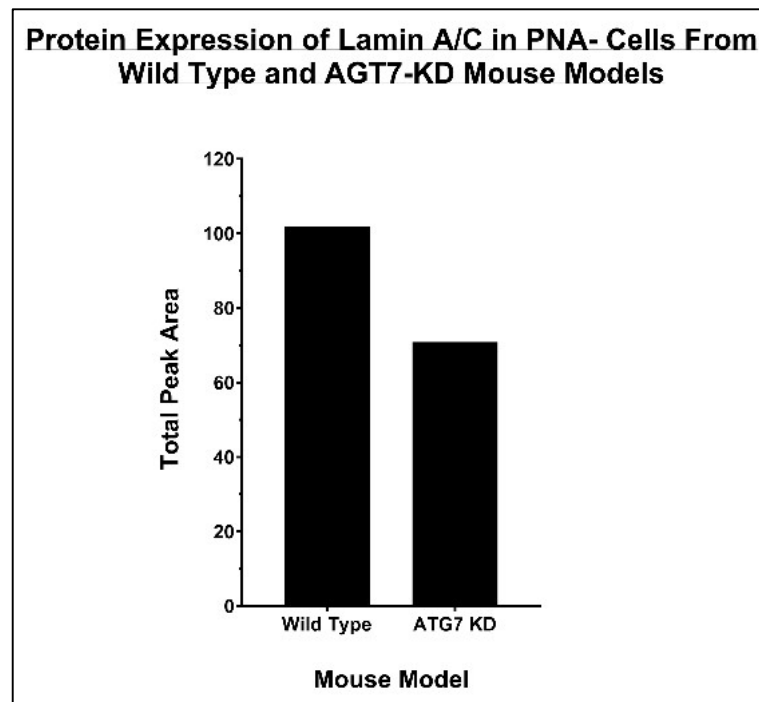


Figure 5-2: Expression of lamin A/C protein in PNA- cells isolated from wild-type and *ATG7*-KD mouse models (n=1). Expression of lamin A/C measured by calculating total peak area of lamin A/C peptide transitions by LC-MRM-MS, normalised by total lysate loaded (per μg of protein).

Figure 5-2 suggests that lamin A/C expression was decreased in PNA- cells isolated from the *ATG7* knock-down mouse compared to PNA- cells from the wild-type mouse. This result was not expected as the $\text{C}\gamma 1$ -cre mouse model system should only allow knock-out of *ATG7* in cells expressing the Ig $\gamma 1$ constant region gene segment, which only occurs in activated B-cells within the GCs, which are enriched within the PNA+ fraction. Therefore, PNA- cells should not be affected by *ATG7* gene knockout in this model.

Next, the protein expression of lamin B1 was investigated. Figure 5-3 below shows the expression of lamin B1 in PNA- cells isolated from wild-type and *ATG7* knock-down mice.

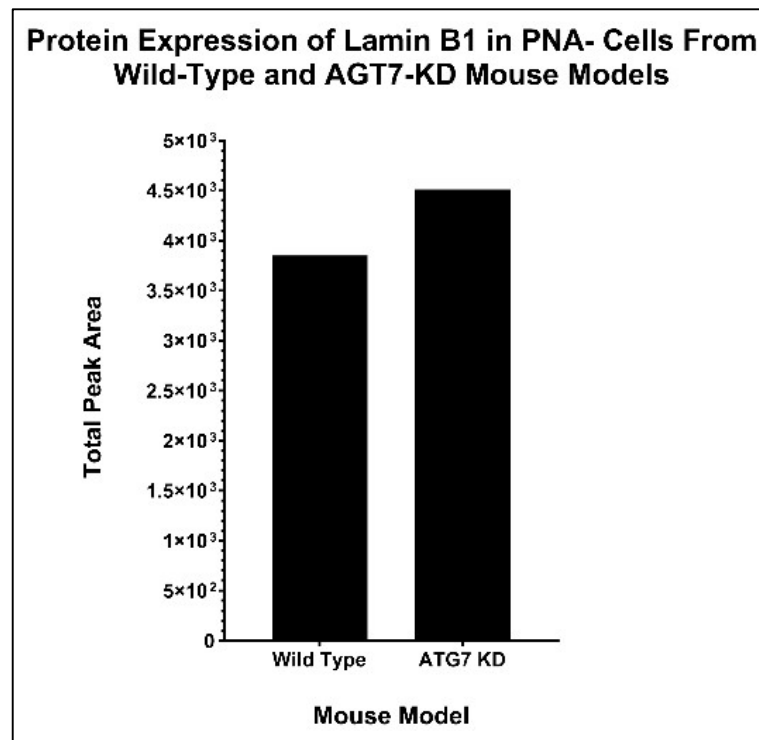


Figure 5-3: Expression of lamin B1 protein in PNA- cells isolated from wild-type and *ATG7*-KD mouse models (n=1). Expression of lamin B1 measured by calculating total peak area of lamin B1 peptide transitions by LC-MRM-MS, normalised by total lysate loaded (per μg of protein).

The preliminary results in Figure 5-3 above suggest that lamin B1 expression is slightly higher in *ATG7* knock-down PNA- cells compared to PNA- cells from the wild-type animal, when the data was normalised by total protein load. As with the results obtained in Figure 5-2 examining the expression of lamin A/C in these two samples, this result was not expected. The expected result would be that lamin B1 has the same level of expression in PNA- cells isolated from the wild-type animal and from the *ATG7*-KD animal, since no *ATG7* knock-down should occur in PNA- cells as they should lack *Cy1*-cre expression. As these results are preliminary, no conclusive statements can be made on the expression of lamin B1 in these sample types.

Finally, the protein expression of lamin B2 was also investigated in PNA- cells from one wild-type mouse and one ATG7 knock-down mouse. The results are shown below in Figure 5-4.

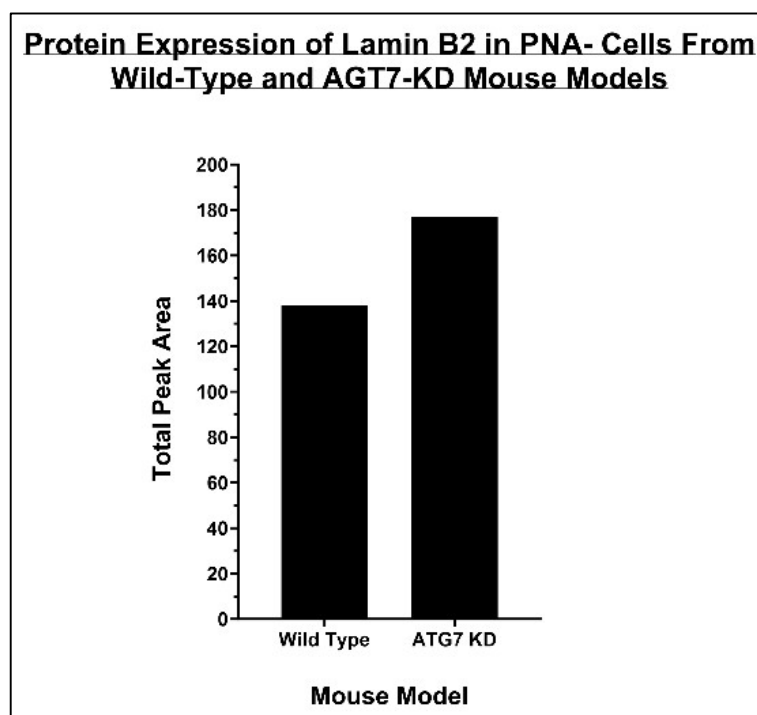


Figure 5-4: Expression of lamin B2 protein in PNA- cells isolated from wild-type and *ATG7*-KD mouse models (n=1). Expression of lamin B2 measured by calculating total peak area of lamin B2 peptide transitions by LC-MRM-MS, normalised by total lysate loaded (per μg of protein).

Preliminary results shown in Figure 5-4 above suggests that lamin B2 expression shows a similar pattern to lamin B1 expression when comparing these two samples, with a slightly higher protein expression observed in the *ATG7* knock-down PNA- cells compared to wild-type PNA- cells, when normalised by total protein load. The expected result was that PNA- cells isolated from the WT animal would have a similar expression of lamin B2 than PNA- cells isolated from the *ATG7*-KD animal. As was explained previously when considering the results of lamin B1 expression in these samples, this result of increased lamin B2 expression in *ATG7*-KD PNA- cells was not expected due to how the Cy1-cre mouse model system works.

When considering the PNA+ GC fractions, unfortunately, no transitions measuring any unique peptides of any nuclear lamin isoforms could be accurately quantified in either of the PNA+ GC samples (wild-type mouse or *ATG7* knock-down mouse). Despite this, lamin A/C and lamin B1, but not lamin B2, were successfully detected, as evidenced by a S:N of greater than 3:1 in more than one transition for peptides unique to each protein isoform. This suggests these proteins are present within the cells, but below the limit of quantitation for this assay. The aim of this section was to assess if macroautophagy is a mechanism which is at least partially responsible for lamin B1 removal in GCs in secondary lymph nodes. This was meant to be achieved by blocking the macroautophagy process via knock-down of *ATG7* and investigating if lamin B1 levels increase in PNA+ activated B-cells within GCs compared to wild-type PNA+ GC cells, however due to assay sensitivity limits and sample concentration limits, this aim could not be reliably addressed, as lamin B1 expression was not quantifiable in PNA+ cells from the *ATG7*-KD animal.

5.4.2.1. Summary

To summarise, lamin A/C expression decreases in *ATG7*-KD PNA- cells compared to PNA- cells from the wild-type animal (Figure 5-2). One hypothesis behind the lower expression of lamin A/C in the *ATG7*-KD PNA- cells is that it may be due to a greater number of apoptotic cells within this sample compared to the wild-type PNA- sample. Lamin A/C are known to be cleaved by caspases during apoptosis, and the detection of fragments of lamin A are commonly used as a marker of apoptosis (Buendia et al., 1999; Kihlmark et al., 2001). Inspection of the details provided for each animal in Appendix VI Table 8-XI suggest that apoptosis does not seem to be the cause of the lower lamin A/C expression in *ATG7*-KD PNA- sample, as this sample contained a greater percentage of viable cells (88.8%) while the PNA- cell sample from the wild-type (A121) animal were contained 81.7% viable cells.

The expression of lamin B1 and lamin B2 in WT and *ATG7*-KD PNA- cells shows a similar pattern of expression, as both nuclear lamin isoforms seem to be upregulated in the

ATG7-KD PNA⁻ cells (Figure 5-3 and Figure 5-4). The expected result was that PNA⁻ cell samples isolated from *ATG7*-KD and wild-type mouse models would have the same *ATG7* expression and therefore the same expression of all nuclear lamin isoforms as the *Cy1*-cre model of expression should only allow knock-out of *ATG7* in cells expressing the Ig γ 1 constant region gene segment. Since only B-cells transcribe the Ig γ 1 constant region gene segment, this may imply that either the MACS separation of PNA⁻ and PNA⁺ cells was somewhat inefficient, or there may be potential issues with the efficiency of the Cre-lox system used in these mouse models, or this may simply a result of biological variation between animals. Since these results are from one animal in each subject group (WT and *ATG7*-KD), the findings should be considered preliminary and multiple biological replicates in each subject group are required to confirm the results shown above.

The most important biological findings of this section would be achieved through studying the expression of nuclear lamin isoforms in PNA⁺ cells isolated from the *ATG7*-KD mouse model and comparing these to the PNA⁺ cells from WT animals. Unfortunately, the nuclear lamin isoforms measured in this study by LC-MRM-MS were not within a quantifiable range to be confidently assessed in these samples. Despite this, it was possible to measure both lamin A/C and lamin B1 within a detectable range in these samples, indicating that both isoforms are present.

For the expression of nuclear lamin protein expression to be confidently assessed in PNA⁻ and PNA⁺ cells from mouse models, several samples from multiple animals could be pooled together to create representative samples of PNA⁺ and PNA⁻ cell fractions however, this is not advised due to biological variabilities between samples, and the number of animals needed for this type of analysis may be significant. The limitation of using mouse samples when investigating GC responses is the low number of PNA⁺ GC cells available from each animal.

5.4.3. Nuclear lamin expression in a LMNB1-KD mouse model

Next, the protein expression of nuclear lamins was examined in PNA- and PNA+ cells from *LMNB1*-KD mouse models. Similarly to the *ATG7*-KD mouse model, there was not an identically matched wild-type mouse that has been immunised in the same way as the *LMNB1* knock-down animal. The *LMNB1*-knock-down mouse model was immunised in the same way as the *ATG7* knock-down mouse model (8 days of NP-Alum immunisation) and the results were compared against the same wild-type mouse as the data for the *ATG7*-KD mouse (A121). In addition, similarly to the analysis conducted for the *ATG7*-KD mouse model, only one *LMNB1*-KD mouse and one WT mouse were used for this study (LCRE39 and A121 respectively). Due to this limited availability of biological replicate samples, no statistical analysis was performed on this data, and no conclusive results can be reported.

Firstly, to examine the expression of nuclear lamin isoforms in *LMNB1*-KD mouse models upon B-cell activation, PNA+ and PNA- cells were analysed by LC-MRM-MS/MS. No nuclear lamin isoforms were measured above the limit of detection (S:N greater than 3:1) when analysed by LC-MRM-MS, and no transitions for any nuclear lamin peptides were measured with a S:N of greater than 10:1 in either sample. When investigating the detection of each nuclear lamin isoform in these samples, two transitions used to measure lamin A/C peptides gave an S:N of greater than 3:1. In addition, lamin B1 was detectable in both PNA+ and PNA- cells, with four transitions measured at a S:N of greater than 3:1. Lamin B2 was undetectable in these samples using the targeted LC-MRM-MS assay, as no transitions were measured by LC-MRM-MS with a S:N of greater than 3:1, which suggests lamin B2 either may not be present within these samples, or the protein expression was below the limit of detection for this assay.

When investigating the expression of nuclear lamin isoforms in PNA- cells in the WT animal and *LMNB1*-KD mouse model, only one transition used to measure the expression of lamin A/C resulted in a S:N of greater than 10:1 in PNA- samples from

both animals. This was deemed unsuitable for confident reporting of the expression of lamin A/C in these samples, and two or more transitions should be reported with an S:N of greater than 10:1 for this analysis to be possible.

Conversely, the protein expression of lamin B1 was quantified in these samples using several transitions in which the peak area recorded gave an S:N of greater than 10:1, and all transitions chosen for quantitation analysis gave a peak area ratio CV of less than 20%. Figure 5-5 below shows the results of lamin B1 protein expression analysis in PNA- cells isolated from WT and *LMNB1*-KD mouse models.

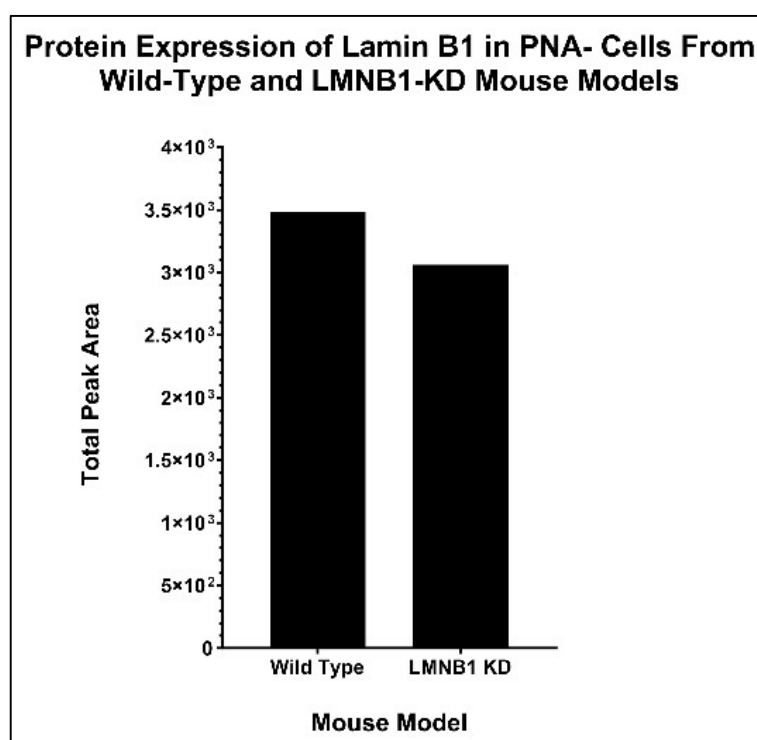


Figure 5-5: Expression of lamin B1 protein in PNA- cells isolated from wild-type and *LMNB1*-KD mouse models (n=1). Expression of lamin B1 measured by calculating total peak area of lamin B1 peptide transitions by LC-MRM-MS, normalised by total lysate loaded (per μg of protein).

The results in Figure 5-5 may suggest that the protein expression of lamin B1 was lower in PNA- cells isolated from the *LMNB1*-KD mouse compared to PNA- cells isolated from the WT animal. Similarly to the results obtained for *ATG7*-KD mouse models, the *Cy1*-cre model of gene knock-out in the *LMNB1*-KD mouse model should only occur in activated B-cells due to the selective expression Cre recombinase expression under the *Cy1* promoter in B-cells. The expected result when comparing the expression of lamin B1 in PNA- cells isolated from wild-type and *LMNB1*-KD mouse models was that the expression was similar in both samples.

When comparing the PNA+ GC fractions of *LMNB1*-KD mouse models and WT animals, unique peptides for lamin B1 and lamin B2 could not be accurately quantified as no transitions of these peptides were measured at an S:N of greater than 10:1. When examining the expression of lamin A/C in these samples, the two transitions which gave a S:N of greater than 10:1 did not have a peak area ratio CV of less than 20% across both samples analysed and therefore were deemed unsuitable to use for quantitation purposes. When *LMNB1* is conditionally knocked out through activation of B-cells and transcription of *Cy1*, lamin B1 should not be expressed in B-cells within the PNA+ fraction. It would be interesting to see if lamin B1 expression is lower in PNA+ GC cells from the *LMNB1*-KD mouse model compared to the wild-type mouse model, however this was not possible due to the low concentration of protein extracted from the PNA+ GC fraction in the *LMNB1*-KD mouse.

Studies using reciprocal knock-in mice (one set of mice producing lamin B2 from the *Lmnb1* locus (*Lmnb1*^{B2/B2}) and another set of mice producing lamin B1 from the *Lmnb2* locus (*Lmnb2*^{B1/B1})) showed that *Lmnb1*^{B2/B2} mice produced increased amounts of lamin B2 but no lamin B1 and still died shortly after birth due to neuronal layering abnormalities within the cerebral cortex, however these defects were less severe than those seen in *Lmnb1*-knockout mice, suggesting that increases in lamin B2 may lessen the impact of the abnormalities associated with lamin B1 deficiency. Similarly, the *Lmnb2*^{B1/B1} mice showed increased amounts of lamin B1 but neurodevelopmental

defects were still present due to lamin B2 deficiency. It was therefore concluded that lamin B1 and B2 have unique roles in the developing brain and increased production of one B-type lamin does not fully compensate the loss of the other (J. M. Lee et al., 2014). It would be interesting to see if this mechanism of partial compensation of B-type lamins is observed in B-cells, which are thought to have an increased dependency on lamin B1 during B-cell activation (Klymenko et al., 2018).

5.4.3.1. Summary

To summarise the preliminary findings of this section, it was shown that lamin B1 expression was suggested to be lower in *LMNB1*-KD PNA⁻ cells compared to PNA⁻ cells from the WT animal (Figure 5-5). The expression of lamin A/C and lamin B2 in WT and *LMNB1*-KD PNA⁻ cells could not be determined, as there were no transition with an S:N of greater than 10:1 that were measured in both samples. As before for the *ATG7*-KD study, the expected result in this section was that PNA⁻ cell samples isolated from the *LMNB1*-KD and WT mouse models would have the same *LMNB1* expression, as the *Cy1*-cre model of expression should only allow knock-out of *LMNB1* in B-cells which selectively express the Ig γ 1 constant region gene segment and are enriched within the PNA⁺ sample. Since the expression of lamin B1 in these two samples was different, this may suggest inefficient MACS separation of PNA⁻ and PNA⁺ cells, a potential issue with the Cre-lox mouse model, or a result of biological variation, as discussed previously in Section 5.4.2.1. These result in this section were obtained from one animal in each subject group and therefore the findings should be considered preliminary. More biological replicates are necessary for each subject group to confirm the results shown in Figure 5-5.

Unfortunately, as with the *ATG7*-KD mouse model, the most significant biological findings of this section would be achieved through studying the expression of nuclear lamin isoforms in PNA⁺ GC cells isolated from the *LMNB1*-KD mouse model and comparing these to the PNA⁺ GC cells from the WT animal. The nuclear lamin isoforms measured in the study of the *LMNB1*-KD mouse model by LC-MRM-MS were not within

a quantifiable range and could not be confidently assessed in these samples. Despite this, it was still possible to measure both lamin A/C and lamin B1 within a detectable range, suggesting that these isoforms are present within these samples.

5.4.4. Nuclear lamin expression in an ADLD mouse model

When investigating the nuclear lamin protein expression in the A121 WT mouse, the preliminary data in Figure 5-1 and the previous literature suggest that lamin B1 is downregulated in PNA+ cells compared to PNA- cells (Klymenko et al., 2018). To see if this relationship is maintained in ADLD mouse models, PNA- and PNA+ samples isolated from three wild-type animals and three ADLD mouse model animals were analysed by LC-MRM-MS/MS.

When comparing PNA- and PNA+ cells in the three ADLD animals, the peak area of one transition unique to lamin A/C was above the limit of detection but below the limit of quantitation when analysing all three PNA+ and PNA- ADLD mouse samples as a dataset. All other transitions measured were below the limit of detection in at least one sample. Therefore, the samples in which transitions consistently measured below the limit of detection was removed from analysis (A116 PNA+ and A118 PNA+). In addition, one PNA- sample from an ADLD mouse did not inject properly into the LC system as not all the sample was taken up by the needle. Therefore, the amount of protein injected for that sample could not be determined and so the expression of each nuclear lamin isoform in each sample was performed with only two ADLD animals for this experiment.

To accurately quantify lamin B1 in PNA- and PNA+ samples from the ADLD mouse model, two PNA- samples and one PNA+ sample were chosen. Figure 5-6 below shows the expression of lamin B1 as measured by LC-MRM-MS/MS.

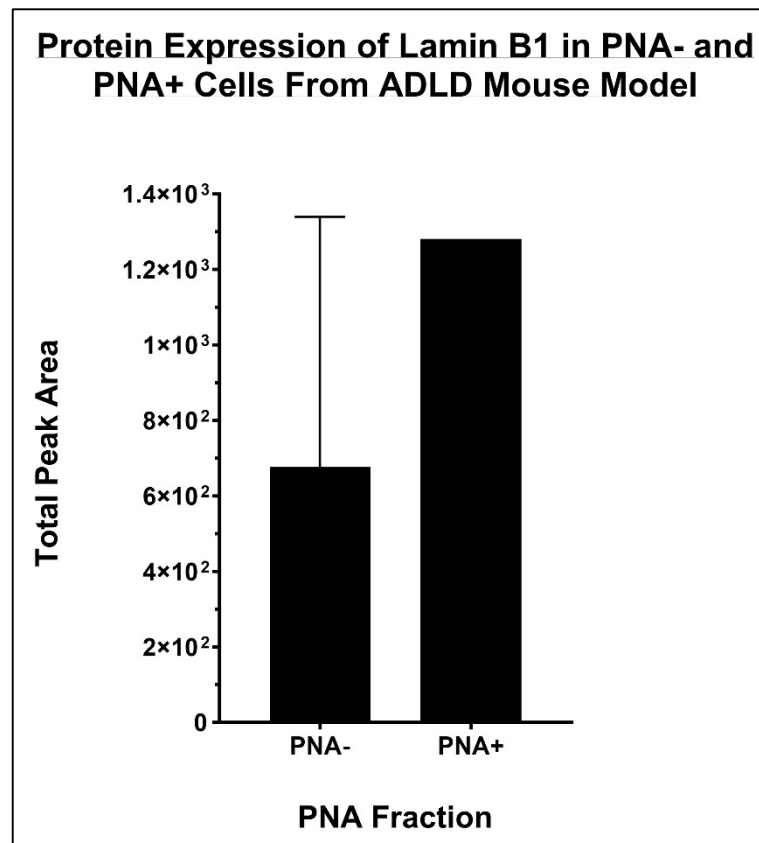


Figure 5-6: Mean expression of lamin B1 protein in PNA- and PNA+ cells isolated from an ADLD mouse model (PNA- n=2, PNA+ n=1). Expression of lamin B1 measured by calculating the total peak area of lamin B1 peptide transitions by LC-MRM-MS and normalising to total protein load. Error bars indicate standard deviation.

Figure 5-6 above shows the expression of lamin B1 in PNA- and PNA+ samples isolated from ADLD mouse models. The results suggest that PNA+ cells contain more lamin B1 than PNA- cells in ADLD mouse models. This result was not expected and does not follow the same expression pattern as that observed in the WT animal, which show a decrease in lamin B1 upon B-cell activation (Figure 5-1). The reason for this could be that lamin B1 is overexpressed in ADLD compared to WT animals, and the downregulation of lamin B1 upon B-cell activation may be disrupted due to the aberrant expression of lamin B1 in ADLD. This experiment was only performed on a small number of animals therefore the results are not conclusive. More biological replicates are necessary to confirm the results of this study.

No transitions measured for unique lamin A/C or lamin B2 peptides were within quantifiable range when analysing both PNA+ and PNA- fractions in three PNA- and one PNA+ sample, and therefore their protein expression in these samples could not be assessed.

The protein expression of nuclear lamins was also investigated in ADLD PNA- samples compared to matched WT samples. Lamin A/C was quantifiable in one of three wild-type mice and one of three ADLD mice by LC-MRM-MS/MS. Figure 5-7 below shows the expression of lamin A/C in PNA- cells isolated from one WT animal and one ADLD mouse.

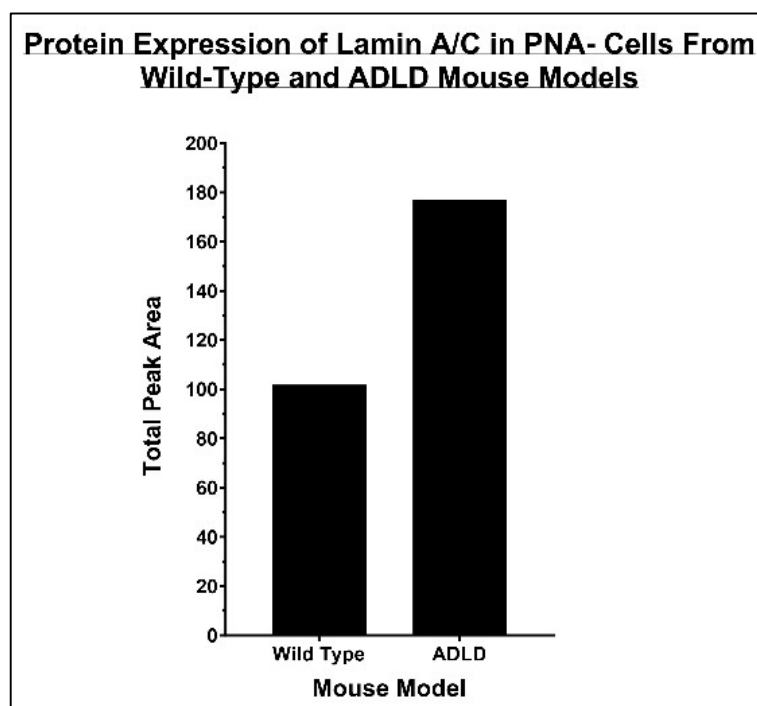


Figure 5-7: Expression of lamin A/C protein in PNA- cells isolated from wild-type and ADLD mouse models (n=1). Expression of lamin A/C measured by calculating total peak area of lamin A/C peptide transitions by LC-MRM-MS, normalised by total lysate loaded (per μg of protein).

The preliminary results in Figure 5-7 suggest that lamin A/C expression was greater in PNA- cells from the ADLD mouse model compared to PNA- cells isolated from the WT mouse. The previous literature suggests that lamin A/C expression does not change in ADLD (Nmezi et al., 2020), therefore these results were not expected. As the results only show preliminary analysis of these sample types, no conclusive statements can be made regarding the expression of lamin A/C in ADLD.

For the examination of lamin B1 expression in ADLD, analysis of one PNA- WT mouse sample and one PNA- ADLD sample did not yield any transitions with a peak area S:N greater than 10:1 for transitions related to lamin B1 peptides, so this sample was excluded from the analysis. Figure 5-8 below shows the protein expression of lamin B1 in PNA- cells isolated from wild type and ADLD mouse models.

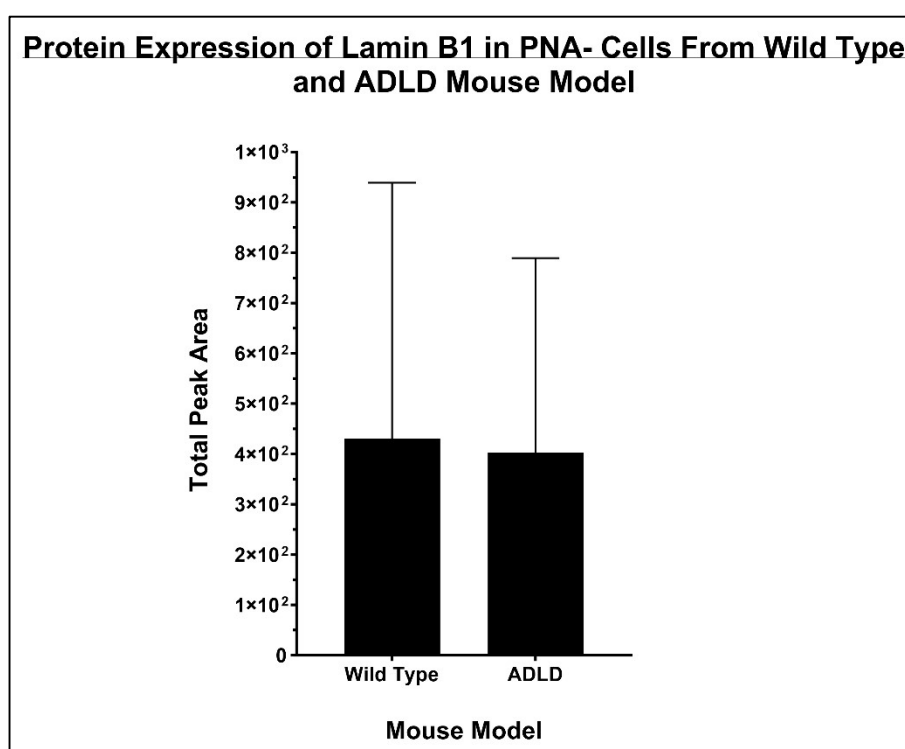


Figure 5-8: Mean expression of lamin B1 protein in PNA- cells isolated from wild type and ADLD mouse models (n=2). Expression of lamin B1 measured by calculating total peak area of lamin B1 peptide transitions by LC-MRM-MS, normalised by total lysate loaded (per µg of protein). Error bars indicate standard deviation.

Figure 5-8 shows the expression of lamin B1 in PNA- cells isolated from WT and ADLD mouse models. The preliminary result of the analysis suggests that the expression of lamin B1 was similar in PNA- cells isolated from both groups. This result was not expected, as previous reports have shown that this ADLD mouse model contains up to a four-times greater expression of lamin B1 (Heng et al., 2013). The reasons behind both mouse models showing similar lamin B1 protein expression could be due to the low number of biological replicates. Further biological replicates are necessary to more comprehensibly compare lamin B1 protein expression in PNA- cells from ADLD mouse models and their WT counterparts.

Finally, the expression of the lamin B2 isoform was examined in these samples by LC-MRM-MS and the results are shown below in Figure 5-9. The lamin B2 isoform could only be quantified in one wild-type mouse and one ADLD mouse model (A121 and A116 respectively).

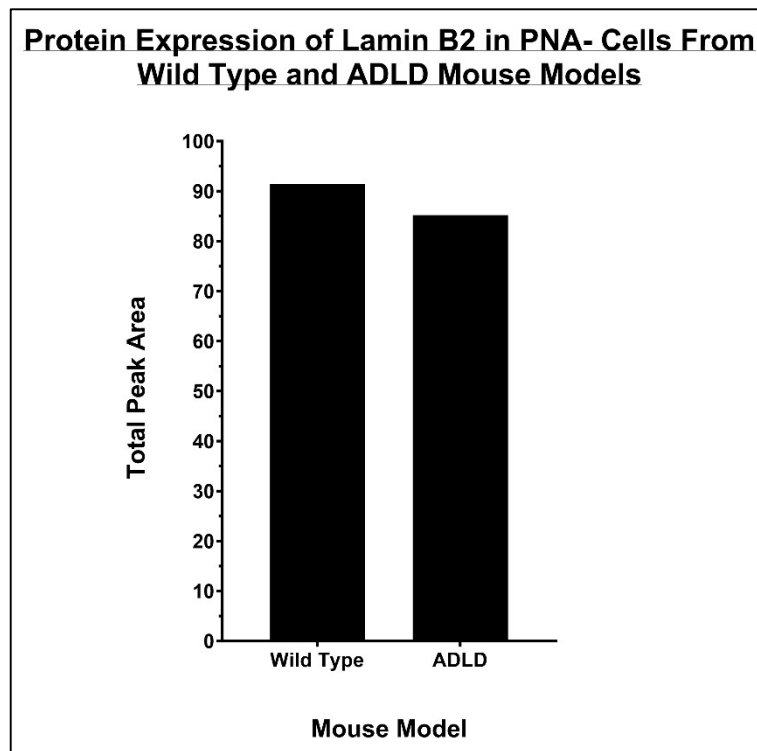


Figure 5-9: Expression of lamin B2 protein in PNA- cells isolated from wild-type and ADLD mouse models (n=1). Expression of lamin B2 measured by calculating total peak area of lamin B2 peptide transitions by LC-MRM-MS, normalised by total lysate loaded (per μg of protein).

The results in Figure 5-9 suggest that there seems to be no difference in the expression of lamin B2 when comparing PNA- cells isolated from the wild-type mouse model and the ADLD mouse model. This suggests that, in ADLD, the expression of lamin B2 is not affected by the increased expression of lamin B1. This result was expected, as ADLD is a disease of overexpressed lamin B1 only, however, previous studies have not investigated the expression of lamin B2 in ADLD.

Finally, a comparison between nuclear lamin isoform expression was conducted in PNA+ fractions from ADLD and WT mice. Figure 5-10 shows the protein expression of lamin A/C in PNA+ fractions isolated from one animal in each biological group, as determined by LC-MRM-MS analysis.

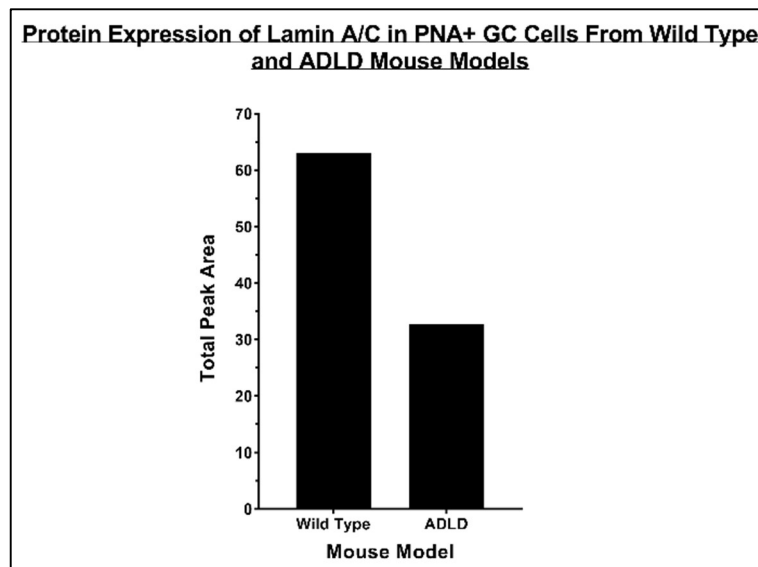


Figure 5-10: Expression of lamin A/C protein in PNA+ GC cells isolated from wild-type and ADLD mouse models (n=1). Expression of lamin A/C measured by calculating total peak area of lamin A/C peptide transitions by LC-MRM-MS, normalised by total lysate loaded (per μg of protein).

The results in Figure 5-10 suggest that lamin A/C is downregulated in the PNA+ fraction isolated from the ADLD mouse model compared to the WT mouse. From what is understood about the role of lamin A/C, the results shown in Figure 5-10 may suggest that activated B-cells from the ADLD mouse model have a less rigid nuclear envelope than those from the WT mouse. Previous studies suggest that lamin A/C does not alter in ADLD when investigating lamin expression in fibroblasts of a DOX-induced *LMNB1* overexpressing mouse model of ADLD (Nmezi et al., 2020), so these findings contradict the current literature in this field.

Next, the protein expression of lamin B1 in PNA+ cells isolated from WT and ADLD mouse models was examined by LC-MRM-MS and the results are shown below in Figure 5-11.

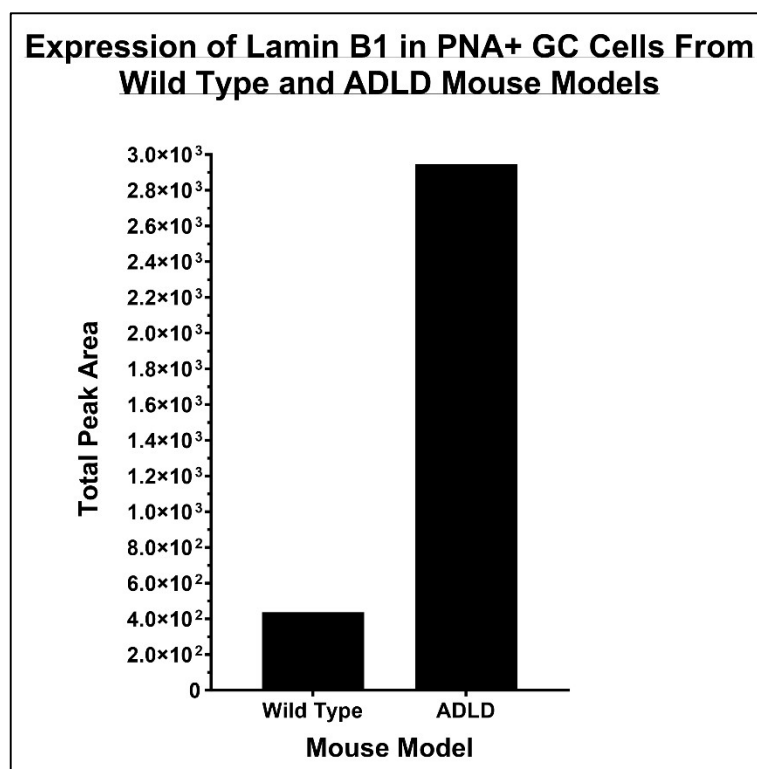


Figure 5-11: Expression of lamin B1 protein in PNA+ GC cells isolated from wild-type and ADLD mouse models (n=1). Expression of lamin B1 measured by calculating total peak area of lamin B1 peptide transitions by LC-MRM-MS, normalised by total lysate loaded (per μg of protein).

The results in Figure 5-11 suggest that the expression of lamin B1 is higher in PNA+ cells isolated from the ADLD mouse model compared to the same fraction isolated from a wild-type mouse. This result was expected as ADLD is caused by the overexpression of lamin B1, and the ADLD mouse model used in this study was shown to produce up to four times the amount of lamin B1 compared to WT animals (Heng et al., 2013).

The lamin B2 protein was not detectable by LC-MRM-MS in any PNA+ samples from ADLD mouse models as no transitions for lamin B2 peptides were detected with a S:N of greater than 3:1, suggesting that lamin B2 is either absent from these cells or below the limit of detection in these samples.

5.4.4.1. Summary

ADLD is a disease relating to the overexpression of lamin B1 in humans. Multiple mouse models have been developed to recapitulate this disease phenotype, and the model used in this study has previously been shown to express up to four times the amount of lamin B1 compared to their wild-type counterparts (Heng et al., 2013).

This study measures the expression of the lamin B1 isoform in PNA- and PNA+ cells isolated from an ADLD mouse model by utilising the LC-MRM-MS assay developed and optimised in Chapter 3. The results suggest that lamin B1 expression was higher in PNA+ cells compared to PNA- cells in ADLD mice (Figure 5-6). This is in contrast to what was observed in the wild-type mouse model where lamin B1 protein expression was lower in activated B-cells compared to other cells found within spleen (Figure 5-1) and may suggest that lamin B1 overexpression in ADLD leads to a greater expression of lamin B1 in activated B-cells where lamin B1 has been theorised to control gene expression in SHM (Klymenko et al., 2018). Thus, a higher expression of lamin B1 in PNA+ cells may lead to aberrant gene expression during B-cell activation. This theory of cell-specific effects of lamin B1 overexpression in ADLD has been previously reported in the literature (Chen et al., 2019; Yang et al., 2011a; Yue et al., 2019).

When comparing the expression of nuclear lamins in PNA- cells from WT animals and the ADLD mouse model, preliminary data suggests that lamin A/C expression is greater in ADLD mouse models (Figure 5-7) while PNA+ cells from ADLD mouse models were suggested to contain a lower expression of lamin A/C compared to the WT counterparts (Figure 5-10). A 2020 study by Nmezi and colleagues suggested that lamin A/C was unaltered in ADLD (Nmezi et al., 2020), which suggests that the results of this chapter oppose the current literature in this area. Furthermore, lamin B1 expression was suggested to be similar in PNA- cells from WT and ADLD animals (Figure 5-8). This does not correlate with what is known about the ADLD model used in this study, since this model was shown to express up to four times the amount of lamin B1 than wild-type animals (Heng et al., 2013). The results shown in Figure 5-8 may be due to

variability within this specific ADLD animal or a genuine phenomenon where lamin B1 protein expression is similar amongst PNA- cells in WT animals and mouse models representative of the human ADLD phenotype. Analysis of more samples would provide a more reliable conclusion to the protein expression of lamin B1 observed in PNA- samples from the ADLD mouse model and could confirm if either of these outcomes may be responsible for the results obtained in this study. Conversely, the results of this chapter suggest the expression of lamin B2 was similar in ADLD and wild type PNA- cells (Figure 5-9). Although there is no literature on the expression of lamin B2 in ADLD, this result was expected as ADLD is thought to only affect the expression of lamin B1 and not other nuclear lamin isoforms.

Finally, the expression of B-type lamins in PNA+ GC cells from ADLD mouse models was compared to that observed in WT PNA+ GC cells. Lamin B1 expression in ADLD PNA+ cells was higher than PNA+ GC cells isolated from wild-type animals (Figure 5-11). This was expected and aligns with the previous literature showing an increase in lamin B1 in the ADLD mouse model used in this study (Heng et al., 2013). Conversely, lamin B2 expression could not be determined in PNA+ GC samples from ADLD animals, possibly due to expression below the limit of detection for this assay, but this could also be due to the absence of lamin B2 expression in activated B-cells in ADLD. There is currently no literature investigating the expression of lamin B2 in ADLD and therefore neither theory as to why lamin B2 could not be quantified in these samples can be confirmed. As mentioned previously, mass spectrometry analysis has shown that lamin B2 is expressed at much lower levels than lamin B1 in haematopoietic cells (Shin et al., 2013) which may explain the challenges faced in reliably quantifying this isoform in these samples. Analysis of WT animals suggests that lamin B2 is present within activated B-cells due to peak areas of transitions being measured above an S:N threshold of 3:1 in PNA+ GC cells (data not shown), which may also suggest that lamin B2 expression decreases in ADLD, as lamin B2 transitions were measured below the limit of detection in ADLD PNA+ GC samples.

5.5. Conclusion

To conclude, LC-MRM-MS/MS analysis was used to quantify the protein expression of different nuclear lamin isoforms in biological samples isolated from WT mice and mouse models of disease. The results report limited success in the detection and quantitation of nuclear lamin isoforms in PNA+ and PNA- cells from mouse models of disease.

Preliminary data on PNA+ and PNA- GC cells in wild-type animals suggests that lamin B1 expression decreases upon B-cell activation, which supports previous findings however, the relative protein expression of lamin A/C and B2 could not be reported.

With respect to the involvement of Atg7 in the decreased lamin B1 expression observed during B-cell activation, lamin A/C protein expression was suggested to decrease in *ATG7*-KD PNA- cells compared to PNA- cells from the wild-type animal (Figure 5-2), while the expression of both B-type lamins shows a similar pattern of upregulation in *ATG7*-KD PNA- cells compared to PNA- WT cells (Figure 5-3 and Figure 5-4). Meanwhile, in *LMNB1*-KD PNA- cells, the expression of lamin B1 expression was suggested to be lower compared to PNA- cells from WT animals (Figure 5-5) and the expression of lamin A/C and lamin B2 could not be determined. Neither of these results observed in PNA- cell fractions were expected due to the assumed exclusive expression of Cγ1 in activated B-cells, and further experimentation is needed to elucidate the reasons associated with these observations. Due to the absence of quantifiable MS signal response for all nuclear lamin isoforms in PNA+ cells

In ADLD mouse models, preliminary results suggest that lamin B1 expression was higher in PNA+ cells compared to PNA- cells (Figure 5-6). This was not expected due to previous findings in this chapter and previous studies suggesting lamin B1 is downregulated upon B-cell activation. Conversely, this may suggest that lamin B1 overexpression in ADLD has a greater influence on activated B-cells than other

splenocytes, and the effects on the immune response must be explored further. Lamin A/C expression in PNA⁻ cells was suggested to be greater in ADLD mouse models compared to WT animals (Figure 5-7), while PNA⁺ cells from ADLD mouse models were suggested to contain a lower expression of lamin A/C compared to the WT counterparts (Figure 5-10). Lamin B1 and lamin B2 proteins were suggested to be similar in expression when comparing PNA⁻ cells from ADLD and WT animals. This suggests further research is needed to investigate the expression of B-type lamins in ADLD.

Overall, this chapter provides preliminary data into the expression of different nuclear lamin isoforms in mouse models of ADLD, which contributes to insights into laminopathies and how the immune system may be altered in lamin B1-overexpressing disease phenotypes, which may affect long-term survival of patients with these genetic disorders.

5.6. Future Work

One of the main limitations of the studies included in this chapter is the low availability of mouse samples where nuclear lamin isoforms could be detected above the limit of quantitation. PNA- and PNA+ samples from three wild-type animals and three ADLD mouse model animals were available for the experiments investigating nuclear lamin expression in ADLD, which is enough for statistical analyses to be conducted but some samples did not detect nuclear lamin peptides above the limit of detection and therefore could not be used to determine the protein abundance of these isoforms in these samples. In addition, only PNA- and PNA+ samples were available from one *ATG7*-KD mouse model and one *LMNB1*-KD mouse model. These limitations meant that no statistical analysis could be performed on the results and therefore no conclusive statements could be made about the findings. It would be beneficial to include more animals in these studies; however it is important to consider the ethical implications of the use and destruction of animals in scientific research. Additionally, small animals such as mice can only generate a small number of PNA- and PNA+ cells due to their size, which leads to the conclusion that this assay is not sensitive enough for analysis of nuclear lamin expression in mice.

Another important consideration in this chapter is the lack of matching wild-type mice involved in the *ATG7*-KD and the *LMNB1*-KD studies. Both models were immunised for 8 days with NP-CGG and aluminium adjuvants (NP-Alum) in contrast to the wild-type mouse model used for comparison which were immunised by OVA and LPS for 4 days. As mentioned previously, wild-type animals culled at 4 days post-immunisation were used for comparisons in all experiments (*ATG7*-KD, *LMNB1*-KD and ADLD studies) in this chapter as GC B cells are present in high abundance at both 4 days and 8 days post-immunisation (Shinall et al., 2000). This also allowed for the reduction in the number of animals needed in these experiments and also reduced the number of experiments needed for this type of analysis into nuclear lamins in animal models. Furthermore, OVA immunisation and NP-CGG immunisation work in similar ways and are both commonly used to study germinal centre responses during immune system activation

in mouse models (Klymenko et al., 2018; Kouyama et al., 2007). Despite this, for true comparability, future work should include more representative wild-type control animals for the *ATG7*-KD and the *LMNB1*-KD studies by using wild-type animals immunised for 8 days with NP-Alum to give true matched control for the comparison of nuclear lamin protein expression.

Considering the concentration of protein loaded in these experiments, the amount of protein loaded was limited by the number of cells harvested from each mouse, and the cells available in each cell fraction, as a result of MACS separation. The concentration of each sample was limited by the number of cells in each sample, and also the sample preparation method. Whole cell lysates were produced from each cell pellet of each fraction and an in-gel digest was performed. To increase the concentration of nuclear proteins within each sample, the sample complexity could be reduced by subcellular fractionation. Subcellular fractionation must be performed on cell pellets soon after harvesting, therefore the most suitable time for fractionation would be immediately after MACS separation of GC cells. The limited concentration of protein may also result from the low number of cells in each GC fraction. This could be overcome by pooling samples from multiple mice to increase the total protein content in each sample. Again, it is important to consider the ethics surrounding the use of more animals and to balance this with the value of the data obtained. While pooling samples from different animals is not recommended due to biological variabilities between each animal, it may be argued that the use of pooled samples could potentially balance out the effects of biological variation seen above and allow for the differences in nuclear lamin protein expression in these samples to be seen more clearly.

The results of this chapter have shown that the detection of nuclear lamin isoforms by LC-MRM-MS is possible, and that the method developed and optimised in Chapter 2 can successfully detect and quantify lamin A/C, lamin B1 and lamin B2 in mouse GC cells. To convert this method to a fully quantifiable assay, the use of heavy-labelled nuclear lamin peptides could be utilised in future studies. In addition, the use of

spiked-in, heavy-labelled peptides would allow for the determination of assay parameters such as the lower limit of detection (LLOD) and lower limit of quantitation (LLOQ). Understanding and defining these parameters for this particular assay would also allow for more accurate reporting of the expression of each nuclear lamin isoform in each sample. If a sample that was analysed resulted in the absence of any MS signal for any isoform, it could be reported that the sample contains a concentration of that isoform that is below the limit of detection, which would be stated in nanograms per microliter. This would potentially save analysing samples where the quantity of nuclear proteins is below the limit of detection, potentially saving time, money and animals.

Numerous studies have shown that lamin A and lamin C have unique roles within the cell (Al-Saaidi & Bross, 2015). In this chapter, there was no differentiation between lamin A and lamin C isoforms, which has been shown previously in human cell lines (Al-Qahtani et al., 2019; Galal et al., 2021). In this LC-MRM-MS assay, several peptides were unique to lamin A or lamin C isoforms. Unfortunately, during the analysis of all mouse samples, none of these peptides were present at levels above the limit of detection and therefore, lamin A and lamin C expression could not be investigated independently, hence the reporting of lamin A/C throughout this chapter. Future work involving a greater protein load, or the pooling of several animals into one sample, may result in the unique lamin A and lamin C peptides being measured above the limit of quantitation, allowing for the individual analysis of each isoform in PNA- and PNA+ cells from a variety of mouse models.

Finally, using conditional knock-down mouse models for *LMNB1* and *ATG7* allowed for the brief exploration of lamin B1 expression in activated B cells, and how autophagy may contribute to lamin B1 downregulation seen previously upon B cell activation (Klymenko et al., 2018). Considering these preliminary results, it is important to consider the unexpected result seen in the knockout experiments which use the Cre-lox system. The Cre-lox system has been used successfully in many studies to investigate gene knockout, however there are some limitations to the method, which

should be discussed. In the mouse models described in this chapter (C57BL/6 Cy1-cre-ATG7 and C57BL/6 Cy1-cre-LMNB1), Cre expression is highly specific for activated B - cells due to their expression of Ig $\gamma 1$ constant region gene segment (Cy1). A rare but possible explanation for the results seen in Figure 5-2, Figure 5-3, Figure 5-4 and Figure 5-5, where there are differences in the expression of nuclear lamin isoforms present in PNA- fractions from wild-type animals and knock-down animals, is low level or ectopic activity of Cre in transitional or pre-GC B cells which are found within the PNA- cell fraction. If expression of Cy1 is found earlier than the B-cell activation stage (for example at a transitional stage of B-cell activation or during B-cell development), this could potentially mean knock-out of *LMNB1* or *ATG7* in the PNA- fraction which may lead to unexpected results. Considering this, studies have shown a small fraction of post-GC B-cells were involved in Cre-mediated recombination in Cy1-cre mice (Casola et al., 2006b). In addition, low-level and ectopic expression of Cre has been observed in previous studies (Song & Palmiter, 2018). To investigate the possibility of aberrant Cre expression in PNA- cells, the measurement of Cre expression via qPCR could be performed in PNA- cells. Furthermore, Cre toxicity has been shown in some systems and have resulted in reduced cell proliferation (Loonstra et al., 2001; Schmidt et al., 2000). Additionally, pseudo-lox sites have been identified within the mouse genome which may lead to off-target recombination effects which have been shown to occur with low efficiency (Semprini et al., 2007; Thyagarajan et al., 2000).

6. Concluding Remarks

Nuclear lamins are thought to have a role in cancer (Section 1.4), neurodegenerative diseases (see Section 1.6.2) and in the adaptive immune response (Section 1.3). They also play a role in the cell's ability to sense the mechanical stiffness of the microenvironment through interactions with the cytoskeleton (Section 1.2).

The overall aim of this thesis was to develop a targeted LC-ESI-MRM-MS/MS-based assay to detect, quantify and differentiate between the four main mammalian protein isoforms of nuclear lamins (lamin A, lamin B1, lamin B2 and lamin C) in biological samples of human and mouse origin, to explore the role of these isoforms in the different biological processes described above. To do this, several objectives were outlined. The first objective was to use Western Blot and LC-MS^e analysis to detect and quantify nuclear lamin isoforms in human cell lines in Chapter 2. This chapter initially investigated the expression of nuclear lamin isoforms across three cell lines representative of different cellular stiffnesses to examine the role of nuclear lamins in mechanosensing. The results of these experiments aligned with what was previously published in this field and suggested that lamin A and lamin C scale with increasing tissue stiffness. This was also similar for lamin B2, with semi-quantitative Western Blot analysis suggesting there was no difference in the expression of this isoform across the cell lines investigated. With respect to lamin B1, mixed results were observed which could pertain to the embryonic origin of HEK293T cells.

The initial proteomics analysis of cell lines in Chapter 2 demonstrated that most nuclear lamin isoforms were detectable and quantifiable by Western Blot (WB) analysis, while LC-MS^e analysis yielded mixed results in the detection of some isoforms. Lamin B2 was not detectable via LC-MS^e analysis in any cell lines examined, despite being detected via WB. The reason for this may be due to the ion matching requirements used for LC-MS^e data analysis, which may have limited the detection of some protein

isoforms via this method. Reducing the ion matching requirements to match those used in the MRM detection of nuclear lamin isoforms (1 fragment per peptide, 2 peptides per protein) led to the detection of lamin B2 in some samples (data not shown), highlighting the importance of understanding the MS data analysis parameters when investigating the expression of low abundant proteins. In addition, use of whole cell lysates may have led to ion suppression due to the high complexity of the samples and subsequent interference from ions with a similar m/z to the target peptide ions.

As a result of limitations resulting from WB and LC-MS^e analysis, a significant portion of this thesis was dedicated to the development of a targeted LC-ESI-MRM-MS/MS assay to examine the relative expression of different nuclear lamin isoforms in various types of biological samples. Chapter 3 explored the design and optimisation of this assay using synthetic peptides of nuclear lamin peptide sequences, while Chapter 4 and Chapter 5 utilised this assay in the evaluation of nuclear lamins in human cell lines and mouse models respectively. Optimisation of nine MS instrument parameters which included two collision-induced dissociation (CID) fragmentation parameters (collision energy (CE) and fragmentor voltage (FV)) and seven ESI source parameters (capillary voltage, nozzle voltage, nebuliser pressure, sheath gas flow rate and temperature, and desolvation gas flow rate and temperature) was performed. Adjustments to four of these parameters (CE, FV, nebuliser pressure and nozzle voltage) resulted in the development of a fully optimised LC-ESI-MRM-MS/MS method with increased sensitivity and selectivity for the measurement of nuclear lamin peptides. The results of Chapter 3 emphasise the importance of method development and optimisation, particularly when analysing low abundant analytes, as it was shown that substantial increases in peak area response were achieved through the selection of assay-specific instrument parameters.

The LC-MRM-MS/MS assay developed in Chapter 3 allowed for the evaluation of each nuclear lamin isoform within the same sample in a single injection. This multiplexing capability was achieved using liquid chromatography separation of isoform-specific

peptide ions with specific retention times based on their hydrophobicity. This allowed for a scheduled MS detection method to be used which selectively detected ions of specified m/z values at specific times during the LC gradient. Analysis of all nuclear lamin isoforms within one experiment is not possible during Western Blot analysis as it was not possible to separate protein bands of lamin B1 and lamin B2 given their similar molecular weights (66 kDa and 68 kDa respectively but observed molecular weights can range from 66 – 70 kDa), therefore multiple blots are needed to analyse all nuclear lamin isoforms within a single sample. With Western Blot analysis, the intensity of the protein bands corresponding to the expression of lamin A and lamin C observed when using an anti-lamin A/C antibody resulted in the visualisation of two proteins bands, one band for each isoform (data not shown). This allowed for the differentiation between lamin A and lamin C and quantification of each isoform individually, which was not possible during LC-MRM-MS/MS analysis as isoform-specific peptides were not measured above the limit of quantitation in samples from humans and mice. This suggests a combined approach using both WB and LC-MRM-MS/MS analysis is more suitable for analysis of these types of samples. To differentiate between lamin A and C isoform expression by MRM-MS, absolute quantitation of lamin A- and lamin C-specific peptides could be performed using heavy-labelled isotopes.

With respect to absolute quantitation, the design and success of the MRM-MS method in selectively quantifying each nuclear lamin isoform in a single sample means that this assay can be translated from a relative quantitation assay to an absolute quantitation assay with ease. Considerations regarding the specific peptides used for absolute quantitation of nuclear lamin isoforms include the presence of PTM sites within the peptide sequences. Peptides containing highly modified amino acids are not considered suitable for absolute quantitation assays for biomarker analysis due to the transient modifications which can alter the peptide chromatographic retention time and/or m/z of the peptide ion, leading to variations in the detection of unmodified peptide ions between samples. Nuclear lamins are highly modified due to their vast array of roles within the cell and therefore finding peptides which are not modified in some way can be challenging. In addition, MS sample preparation protocols include the

oxidation of methionine residues and methylation of cysteine residues, and these reactions are not 100% efficient. The disqualification of peptide sequences containing PTM sites drastically limits the number of peptide targets which could potentially be used for the absolute quantitation of lamins in human clinical samples by LC-MRM-MS/MS. Furthermore, to validate this method for absolute quantitation, the European Medicines Association (EMA) require comprehensive method validation including recording the accuracy, precision, stability, linearity, matrix effects, and measurements of the lower and upper limits of detection (LLOD and ULOD) and quantitation (LLOQ and ULOQ) of the target biomarker (European Medicines Agency, 2022).

Despite issues with the limits of detection, the assay developed in Chapter 3 has significant potential as a technique used to quantify nuclear lamin isoforms in clinical samples with minor modifications needed to translate this relative quantitation assay into an absolute quantitation method for biomarker quantitation. Firstly, the assay was designed to detect nuclear lamin isoforms in biological samples of both human and mouse origin. This is important as many biomedical research studies begin with the investigation of a disease phenotype in mouse models before being translated to clinical samples due to physiological and genetic similarities between the two organisms. The ability to detect and quantify nuclear lamin isoforms in both human and mouse biological samples in one assay means that this assay is fully optimised for investigation into mouse models and upon translation of research into clinical human samples.

One of the aims of this thesis was to investigate the protein expression of nuclear lamins in human cell lines representative of B-cell malignancies. This was conducted in a Burkitt lymphoma cell line (BL-2) and two multiple myeloma cell lines (OPM-2 and U266) representative of different disease subtypes. The results of WB analysis, LC-MS^e and targeted LC-MRM-MS/MS seemed to suggest that the OPM-2 cell line, which was representative of IgG-lambda-secreting multiple myeloma, expressed a notably highest amount of lamin A/C protein compared to the other cell lines examined. This suggests

that lamin A/C may have the potential to be used as a biomarker of disease in this cancer. Several studies on a variety of solid tumours have shown that the expression of nuclear lamins is distinctive in different subtypes of cancers within the same tissue type (Section 1.4), therefore it is plausible that lamin A/C could be specifically upregulated in IgG-MM compared to other MM subtypes, which makes it an attractive biomarker candidate for this disease.

To further validate lamin A/C as a potential biomarker in IgG subtype MM, future work should emphasize the evaluation of other MM subtypes for their expression of lamin A/C, along with providing quantitative measurements on the lamin A/C protein expression across different B-cell malignancies. In addition, studies have reported the transformation of MM to plasma cell leukemia (PCL). PCL is a rare and aggressive cancer which can occur as a primary disease or as a secondary disease via transformation from multiple myeloma. Previous literature suggests that 2-4% of multiple myelomas transform into secondary PCL (sPCL) (Swaminathan & Varadi, 2022) and the prognosis and overall survival remain poor (Bommannan et al., 2016). It would be interesting to investigate if the expression of lamin A/C changes upon transformation of MM into sPCL. During sPCL, malignant plasma cells escape from the bone marrow (BM) microenvironment and enter the peripheral blood stream. Considering this, it would be interesting to investigate if the expression of lamin A/C decreases upon transformation due to the need for the cells to migrate out of the BM, or if lamin A/C expression increases due to changes within the microenvironment between the BM and circulatory system. It would also be of interest to examine if lamin A/C expression correlates with prognostic factors such as the concentration of free light chain proteins, or clinical complications such as renal damage, anaemia or infection. For this to be possible, the protein expression of lamin A/C would have to be accurately measured and quantified in clinical samples.

One of the biological questions of this thesis was to investigate if lamin B1 downregulation upon B-cell activation correlated with the increased expression of the

key regulating enzyme AID. The theory tested was that the expression of AID during B-cell activation may directly or indirectly result in a decrease in lamin B1 protein expression. The WB results when comparing the BL-2 cell line with and without *AICDA* gene knockout seemed to suggest that there was no difference in the expression of any nuclear lamin isoforms upon *AICDA* gene knock-out. LC/MS^e and MRM data for lamin B1 agree there was no notable difference. MRM data for lamin A/C and lamin B2 also suggest no difference in these isoforms when comparing BL-2 AID^{+/+} and BL-2 AID^{-/-} cell lines. Furthermore, targeted MRM analysis allowed for the quantitation of lamin isoforms A/C and lamin B2, which could not be seen by LC/MS^e analysis. Together, these results may suggest that AID expression does not affect the protein expression of nuclear lamins in activated B-cells. Therefore, the decrease in lamin B1 in SHM does not appear to be due to a direct relationship between lamin B1 and AID expression, and further investigation into the causes of lamin B1 downregulation during the immune response is required.

Regarding nuclear lamin expression in mouse models, the transgenic mouse model of ADLD used in this study was previously shown to recapitulate human ADLD (Heng et al., 2013) and preliminary results shown in Chapter 5 may suggest that lamin A/C expression was upregulated in activated B-cells from ADLD animals compared to wild-type animals. Furthermore, lamin B1 expression was suggested to be higher in PNA+ cells from an ADLD mouse model compared to a WT animal, which was unexpected. Conditional knock-down mouse models of *ATG7* and *LMNB1* provided limited data on the differences in nuclear lamin expression compared to WT animals and unfortunately yielded no data with respect to lamin involvement in the adaptive immune response in these models due to the low sample volume and MRM assay sensitivity limits.

In summary, the investigation into the protein expression of nuclear lamin isoforms in mouse biological samples by LC-MRM-MS analysis was inconclusive as this MS method was not sensitive enough to analyse the types of samples relevant to this study. The samples used consisted of cells residing in the spleens of each animal, of which only a

small portion were activated B-cells. Sample preparation involved cutting the spleens in half before extracting cells, performing MACS separation, performing live-dead staining, sample lysis and in-gel digestion before MS analysis. At each point of this lengthy protocol, sample loss may have occurred to some degree. Further experimentation into the expression of nuclear lamin isoforms during the immune response in mouse models requires a more sensitive method of protein quantitation than the LC-MRM-MS/MS assay used in this thesis. This could include nano-LC-MRM-MS, which provides greater sensitivity with ultra-high performance liquid chromatography, increased instrument sensitivity and decreased sample flow rates for overall increased assay sensitivity. Alternatively, whole mouse spleens could be used which would potentially double the sample volume for analysis. As these animals were used in multiple studies, only half of the spleen was available for analysis for this thesis. Furthermore, increasing the concentration of protein analysed may lead to an increase in target ion abundance. Use of fractionation protocols such as subcellular fractionation with the in-gel digestion protocol used, or 2-dimensional difference gel electrophoresis (2D-DIGE) prior to MS analysis may also increase the chance of detection of these isoforms by decreasing the sample complexity.

To conclude, a combination of proteomic techniques including Western Blot analysis, untargeted LC-MS^e and targeted LC-MRM-MS/MS was used in this thesis to investigate the protein expression of four main nuclear lamin isoforms in human and mouse samples. The use of multiple proteomics techniques including the development, optimisation and utilisation of a targeted mass spectrometry assay has led to the production of preliminary data which demonstrated how aberrant expression of different isoforms may lead to malignant transformation and neurodegenerative disease. This targeted assay demonstrates potential as a method of differentiating between nuclear lamin isoforms, and future work focussing on the translation of this semi-quantitative assay to an absolute quantitation assay via the introduction of heavy-labelled isoform-specific peptides and further method validation as appropriate to comply with the EMA guidelines could result in nuclear lamin isoforms being used as a biomarker of disease in haematological malignancies and ADLD.

7. References

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8. Appendices

8.1. Appendix I

Table 8-I: Ethics approval details for the studies in this thesis.

Title of Research Study	Ethics Review Reference	Approval Date	Thesis Chapter (s)
Analysis of nuclear lamina dynamics in blood cancer models	ER9917820	18/10/2018	Chapter 2 Chapter 4
Analysis of nuclear lamina dynamics in blood cancer models	ER19432525	5/11/2019	Chapter 5

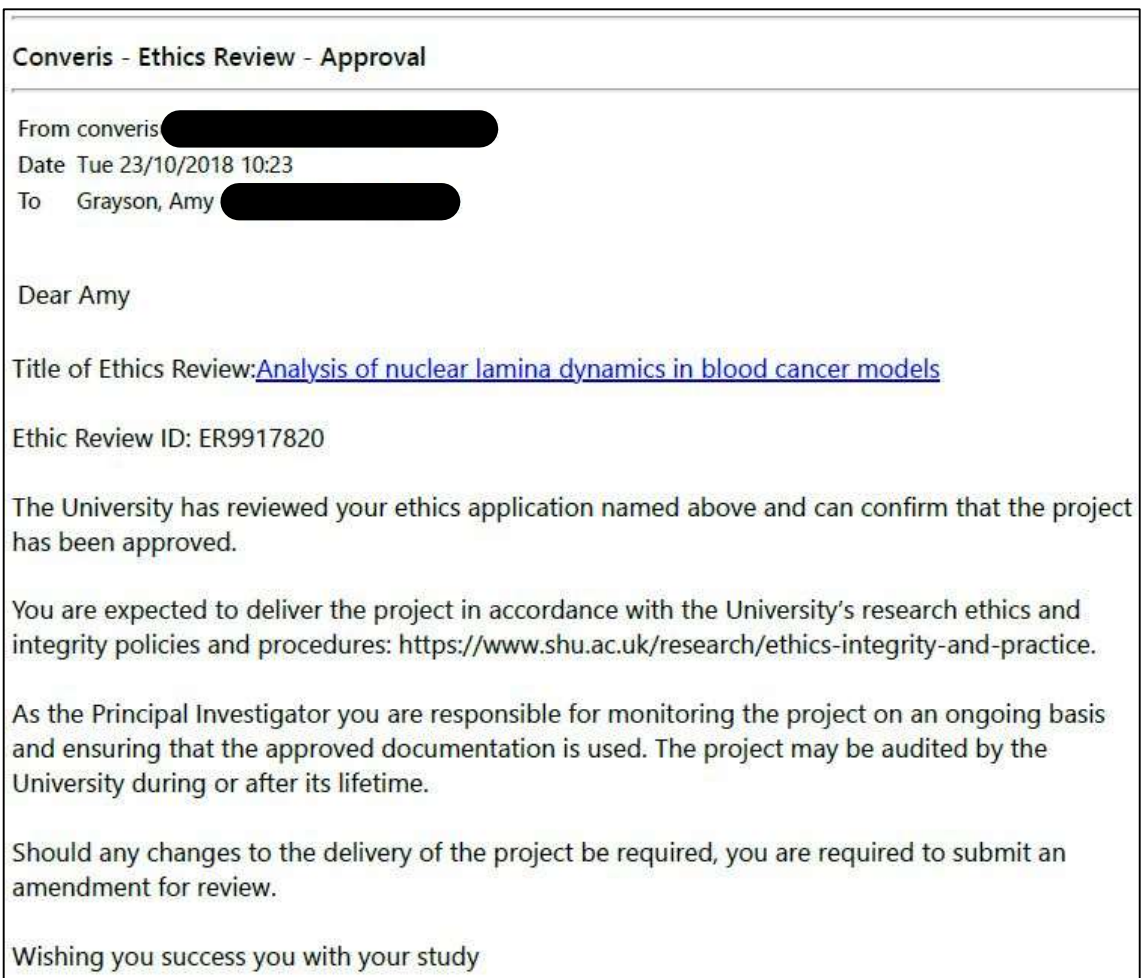


Figure 8-1: Confirmation of ethics application approval from SHU Converis for Research Study ER9917820.

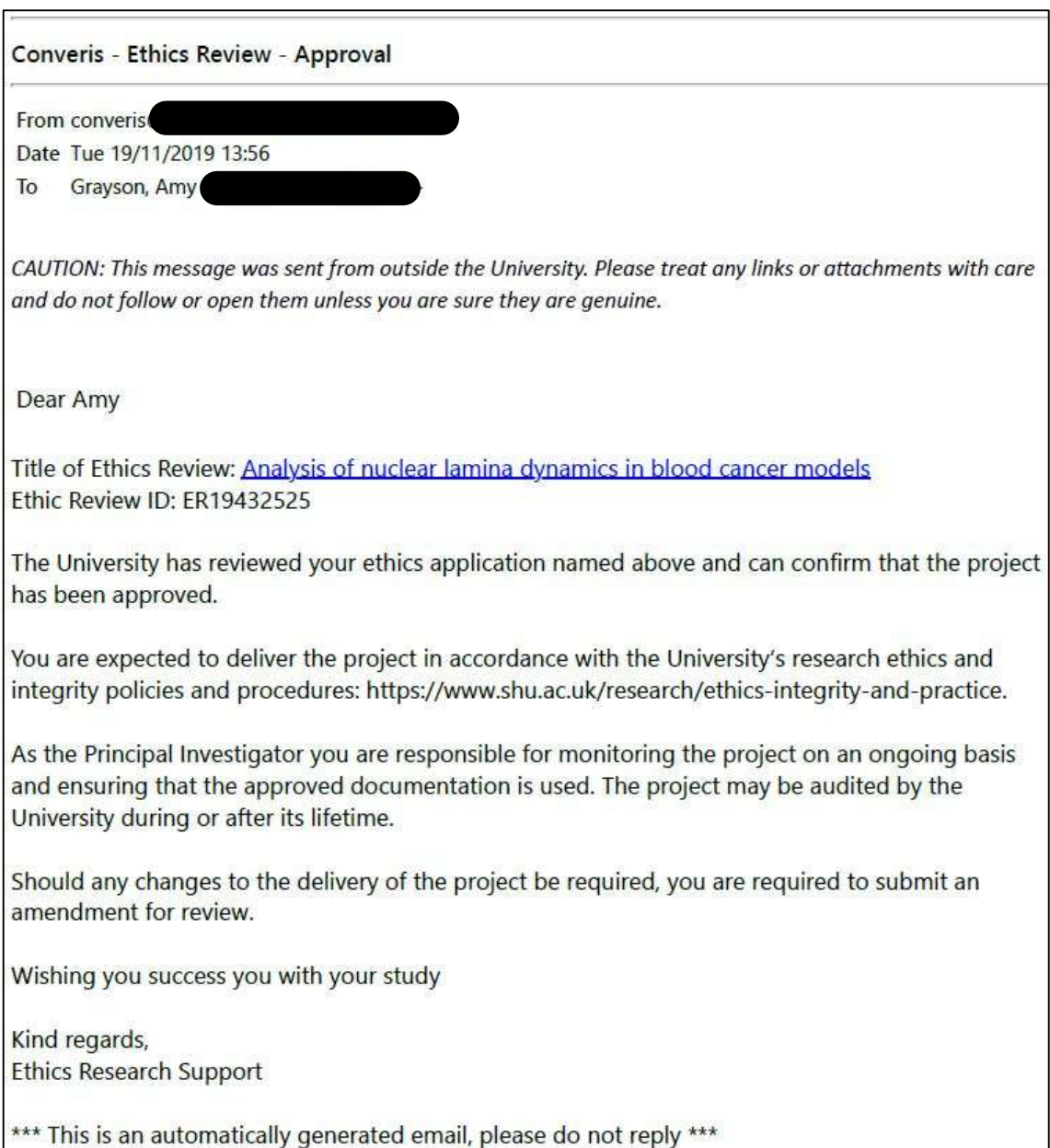


Figure 8-2: Confirmation of ethics application approval from SHU Converis for Research Study ER19432525.

8.2. Appendix II

Human	1	METPSQRRATRSGAQASSTPLSPTRITRLQEKEDLQELNDR LAVYIDRVRSLETENAGLR	60
Mouse	1	METPSQRRATRSGAQASSTPLSPTRITRLQEKEDLQELNDR LAVYIDRVRSLETENAGLR	60
Human	61	LRITSEEVVSREVSIGKAAEAEELGDARKTLDVAKERARLQLELSKVREEFKELKARN	120
Mouse	61	LRITSEEVVSREVSIGKAAEAEELGDARKTLDVAKERARLQLELSKVREEFKELKARN	120
Human	121	TKKEGDLIAAQARLKDL EALLNSKEAALSTALSEKRTLEGELHDLRGQVAKLEAALGEAK	180
Mouse	121	TKKEGDLIAAQARLKDL EALLNSKEAALSTALSEKRTLEGELHDLRGQVAKLEAALGEAK	180
Human	181	KQLQDEMLRRVDAENRLQTMKEELDFQKNYSEELRETKRRHETRLVEIDNGKQREFESR	240
Mouse	181	KQLQDEMLRRVDAENRLQTMKEELDFQKNYSEELRETKRRHETRLVEIDNGKQREFESR	240
Human	241	LADALQELRAQHEDQVEQYKKELEKTYSAKLDNARQSAERNLNLVGA AHEELQQSRIRID	300
Mouse	241	LADALQELRAQHEDQVEQYKKELEKTYSAKLDNARQSAERNLNLVGA AHEELQQSRIRID	300
Human	301	SLSAQLSQLQKQLAAKEAKLRDLED SLARERDTSRRL LAEKEREMAEMRARMQQQLDEYQ	360
Mouse	301	SLSAQLSQLQKQLAAKEAKLRDLED SLARERDTSRRL LAEKEREMAEMRARMQQQLDEYQ	360
Human	361	ELLDIKLALDMEIHAYRKLLEGE EERLRLSPSPTSQRSRGRASSHSSQTGGGSVTKKRK	420
Mouse	361	ELLDIKLALDMEIHAYRKLLEGE EERLRLSPSPTSQRSRGRASSHSSQTGGGSVTKKRK	420
Human	421	LESTESRSSFSQHARTSGRVAVEEVDEEGKFVRLRNKSNEDQSMGNWQIKRQNGDDPLLT	480
Mouse	421	LESTESRSSFSQHARTSGRVAVEEVDEEGKFVRLRNKSNEDQSMGNWQIKRQNGDDPLMT	480
Human	481	YRFPPKFTLKAGQVVTIWAAGAGATHSPPTDLVWKAQNTWGCNSLRTALINSTGEEVAM	540
Mouse	481	YRFPPKFTLKAGQVVTIWAAGAGATHSPPTDLVWKAQNTWGCNSLRTALINSTGEEVAM	540
Human	541	RKLVRSVTVVED--DEDEDGDDLHHHHGSHCSSGDP AEYNLRSRTVLCGTCGQPADKA	598
Mouse	541	RKLVRSLTMVEDNEDDEDGEELLHHH GSHCSSGDP AEYNLRSRTVLCGTCGQPADKA	600
Human	599	SASGSGAQVGGPISSGSSASSVTVTRSYRSVGGSGGGSFGDNLVTRSY	646
Mouse	601	-AGGAGAQVGGSISSGSSASSVTVTRSYRSVGGSGGGSFGDNLVTRSY	647

Figure 8-3: Comparison of human (*Homo sapiens* (P02545-1)) and mouse (*Mus musculus* (P48678-1)) lamin A amino acid sequences. Figure created using NIH National Library of Medicine protein Basic Local Alignment Search Tool (BLAST®) (BLASTp or protein-protein BLAST) search tool to compare FASTA sequences. Tryptic peptides included in the MRM method optimisation in Chapter 3 are highlighted.

Identical amino acids are located between the two sequences. Numbers flanking the sequences indicate the amino acid number at the start and end of the line.

Human	1	METPSQRRATRSGAQASSTPLSPTRITRLQEKEDLQELNDR LAVYIDRVRSLETENAGLR	60
Mouse	1	METPSQRRATRSGAQASSTPLSPTRITRLQEKEDLQELNDR LAVYIDRVRSLETENAGLR	60
Human	61	LRITSEEEVVSREVSIGKAAYEAE LGDARKTLDVAKERARLQELSKVREEFKELKARN	120
Mouse	61	LRITSEEEVVSREVSIGKAAYEAE LGDARKTLDVAKERARLQELSKVREEFKELKARN	120
Human	121	TKKEGDLIAAQARLKDLEALLNSKEAALSTALSEKRTLEGELHDLRGQVAKLEAALGEAK	180
Mouse	121	TKKEGDLIAAQARLKDLEALLNSKEAALSTALSEKRTLEGELHDLRGQVAKLEAALGEAK	180
Human	181	KQLQDEMLRRVDAENRLQTMKEELDFQKNITYSEELRETKRRHETRLVEIDNGKQREFESR	240
Mouse	181	KQLQDEMLRRVDAENRLQTMKEELDFQKNITYSEELRETKRRHETRLVEIDNGKQREFESR	240
Human	241	LADALQELRAQHEDQVEQYKKELEKTYSAKLDNARQSAERNNSLVGAAHEELQQSRIRID	300
Mouse	241	LADALQELRAQHEDQVEQYKKELEKTYSAKLDNARQSAERNNSLVGAAHEELQQSRIRID	300
Human	301	SLSAQLSQLOKQLAAKEAKLRDLED SLARERDTSRRL LAEKEREMAEMRARMQQQLDEYQ	360
Mouse	301	SLSAQLSQLOKQLAAKEAKLRDLED SLARERDTSRRL LAEKEREMAEMRARMQQQLDEYQ	360
Human	361	ELLDIKLALDMEIHAYRKLLEGE EERLRLSPSPTSQR SRGRASSHSSQTQGGGSVTKKRK	420
Mouse	361	ELLDIKLALDMEIHAYRKLLEGE EERLRLSPSPTSQR SRGRASSHSSQTQGGGSVTKKRK	420
Human	421	LESTESRSSFSQHARTSGRVAVEEVDEEGKFVRLRNKSNEDQSMGNWQIKRQNGDDPLLT	480
Mouse	421	LESTESRSSFSQHARTSGRVAVEEVDEEGKFVRLRNKSNEDQSMGNWQIKRQNGDDPLMT	480
Human	481	YRFPPKFTLKAGQVVTIWAAGAGATHSPPTDLVWKAQNTWGCNSLR TALINSTGEEVAM	540
Mouse	481	YRFPPKFTLKAGQVVTIWAAGAGATHSPPTDLVWKAQNTWGCNSLR TALINSTGEEVAM	540
Human	541	RKLVRSVTVVED--DEDEDGDLLHHHVS GSRR	572
Mouse	541	RKLVRSLTMVEDNEDDEDGEELLHHHRVSGSRR	574

Figure 8-4: Comparison of human (*Homo sapiens* (P02545-2)) and mouse (*Mus musculus* (P48678-2)) lamin C amino acid sequences. Figure created using NIH National Library of Medicine protein Basic Local Alignment Search Tool (BLAST®) (BLASTp or protein-protein BLAST) search tool to compare FASTA sequences. Tryptic peptides included in the MRM method optimisation in Chapter 3 are highlighted. Identical amino acids are located between the two sequences. Numbers flanking the sequences indicate the amino acid number at the start and end of the line.

Human	1	MATATPVPP-RMGSRAGGPTTPLSPTRLRLQKEELRELNDRLAVYIDKVRSLLETENSA	59
Mouse	1	MATATPVQQQRAGSRASAPATPLSPTRLRLQKEELRELNDRLAVYIDKVRSLLETENSA	60
Human	60	LQLQVTEREEVRGRELTLGLKALYETELADARRALDDTARERAKLQIELGKCKAEHDQLLL	119
Mouse	61	LQLQVTEREEVRGRELTLGLKALYETELADARRALDDTARERAKLQIELGKCKAEHDQLLL	120
Human	120	NYAKKESDLNGAQIKLREYEAALNSKDAALATALGDKKSLLEGDLEDLKDQIAQLEASLAA	179
Mouse	121	NYAKKESDLNGAQIKLREYEAALNSKDAALATALGDKKSLLEGDLEDLKDQIAQLEASLSA	180
Human	180	AKKQLADETLTKVDLENRCQSLTEDLEFRKSMYEEIEINETRRKHETRLVEVDSGRQIEYE	239
Mouse	181	AKKQLADETLTKVDLENRCQSLTEDLEFRKSMYEEIEINETRRKHETRLVEVDSGRQIEYE	240
Human	240	YKLAQALHEMREQHDAQVRLYKEELEQTYHAKLENARLSSEMNTSTVNSAREELMESRMR	299
Mouse	241	YKLAQALHEMREQHDAQVRLYKEELEQTYHAKLENARLSSEMNTSTVNSAREELMESRMR	300
Human	300	IESLSSQLSNLQKESRACLERIQELEDLLAKEKDNSRRMLTDKEREMAEIRDQMQQQLND	359
Mouse	301	IESLSSQLSNLQKESRACLERIQELEDLLAKEKDNSRRMLSDREREMAEIRDQMQQQLSD	360
Human	360	YEQLLDVKLALDMEISAYRKLLGEGERLKLSPSPSSRVTVSRASSSRVTRTRGKRKR	419
Mouse	361	YEQLLDVKLALDMEISAYRKLLGEGERLKLSPSPSSRVTVSRASSSRVTRTRGKRKR	420
Human	420	DVEESEASSSVSISHSASATGNVCIEEIDVDGKFIRLKNNTSEQDQPMGGWEMIRKIGDTS	479
Mouse	421	DVEESEASSSVSISHSASATGNVCIEEIDVDGKFIRLKNNTSEQDQPMGGWEMIRKIGDTS	480
Human	480	VSYKYTSRYVLKAGQTVTIWAANAGVTASPTDLIWKNNQNSWGTGEDVKVILKNSQGEV	539
Mouse	481	VSYKYTSRYVLKAGQTVTIWAANAGVTASPTDLIWKNNQNSWGTGEDVKVILKNSQGEV	540
Human	540	AQRSTVFKTTIP-EEEEEEEEAGVVEEELFHQQGTPRASNRSICAIM	586
Mouse	541	AQRSTVFKTTIPEEEEEEEEEIGVAVEEERFHQQGAPRASNRSICAIM	588

Figure 8-5: Comparison of human (*Homo sapiens* (P20700)) and mouse (*Mus musculus* (P14733)) lamin B1 amino acid sequences. Figure created using NIH National Library of Medicine protein Basic Local Alignment Search Tool (BLAST®) (BLASTp or protein-protein BLAST) search tool to compare FASTA sequences. Tryptic peptides included in the MRM method optimisation in Chapter 3 are highlighted. Identical amino acids are located between the two sequences. Numbers flanking the sequences indicate the amino acid number at the start and end of the line.

Human	25	LPGRAGGPATPLSPTRL SRLQEKEELRELNDRLAHYIDRVRALELENDRLLLKISEKEEV	84
		LP AG PATPLSPTRL SRLQEKEELRELNDRLAHYIDRVRALELENDRLLL+ISEKEEV	
Mouse	4	LPPHAG-PATPLSPTRL SRLQEKEELRELNDRLAHYIDRVRALELENDRLLLRISEKEEV	62
Human	85	TTREVSIGIKALYESELADARRVLDETARERARLQIEIGKLRAELDEVNKSAKKREGELTV	144
		TTREVSIGIK LYESELADARRVLDETARERARLQIEIGK++AEL+E KSAKKREGELTV	
Mouse	63	TTREVSIGIKTYESELADARRVLDETARERARLQIEIGKVQAELEEARSAKKREGELTV	122
Human	145	AQGRVKDLESLEFHRSEVELAAALSDKRGLESDVAELRAQLAKAEDGHAVAKKQLEKETLM	204
		AQGRVKDLESLEFHRSE ELA ALSDK+GLE++VAELRAQLAKAEDGHAVAKKQLEKETLM	
Mouse	123	AQGRVKDLESLEFHRSEALATALSDKQGLETEVAELRAQLAKAEDGHAVAKKQLEKETLM	182
Human	205	RVDLENRCQSLQEELDFRKSVFEEVRETRRRHERRLVEVDSSRQQEYDFKMAQALEELR	264
		RVDLENRCQSLQEEL F KSVFEEVRETRRRHERRLVEVDSSRQQEYDFKMAQALE+LR	
Mouse	183	RVDLENRCQSLQEELAFSKSVFEEVRETRRRHERRLVEVDSSRQQEYDFKMAQALEDLR	242
Human	265	SOHDEQVRLYKLELEQTYQAKLDSAKLSSDQNDKAASAAREELKEARMRESLSYQLSGL	324
		SOHDEQVRLY++ELEQTYQAKLD+AKL SDQNDKAA AAREELKEARMR+ESLSYQL GL	
Mouse	243	SOHDEQVRLYRVELEQTYQAKLDNAKLSSDQNDKAAHAAREELKEARMRVESLSYQLLGL	302
Human	325	QKQASAAEDRIREEAMAGERDKFRKMLDAKEQEMTEMRDVMQQQLAEYQELLDVKLAL	384
		QKQASAAE+ I ELEE+AGERDKFRKMLDAKEQEMTE+RD MQQQLAEYQELLD+KLAL	
Mouse	303	QKQASAAENHIHEEEALAGERDKFRKMLDAKEQEMTEVRDAMQQQLAEYQELLDIKLAL	362
Human	385	DMEINAYRKLEGEERLKLSPSPSSRVTVSRATSSSSGSLSATGRL--GRSKRRLEVE	442
		DMEI+AYRKLEGEERLKLSPSPSSR+T+SRATSSSS S + GR KR+RLE E	
Mouse	363	DMEISAYRKLEGEERLKLSPSPSSRITISRATSSSSSSSGVGMVSGQGRGRRRLETE	422
Human	443	EPLGSGPSVLGTGTGGSGGFHLAQQASAGSVSIEEIDLEGKFVQLKNNSSDKDQSLGNWR	502
		+ GS PS + S G LAQQ A+G V+I+E+D EG+FV+LKN+SDKDQSLGNWR	
Mouse	423	DTSGS-PS---RASRVSSGSRLAQQTVATGVNIDEVDPEGRFVRLKNNSSDKDQSLGNWR	478
Human	503	IKRQVLEGEIAYKFTPKYILRAGQMTVWAAGAGVAHSPSTLVWKGQSSWGTGESFRT	562
		IKRQVLEGE+IAYKFTPKY+LRAGQ VTVWAAGAG HSPSTLVWK Q++WG GESFRT	
Mouse	479	IKRQVLEGEIAYKFTPKYVLRAGQTVTVWAAGAGATHSPSTLVWKSQTNWGPGESFRT	538
Human	563	VLVNADGEEVAMRTVKKSSVMRENENGE EEEEEAEFG EEDLFHQQGDPRTTSRGCYVM	620
		LV+ADGEEVA++ K SSV EEEEEAEFG EEDLFHQQGDPRTTSRGC +M	
Mouse	539	ALVSADGEEVAVKAAKHSSVQGRENGE EEEEEAEFG EEDLFHQQGDPRTTSRGCRLM	596

Figure 8-6: Comparison of human (*Homo sapiens* (Q03252)) and mouse (*Mus musculus* (P21619)) lamin B2 amino acid sequences. Figure created using NIH National Library of Medicine protein Basic Local Alignment Search Tool (BLAST®) (BLASTp or protein-protein BLAST) search tool to compare FASTA sequences. Tryptic peptides included in the MRM method optimisation in Chapter 3 are highlighted. Identical amino acids are located between the two sequences. Numbers flanking the sequences indicate the amino acid number at the start and end of the line.

8.3. Appendix III

Table 8-II: Details of human lamin A/C peptides (P02545) identified through Progenesis QI for Proteomics software in cell lines representative of different stiffnesses.

Sequence	Modifications	Retention time (min)	Charge	m/z	Measured mass	Mass error (ppm)	Score
VAVEEVDEEGKFVR		20.23895	3	535.94573	1604.81536	6.69041	5.3749
IDSLSAQLSQLQK		23.29361667	2	715.89885	1429.78315	3.86522	5.0264
NSNLVGAAHEELQQSR		19.20393333	3	584.96399	1751.87014	8.63127	5.1343
SGAQASSTPLSPTR		16.35631667	2	680.34962	1358.68469	4.17043	5.0401
SVGGSGGGSFGDNLVTR		20.7455	2	783.88179	1565.74903	3.58213	4.8476
EGELHDLR		20.25325	2	484.74595	967.47735	5.19036	0

Table 8-III: Details of human lamin A/C peptides (P02545) identified through Progenesis QI for Proteomics software in malignant B-cell cell lines.

Sequence	Modifications	Retention time (min)	Charge	m/z	Measured mass	Mass error (ppm)	Score
VRSLETENAGLR		21.65	2	672.86350	1343.71245	-2.43584	5.3308
VDAENRLQTMKEELDFQK		23.54	3	732.03564	2193.08510	5.23261	5.0669
QREFESR		1.62	3	317.82828	950.46300	6.28730	5.5891
NSNLVGAAHEELQQSR		18.85	3	584.96249	1751.86565	6.06392	5.811
NIYSEELR		18.86	2	512.25909	1022.50363	0.29613	5.9006
DLEDGLAR		19.57	2	459.73346	917.45237	7.57056	5.7055
AAVEAELGDAR		18.32	2	583.27925	1164.54394	2.41935	5.5754
IDSLSAQLSQLQK		23.01	2	715.89914	1429.78372	4.26189	5.4961
IDSLSAQLSQLQK		23.03	3	477.60295	1429.78702	6.57166	---
QNGDDPLLTYR		21.31	2	646.31977	1290.62498	3.53016	5.8083
VAVEEVDEEGK		17.13	2	602.29388	1202.57320	5.47120	5.4791
EAALSTALSEKR		17.53	3	425.90249	1274.68565	2.06231	5.0931
EAALSTALSEKR		17.55	2	638.35176	1274.68896	4.65658	---
ITESEEVVSR		15.84	2	574.79086	1147.56718	-4.31066	5.5227

VAVEEVDEEGKFVR		20.04	3	535.94517	1604.81369	5.65064	5.3506
LRITESEEVVSR		21.84	2	709.38236	1416.75017	-4.98165	5.201
LQEKEDLQELNDR		18.16	3	543.94392	1628.80992	5.70798	4.9618
ASASGSGAQVGGPISSGSSASSVTVTR		19.68	3	789.06115	2364.16162	4.18577	5.8066
MQQQLDEYQELLDIK		26.25	2	947.46820	1892.92185	1.49546	5.506
MQQQLDEYQELLDIK		26.25	3	631.98175	1892.92342	2.32219	---

Table 8-IV: Details of human lamin B1 peptides (P20700) identified through Progenesis QI for Proteomics software in malignant B-cell cell lines.

Sequence	Modifications	Retention time (min)	Charge	m/z	Measured mass	Mass error (ppm)	Score
ALYETELADAR		20.81	2	626.31597	1250.61739	2.45324	4.6946
IESLSSQLSNLQK		22.14	2	723.89540	1445.77624	2.56894	4.875
LKNTSEQDQPMGGWEMIR	[11] Oxidation M	23.54	3	712.66611	2134.97650	-0.47860	4.3896
LSSEMNTSTVNSAR		17.34	2	748.85631	1495.69806	2.96680	4.8884
ALYETELADARR		21.39	2	704.36081	1406.70706	-5.94492	4.1876

Table 8-V: Details of human lamin A/C peptides (P02545) identified through Progenesis QI for Proteomics software in BL-2 AID^{+/+} and BL-2^{-/-} cell lines.

Sequence	Modifications	Retention time (min)	Charge	m/z	Measured mass	Mass error (ppm)	Score	Anova
IESLSSQLSNLQK		22.456783	2	723.895463	1445.77637	2.66294125	4.3438	0.54330836
		33		2	4	1		4
NQNSWGTGEDVKVILK		24.041133	2	894.469972	1786.92539	2.27662661	3.961	0.46588645
		33		3	2	7		2

8.4. Appendix IV

Table 8-VI: Optimised MRM transition settings for the relative quantitation of nuclear lamin isoforms in biological samples of human and mouse origin.

Target Protein	Peptide Sequence	Precursor Ion m/z	Precursor Ion Charge	Product Ion m/z	Product Ion Charge	y-ion	Retention Time (min)	Fragmentor Voltage (V)	Collision Energy (V)
Lamin A/C	METPSQR	424.700177	2	717.352593	1	y6	3.54	90	14.2
Lamin A/C	METPSQR	424.700177	2	588.310000	1	y5	3.54	90	11.2
Lamin A/C	METPSQR	424.700177	2	487.262322	1	y4	3.54	90	14.2
Lamin A/C	METPSQR	424.700177	2	390.209558	1	y3	3.54	100	23.2
Lamin A/C	SGAQASSTPLSPTR	680.346780	2	1016.537100	1	y10	5.39	140	25.1
Lamin A/C	SGAQASSTPLSPTR	680.346780	2	945.499986	1	y9	5.39	150	25.1
Lamin A/C	SGAQASSTPLSPTR	680.346780	2	858.467957	1	y8	5.39	120	25.1
Lamin A/C	SGAQASSTPLSPTR	680.346780	2	670.388250	1	y6	5.39	140	22.1
Lamin A/C	SGAQASSTPLSPTR	680.346780	2	373.219394	1	y3	5.39	130	22.1
Lamin A/C	EDLQELNDR	566.267467	2	887.458121	1	y7	5.91	90	18.6
Lamin A/C	EDLQELNDR	566.267467	2	774.374057	1	y6	5.91	120	18.6
Lamin A/C	EDLQELNDR	566.267467	2	646.315479	1	y5	5.91	90	15.6

Lamin A/C	EDLQELNDR	566.267467	2	517.272886	1	y4	5.91	120	15.6
Lamin A/C	EDLQELNDR	566.267467	2	404.188822	1	y3	5.91	110	15.6
Lamin A/C	LAVYIDR	425.245078	2	736.398815	1	y6	7.18	90	14.2
Lamin A/C	LAVYIDR	425.245078	2	665.361701	1	y5	7.18	90	11.2
Lamin A/C	LAVYIDR	425.245078	2	566.293287	1	y4	7.18	90	11.2
Lamin A/C	LAVYIDR	425.245078	2	403.229959	1	y3	7.18	100	14.2
Lamin A/C	SLETENAGLR	545.280377	2	889.437386	1	y8	5.74	90	17.9
Lamin A/C	SLETENAGLR	545.280377	2	760.394792	1	y7	5.74	90	17.9
Lamin A/C	SLETENAGLR	545.280377	2	659.347114	1	y6	5.74	90	17.9
Lamin A/C	SLETENAGLR	545.280377	2	530.304521	1	y5	5.74	90	17.9
Lamin A/C	SLETENAGLR	545.280377	2	345.224480	1	y3	5.74	90	17.9
Lamin A/C	ITESEEVVSR	574.793317	2	1035.495294	1	y9	5.21	90	21.8
Lamin A/C	ITESEEVVSR	574.793317	2	934.447616	1	y8	5.21	110	18.8
Lamin A/C	ITESEEVVSR	574.793317	2	805.405023	1	y7	5.21	90	21.8
Lamin A/C	ITESEEVVSR	574.793317	2	589.330401	1	y5	5.21	120	18.8
Lamin A/C	ITESEEVVSR	574.793317	2	361.219394	1	y3	5.21	130	15.8
Lamin A/C	AA YEAE LGDAR	583.277834	2	860.410836	1	y8	6.14	110	19.1
Lamin A/C	AA YEAE LGDAR	583.277834	2	731.368243	1	y7	6.14	120	19.1

Lamin A/C	AAYEAEELGDAR	583.277834	2	660.331130	1	y6	6.14	120	16.1
Lamin A/C	AAYEAEELGDAR	583.277834	2	531.288536	1	y5	6.14	110	16.1
Lamin A/C	AAYEAEELGDAR	583.277834	2	418.204472	1	y4	6.14	120	13.1
Lamin A/C	TLDSVAK	367.208161	2	632.361367	1	y6	4.46	90	12.4
Lamin A/C	TLDSVAK	367.208161	2	519.277303	1	y5	4.46	90	9.4
Lamin A/C	TLDSVAK	367.208161	2	404.250360	1	y4	4.46	90	12.4
Lamin A/C	TLDSVAK	367.208161	2	317.218332	1	y3	4.46	90	15.4
Lamin A/C	DLEALLNSK	501.776939	2	774.435595	1	y7	9.09	90	10.6
Lamin A/C	DLEALLNSK	501.776939	2	645.393001	1	y6	9.09	90	10.6
Lamin A/C	DLEALLNSK	501.776939	2	574.355888	1	y5	9.09	90	10.6
Lamin A/C	DLEALLNSK	501.776939	2	461.271824	1	y4	9.09	90	10.6
Lamin A/C	DLEALLNSK	501.776939	2	348.187760	1	y3	9.09	90	22.6
Lamin A/C	EAALSTALSEK	560.298235	2	919.509488	1	y9	6.82	90	18.4
Lamin A/C	EAALSTALSEK	560.298235	2	848.472374	1	y8	6.82	90	18.4
Lamin A/C	EAALSTALSEK	560.298235	2	735.388310	1	y7	6.82	90	15.4
Lamin A/C	EAALSTALSEK	560.298235	2	648.356282	1	y6	6.82	90	15.4
Lamin A/C	EAALSTALSEK	560.298235	2	363.187425	1	y3	6.82	110	27.4
Lamin A/C	TLEGELHDLR	591.809302	2	968.479585	1	y8	7.23	140	22.3

Lamin A/C	TLEGELHDLR	591.809302	2	839.436992	1	y7	7.23	130	22.3
Lamin A/C	TLEGELHDLR	591.809302	2	653.372935	1	y5	7.23	140	22.3
Lamin A/C	TLEGELHDLR	591.809302	2	540.288871	1	y4	7.23	140	22.3
Lamin A/C	TLEGELHDLR	591.809302	2	403.229959	1	y3	7.23	140	22.3
Lamin A/C	LEAALGEAK	451.253100	2	788.414859	1	y8	5.79	90	15.0
Lamin A/C	LEAALGEAK	451.253100	2	659.372266	1	y7	5.79	90	12.0
Lamin A/C	LEAALGEAK	451.253100	2	588.335152	1	y6	5.79	90	12.0
Lamin A/C	LEAALGEAK	451.253100	2	517.298038	1	y5	5.79	90	15.0
Lamin A/C	LEAALGEAK	451.253100	2	404.213974	1	y4	5.79	90	21.0
Lamin A/C	QLQDEMLR	516.760766	2	904.455678	1	y7	6.86	110	20.0
Lamin A/C	QLQDEMLR	516.760766	2	791.371614	1	y6	6.86	90	17.0
Lamin A/C	QLQDEMLR	516.760766	2	663.313037	1	y5	6.86	90	17.0
Lamin A/C	QLQDEMLR	516.760766	2	548.286094	1	y4	6.86	90	23.0
Lamin A/C	QLQDEMLR	516.760766	2	419.243500	1	y3	6.86	110	14.0
Lamin A/C	NIYSEELR	512.258913	2	909.467623	1	y7	6.64	90	16.9
Lamin A/C	NIYSEELR	512.258913	2	796.383559	1	y6	6.64	90	10.9
Lamin A/C	NIYSEELR	512.258913	2	633.320230	1	y5	6.64	90	16.9
Lamin A/C	NIYSEELR	512.258913	2	546.288202	1	y4	6.64	90	16.9

Lamin A/C	NIYSEELR	512.258913	2	417.245609	1	y3	6.64	90	25.9
Lamin A/C	LVEIDNGK	444.245275	2	774.399209	1	y7	5.55	90	14.8
Lamin A/C	LVEIDNGK	444.245275	2	675.330795	1	y6	5.55	90	11.8
Lamin A/C	LVEIDNGK	444.245275	2	546.288202	1	y5	5.55	90	11.8
Lamin A/C	LVEIDNGK	444.245275	2	433.204138	1	y4	5.55	90	17.8
Lamin A/C	LVEIDNGK	444.245275	2	318.177195	1	y3	5.55	90	20.8
Lamin A/C	LADALQELR	514.790381	2	915.489421	1	y8	7.96	90	17.0
Lamin A/C	LADALQELR	514.790381	2	844.452307	1	y7	7.96	90	17.0
Lamin A/C	LADALQELR	514.790381	2	729.425364	1	y6	7.96	120	20.0
Lamin A/C	LADALQELR	514.790381	2	658.388250	1	y5	7.96	90	17.0
Lamin A/C	LADALQELR	514.790381	2	545.304186	1	y4	7.96	90	14.0
Lamin A/C	AQHEDQVEQYK	687.817855	2	1175.532743	1	y9	4.06	150	25.3
Lamin A/C	AQHEDQVEQYK	687.817855	2	1038.473831	1	y8	4.06	150	25.3
Lamin A/C	AQHEDQVEQYK	687.817855	2	909.431238	1	y7	4.06	150	28.3
Lamin A/C	AQHEDQVEQYK	687.817855	2	567.277303	1	y4	4.06	150	28.3
Lamin A/C	AQHEDQVEQYK	687.817855	2	438.234710	1	y3	4.06	150	28.3
Lamin A/C	NSNLVGAAHEELQQSR	876.934813	2	1324.660403	1	y12	6.55	150	37.2
Lamin A/C	NSNLVGAAHEELQQSR	876.934813	2	1225.591989	1	y11	6.55	150	37.2

Lamin A/C	NSNLVGAAHEELQQSR	876.934813	2	1097.533411	1	y9	6.55	150	37.2
Lamin A/C	NSNLVGAAHEELQQSR	876.934813	2	1026.496297	1	y8	6.55	150	37.2
Lamin A/C	NSNLVGAAHEELQQSR	876.934813	2	889.437386	1	y7	6.55	150	37.2
Lamin A/C	NSNLVGAAHEELQQSR	584.958967	3	776.397335	2	y14	6.55	90	13.3
Lamin A/C	NSNLVGAAHEELQQSR	584.958967	3	719.375871	2	y13	6.55	120	16.3
Lamin A/C	NSNLVGAAHEELQQSR	584.958967	3	662.833839	2	y12	6.55	120	16.3
Lamin A/C	NSNLVGAAHEELQQSR	584.958967	3	613.299632	2	y11	6.55	90	16.3
Lamin A/C	NSNLVGAAHEELQQSR	584.958967	3	316.179738	2	y5	6.55	120	16.3
Lamin A/C	IDSLSAQLSQLQK	715.896105	2	1002.557835	1	y9	8.52	150	23.2
Lamin A/C	IDSLSAQLSQLQK	715.896105	2	915.525807	1	y8	8.52	150	23.2
Lamin A/C	IDSLSAQLSQLQK	715.896105	2	844.488693	1	y7	8.52	150	20.2
Lamin A/C	IDSLSAQLSQLQK	715.896105	2	716.430115	1	y6	8.52	150	14.2
Lamin A/C	IDSLSAQLSQLQK	715.896105	2	603.346051	1	y5	8.52	150	20.2
Lamin A/C	IDSLSAQLSQLQK	477.599829	3	716.430115	1	y6	8.52	90	9.4
Lamin A/C	IDSLSAQLSQLQK	477.599829	3	603.346051	1	y5	8.52	90	9.4
Lamin A/C	IDSLSAQLSQLQK	477.599829	3	516.314023	1	y4	8.52	90	9.4
Lamin A/C	IDSLSAQLSQLQK	477.599829	3	388.255445	1	y3	8.52	90	18.4
Lamin A/C	IDSLSAQLSQLQK	477.599829	3	358.718696	2	y6	8.52	100	6.4

Lamin A/C	DLEDSTAR	459.729989	2	803.425758	1	y7	6.77	90	18.3
Lamin A/C	DLEDSTAR	459.729989	2	690.341694	1	y6	6.77	90	12.3
Lamin A/C	DLEDSTAR	459.729989	2	561.299101	1	y5	6.77	90	12.3
Lamin A/C	DLEDSTAR	459.729989	2	446.272158	1	y4	6.77	90	21.3
Lamin A/C	DLEDSTAR	459.729989	2	359.240130	1	y3	6.77	90	12.3
Lamin A/C	MQQQLDEYQELLDIK	947.466766	2	1378.710038	1	y11	10.05	150	33.4
Lamin A/C	MQQQLDEYQELLDIK	947.466766	2	1265.625974	1	y10	10.05	150	30.4
Lamin A/C	MQQQLDEYQELLDIK	947.466766	2	601.391939	1	y5	10.05	150	33.4
Lamin A/C	MQQQLDEYQELLDIK	947.466766	2	488.307875	1	y4	10.05	140	30.4
Lamin A/C	MQQQLDEYQELLDIK	947.466766	2	375.223811	1	y3	10.05	150	30.4
Lamin A/C	MQQQLDEYQELLDIK	631.980269	3	858.493110	1	y7	10.05	90	15.0
Lamin A/C	MQQQLDEYQELLDIK	631.980269	3	730.434532	1	y6	10.05	90	15.0
Lamin A/C	MQQQLDEYQELLDIK	631.980269	3	601.391939	1	y5	10.05	120	12.0
Lamin A/C	MQQQLDEYQELLDIK	631.980269	3	488.307875	1	y4	10.05	90	12.0
Lamin A/C	MQQQLDEYQELLDIK	631.980269	3	375.223811	1	y3	10.05	110	9.0
Lamin A/C	LALDMEIHAYR	666.342454	2	1034.472391	1	y8	8.36	150	24.7
Lamin A/C	LALDMEIHAYR	666.342454	2	788.404963	1	y6	8.36	140	24.7
Lamin A/C	LALDMEIHAYR	666.342454	2	659.362370	1	y5	8.36	150	27.7

Lamin A/C	LALDMEIHAYR	666.342454	2	546.278306	1	y4	8.36	140	24.7
Lamin A/C	LALDMEIHAYR	666.342454	2	409.219394	1	y3	8.36	150	24.7
Lamin A/C	LSPSPTSQR	486.759080	2	772.394792	1	y7	4.16	90	16.1
Lamin A/C	LSPSPTSQR	486.759080	2	675.342029	1	y6	4.16	90	22.1
Lamin A/C	LSPSPTSQR	486.759080	2	588.310000	1	y5	4.16	90	19.1
Lamin A/C	LSPSPTSQR	486.759080	2	491.257236	1	y4	4.16	110	25.1
Lamin A/C	LSPSPTSQR	486.759080	2	390.209558	1	y3	4.16	90	25.1
Lamin A/C	SSFSQHAR	460.222665	2	832.406026	1	y7	2.21	100	18.3
Lamin A/C	SSFSQHAR	460.222665	2	745.373997	1	y6	2.21	110	18.3
Lamin A/C	SSFSQHAR	460.222665	2	598.305583	1	y5	2.21	120	18.3
Lamin A/C	SSFSQHAR	460.222665	2	511.273555	1	y4	2.21	90	18.3
Lamin A/C	SSFSQHAR	460.222665	2	383.214977	1	y3	2.21	100	18.3
Lamin A/C	VAVEEVDEEGK	602.290607	2	1033.468411	1	y9	5.39	90	16.7
Lamin A/C	VAVEEVDEEGK	602.290607	2	934.399997	1	y8	5.39	90	19.7
Lamin A/C	VAVEEVDEEGK	602.290607	2	805.357404	1	y7	5.39	90	19.7
Lamin A/C	VAVEEVDEEGK	602.290607	2	676.314811	1	y6	5.39	130	19.7
Lamin A/C	VAVEEVDEEGK	602.290607	2	577.246397	1	y5	5.39	110	25.7
Lamin A/C	TALINSTGEEVAMR	746.377222	2	1093.494249	1	y10	7.46	150	27.1

Lamin A/C	TALINSTGEEVAMR	746.377222	2	979.451321	1	y9	7.46	140	24.1
Lamin A/C	TALINSTGEEVAMR	746.377222	2	892.419293	1	y8	7.46	140	27.1
Lamin A/C	TALINSTGEEVAMR	746.377222	2	791.371614	1	y7	7.46	140	24.1
Lamin A/C	TALINSTGEEVAMR	746.377222	2	377.196550	1	y3	7.46	150	21.1
Lamin A/C	TALINSTGEEVAMR	497.920574	3	791.371614	1	y7	7.46	90	16.1
Lamin A/C	TALINSTGEEVAMR	497.920574	3	734.350150	1	y6	7.46	100	16.1
Lamin A/C	TALINSTGEEVAMR	497.920574	3	605.307557	1	y5	7.46	90	13.1
Lamin A/C	TALINSTGEEVAMR	497.920574	3	476.264964	1	y4	7.46	90	13.1
Lamin A/C	TALINSTGEEVAMR	497.920574	3	377.196550	1	y3	7.46	90	7.1
Lamin A (mouse only)	GSH <u>C</u> SGSGDPAEYNLR	853.862999	2	1265.575670	1	y12	5.37	140	36.5
Lamin A (mouse only)	GSH <u>C</u> SGSGDPAEYNLR	853.862999	2	1178.543642	1	y11	5.37	140	33.5
Lamin A (mouse only)	GSH <u>C</u> SGSGDPAEYNLR	853.862999	2	862.441743	1	y7	5.37	150	36.5
Lamin A (mouse only)	GSH <u>C</u> SGSGDPAEYNLR	853.862999	2	565.309272	1	y4	5.37	150	36.5
Lamin A (mouse only)	GSH <u>C</u> SGSGDPAEYNLR	853.862999	2	402.245943	1	y3	5.37	140	36.5

Lamin A (mouse only)	GSH <u>C</u> SGSGDPAEYNLR	569.577758	3	862.441743	1	y7	5.37	90	15.7
Lamin A (mouse only)	GSH <u>C</u> SGSGDPAEYNLR	569.577758	3	765.388979	1	y6	5.37	90	21.7
Lamin A (mouse only)	GSH <u>C</u> SGSGDPAEYNLR	569.577758	3	694.351865	1	y5	5.37	90	18.7
Lamin A (mouse only)	GSH <u>C</u> SGSGDPAEYNLR	569.577758	3	565.309272	1	y4	5.37	90	12.7
Lamin A (mouse only)	GSH <u>C</u> SGSGDPAEYNLR	569.577758	3	431.724509	2	y7	5.37	90	12.7
Lamin A	TVL <u>C</u> GT <u>C</u> GQPADK	703.823769	2	1206.524169	1	y11	5.21	130	22.8
Lamin A	TVL <u>C</u> GT <u>C</u> GQPADK	703.823769	2	1093.440105	1	y10	5.21	150	22.8
Lamin A	TVL <u>C</u> GT <u>C</u> GQPADK	703.823769	2	933.409457	1	y9	5.21	130	22.8
Lamin A	TVL <u>C</u> GT <u>C</u> GQPADK	703.823769	2	775.340314	1	y7	5.21	120	22.8
Lamin A	TVL <u>C</u> GT <u>C</u> GQPADK	703.823769	2	430.229624	1	y4	5.21	140	31.8
Lamin A	TVL <u>C</u> GT <u>C</u> GQPADK	469.551604	3	775.340314	1	y7	5.21	90	9.1
Lamin A	TVL <u>C</u> GT <u>C</u> GQPADK	469.551604	3	615.309666	1	y6	5.21	90	9.1
Lamin A	TVL <u>C</u> GT <u>C</u> GQPADK	469.551604	3	430.229624	1	y4	5.21	90	12.1
Lamin A	TVL <u>C</u> GT <u>C</u> GQPADK	469.551604	3	388.173795	2	y7	5.21	90	6.1

Lamin A	TVL <u>C</u> GT <u>C</u> GQPADK	469.551604	3	215.618450	2	y4	5.21	90	9.1
Lamin A	SVGGSGGGSFGDNLVTR	783.878975	2	1179.575276	1	y12	7.28	150	28.3
Lamin A	SVGGSGGGSFGDNLVTR	783.878975	2	921.478856	1	y8	7.28	150	31.3
Lamin A	SVGGSGGGSFGDNLVTR	783.878975	2	774.410442	1	y7	7.28	150	28.3
Lamin A	SVGGSGGGSFGDNLVTR	783.878975	2	602.362036	1	y5	7.28	150	34.3
Lamin A	SVGGSGGGSFGDNLVTR	783.878975	2	375.235044	1	y3	7.28	150	31.3
Lamin A	SVGGSGGGSFGDNLVTR	522.921742	3	774.410442	1	y7	7.28	90	17.0
Lamin A	SVGGSGGGSFGDNLVTR	522.921742	3	602.362036	1	y5	7.28	100	23.0
Lamin A	SVGGSGGGSFGDNLVTR	522.921742	3	488.319108	1	y4	7.28	90	17.0
Lamin A	SVGGSGGGSFGDNLVTR	522.921742	3	375.235044	1	y3	7.28	90	11.0
Lamin A	SVGGSGGGSFGDNLVTR	522.921742	3	690.828754	2	y15	7.28	90	8.0
Prelamin A/C	SYLLGNSSPR	547.285462	2	843.468292	1	y8	6.88	90	18.0
Prelamin A/C	SYLLGNSSPR	547.285462	2	730.384228	1	y7	6.88	110	18.0
Prelamin A/C	SYLLGNSSPR	547.285462	2	617.300164	1	y6	6.88	90	18.0
Prelamin A/C	SYLLGNSSPR	547.285462	2	560.278700	1	y5	6.88	110	15.0
Prelamin A/C	SYLLGNSSPR	547.285462	2	446.235772	1	y4	6.88	100	15.0
Lamin B1	LAVYIDK	411.242004	2	708.392667	1	y6	7.03	90	10.7
Lamin B1	LAVYIDK	411.242004	2	637.355553	1	y5	7.03	90	10.7

Lamin B1	LAVYIDK	411.242004	2	538.287139	1	y4	7.03	90	10.7
Lamin B1	LAVYIDK	411.242004	2	375.223811	1	y3	7.03	90	16.7
Lamin B1	SLETENSALQLQVTER	909.465612	2	873.478856	1	y7	7.99	150	32.2
Lamin B1	SLETENSALQLQVTER	909.465612	2	745.420279	1	y6	7.99	150	29.2
Lamin B1	SLETENSALQLQVTER	909.465612	2	632.336215	1	y5	7.99	150	29.2
Lamin B1	SLETENSALQLQVTER	909.465612	2	504.277637	1	y4	7.99	150	26.2
Lamin B1	SLETENSALQLQVTER	909.465612	2	405.209223	1	y3	7.99	150	26.2
Lamin B1	SLETENSALQLQVTER	606.646166	3	745.420279	1	y6	7.99	90	17.0
Lamin B1	SLETENSALQLQVTER	606.646166	3	632.336215	1	y5	7.99	100	14.0
Lamin B1	SLETENSALQLQVTER	606.646166	3	504.277637	1	y4	7.99	100	14.0
Lamin B1	SLETENSALQLQVTER	606.646166	3	405.209223	1	y3	7.99	90	11.0
Lamin B1	SLETENSALQLQVTER	606.646166	3	744.886269	2	y13	7.99	100	11.0
Lamin B1	ALYETELADAR	626.314417	2	1067.500380	1	y9	7.35	120	17.4
Lamin B1	ALYETELADAR	626.314417	2	904.437051	1	y8	7.35	120	20.4
Lamin B1	ALYETELADAR	626.314417	2	775.394458	1	y7	7.35	120	20.4
Lamin B1	ALYETELADAR	626.314417	2	545.304186	1	y5	7.35	130	17.4
Lamin B1	ALYETELADAR	626.314417	2	432.220122	1	y4	7.35	120	17.4
Lamin B1	ALDDTAR	381.193042	2	690.341694	1	y6	3.71	90	12.8

Lamin B1	ALDDTAR	381.193042	2	577.257630	1	y5	3.71	90	9.8
Lamin B1	ALDDTAR	381.193042	2	462.230687	1	y4	3.71	90	12.8
Lamin B1	ALDDTAR	381.193042	2	347.203744	1	y3	3.71	90	18.8
Lamin B1	AEHDQLLLNYAK	707.869891	2	1077.593886	1	y9	7.48	150	25.9
Lamin B1	AEHDQLLLNYAK	707.869891	2	721.424302	1	y6	7.48	150	25.9
Lamin B1	AEHDQLLLNYAK	707.869891	2	608.340238	1	y5	7.48	150	22.9
Lamin B1	AEHDQLLLNYAK	707.869891	2	495.256174	1	y4	7.48	150	28.9
Lamin B1	AEHDQLLLNYAK	707.869891	2	381.213246	1	y3	7.48	150	31.9
Lamin B1	AEHDQLLLNYAK	472.249019	3	721.424302	1	y6	7.48	90	12.2
Lamin B1	AEHDQLLLNYAK	472.249019	3	608.340238	1	y5	7.48	90	9.2
Lamin B1	AEHDQLLLNYAK	472.249019	3	495.256174	1	y4	7.48	90	12.2
Lamin B1	AEHDQLLLNYAK	472.249019	3	381.213246	1	y3	7.48	90	21.2
Lamin B1	AEHDQLLLNYAK	472.249019	3	607.830037	2	y10	7.48	90	12.2
Lamin B1	EYEAALNSK	512.750921	2	732.388644	1	y7	5.12	90	13.9
Lamin B1	EYEAALNSK	512.750921	2	603.346051	1	y6	5.12	90	13.9
Lamin B1	EYEAALNSK	512.750921	2	532.308937	1	y5	5.12	90	10.9
Lamin B1	EYEAALNSK	512.750921	2	461.271824	1	y4	5.12	90	10.9
Lamin B1	EYEAALNSK	512.750921	2	348.187760	1	y3	5.12	90	10.9

Lamin B1	DAALATALGDK	523.279846	2	859.488358	1	y9	7.26	90	14.2
Lamin B1	DAALATALGDK	523.279846	2	788.451245	1	y8	7.26	90	14.2
Lamin B1	DAALATALGDK	523.279846	2	675.367181	1	y7	7.26	90	14.2
Lamin B1	DAALATALGDK	523.279846	2	604.330067	1	y6	7.26	90	11.2
Lamin B1	DAALATALGDK	523.279846	2	319.161211	1	y3	7.26	110	26.2
Lamin B1	SLEGDLEDLK	559.782418	2	918.441468	1	y8	8.47	90	15.4
Lamin B1	SLEGDLEDLK	559.782418	2	789.398875	1	y7	8.47	90	15.4
Lamin B1	SLEGDLEDLK	559.782418	2	617.350468	1	y5	8.47	110	21.4
Lamin B1	SLEGDLEDLK	559.782418	2	504.266404	1	y4	8.47	110	18.4
Lamin B1	SLEGDLEDLK	559.782418	2	375.223811	1	y3	8.47	110	24.4
Lamin B1	QLADETLLK	515.792589	2	789.435260	1	y7	7.57	90	17.0
Lamin B1	QLADETLLK	515.792589	2	718.398146	1	y6	7.57	90	17.0
Lamin B1	QLADETLLK	515.792589	2	603.371203	1	y5	7.57	90	17.0
Lamin B1	QLADETLLK	515.792589	2	474.328610	1	y4	7.57	90	14.0
Lamin B1	QLADETLLK	515.792589	2	373.280932	1	y3	7.57	110	23.0
Lamin B1	<u>C</u> QSLTEDLEFR	699.321916	2	1109.547330	1	y9	8.64	140	25.7
Lamin B1	<u>C</u> QSLTEDLEFR	699.321916	2	1022.515302	1	y8	8.64	150	25.7
Lamin B1	<u>C</u> QSLTEDLEFR	699.321916	2	909.431238	1	y7	8.64	140	25.7

Lamin B1	<u>C</u> QSLTEDLEFR	699.321916	2	808.383559	1	y6	8.64	150	25.7
Lamin B1	<u>C</u> QSLTEDLEFR	699.321916	2	679.340966	1	y5	8.64	140	25.7
Lamin B1	LVEVDSGR	437.735074	2	761.378808	1	y7	5.07	90	14.6
Lamin B1	LVEVDSGR	437.735074	2	662.310394	1	y6	5.07	90	11.6
Lamin B1	LVEVDSGR	437.735074	2	533.267801	1	y5	5.07	90	11.6
Lamin B1	LVEVDSGR	437.735074	2	434.199387	1	y4	5.07	90	11.6
Lamin B1	LVEVDSGR	437.735074	2	319.172444	1	y3	5.07	90	23.6
Lamin B1	QIEYEYK	486.737282	2	844.408711	1	y6	5.79	90	16.1
Lamin B1	QIEYEYK	486.737282	2	731.324647	1	y5	5.79	90	13.1
Lamin B1	QIEYEYK	486.737282	2	602.282054	1	y4	5.79	90	13.1
Lamin B1	QIEYEYK	486.737282	2	439.218725	1	y3	5.79	90	10.1
Lamin B1	LAQALHEMR	534.784575	2	884.440697	1	y7	5.44	130	20.6
Lamin B1	LAQALHEMR	534.784575	2	756.382119	1	y6	5.44	150	20.6
Lamin B1	LAQALHEMR	534.784575	2	685.345005	1	y5	5.44	140	20.6
Lamin B1	LAQALHEMR	534.784575	2	572.260941	1	y4	5.44	90	20.6
Lamin B1	LAQALHEMR	534.784575	2	435.202030	1	y3	5.44	120	26.6
Lamin B1	EQHDAQVR	491.738679	2	853.427490	1	y7	10.23	90	19.2
Lamin B1	EQHDAQVR	491.738679	2	725.368912	1	y6	10.23	140	19.2

Lamin B1	EQHDAQVR	491.738679	2	588.310000	1	y5	10.23	140	22.2
Lamin B1	EQHDAQVR	491.738679	2	473.283057	1	y4	10.23	90	22.2
Lamin B1	EQHDAQVR	491.738679	2	402.245943	1	y3	10.23	150	22.2
Lamin B1	EELEQTYHAK	624.298767	2	989.505071	1	y8	4.65	140	23.4
Lamin B1	EELEQTYHAK	624.298767	2	876.421007	1	y7	4.65	130	23.4
Lamin B1	EELEQTYHAK	624.298767	2	747.378414	1	y6	4.65	150	23.4
Lamin B1	EELEQTYHAK	624.298767	2	619.319837	1	y5	4.65	130	23.4
Lamin B1	EELEQTYHAK	624.298767	2	355.208829	1	y3	4.65	130	29.4
Lamin B1	LSSEMNTSTVNSAR	748.854111	2	949.469748	1	y9	5.46	140	27.2
Lamin B1	LSSEMNTSTVNSAR	748.854111	2	835.426821	1	y8	5.46	150	30.2
Lamin B1	LSSEMNTSTVNSAR	748.854111	2	734.379142	1	y7	5.46	140	27.2
Lamin B1	LSSEMNTSTVNSAR	748.854111	2	647.347114	1	y6	5.46	150	27.2
Lamin B1	LSSEMNTSTVNSAR	748.854111	2	447.231021	1	y4	5.46	140	24.2
Lamin B1	LSSEMNTSTVNSAR	499.571833	3	734.379142	1	y7	5.46	90	13.2
Lamin B1	LSSEMNTSTVNSAR	499.571833	3	647.347114	1	y6	5.46	90	13.2
Lamin B1	LSSEMNTSTVNSAR	499.571833	3	546.299435	1	y5	5.46	90	13.2
Lamin B1	LSSEMNTSTVNSAR	499.571833	3	447.231021	1	y4	5.46	90	13.2
Lamin B1	LSSEMNTSTVNSAR	499.571833	3	333.188094	1	y3	5.46	100	13.2

Lamin B1	EELMESR	447.205292	2	764.360715	1	y6	5.22	90	14.9
Lamin B1	EELMESR	447.205292	2	635.318122	1	y5	5.22	90	14.9
Lamin B1	EELMESR	447.205292	2	522.234058	1	y4	5.22	110	11.9
Lamin B1	EELMESR	447.205292	2	391.193573	1	y3	5.22	90	8.9
Lamin B1	IESLSSQLSNLQK	723.893563	2	1204.653192	1	y11	8.16	150	23.4
Lamin B1	IESLSSQLSNLQK	723.893563	2	1004.537100	1	y9	8.16	150	23.4
Lamin B1	IESLSSQLSNLQK	723.893563	2	917.505071	1	y8	8.16	150	23.4
Lamin B1	IESLSSQLSNLQK	723.893563	2	702.414465	1	y6	8.16	150	23.4
Lamin B1	IESLSSQLSNLQK	723.893563	2	589.330401	1	y5	8.16	150	23.4
Lamin B1	IESLSSQLSNLQK	482.931467	3	702.414465	1	y6	8.16	90	9.6
Lamin B1	IESLSSQLSNLQK	482.931467	3	589.330401	1	y5	8.16	90	9.6
Lamin B1	IESLSSQLSNLQK	482.931467	3	502.298373	1	y4	8.16	90	9.6
Lamin B1	IESLSSQLSNLQK	482.931467	3	388.255445	1	y3	8.16	90	18.6
Lamin B1	IESLSSQLSNLQK	482.931467	3	351.710871	2	y6	8.16	90	6.6
Lamin B1	LALDMEISAYR	641.329013	2	984.445507	1	y8	9.34	110	20.9
Lamin B1	LALDMEISAYR	641.329013	2	869.418564	1	y7	9.34	140	20.9
Lamin B1	LALDMEISAYR	641.329013	2	738.378080	1	y6	9.34	140	20.9
Lamin B1	LALDMEISAYR	641.329013	2	609.335487	1	y5	9.34	130	20.9

Lamin B1	LALDMEISAYR	641.329013	2	496.251423	1	y4	9.34	130	17.9
Lamin B1	NTSEQDQPMGGWEMIR	939.909092	2	1319.587103	1	y11	9.04	140	30.1
Lamin B1	NTSEQDQPMGGWEMIR	939.909092	2	1076.501583	1	y9	9.04	150	33.1
Lamin B1	NTSEQDQPMGGWEMIR	939.909092	2	848.408334	1	y7	9.04	140	33.1
Lamin B1	NTSEQDQPMGGWEMIR	939.909092	2	548.286094	1	y4	9.04	100	30.1
Lamin B1	NTSEQDQPMGGWEMIR	939.909092	2	419.243500	1	y3	9.04	140	30.1
Lamin B1	NTSEQDQPMGGWEMIR	626.941820	3	1076.501583	1	y9	9.04	110	17.8
Lamin B1	NTSEQDQPMGGWEMIR	626.941820	3	848.408334	1	y7	9.04	100	17.8
Lamin B1	NTSEQDQPMGGWEMIR	626.941820	3	548.286094	1	y4	9.04	100	11.8
Lamin B1	NTSEQDQPMGGWEMIR	626.941820	3	419.243500	1	y3	9.04	130	20.8
Lamin B1	NTSEQDQPMGGWEMIR	626.941820	3	538.754429	2	y9	9.04	110	11.8
Lamin B1	IGDTSVSYK	485.248014	2	856.404688	1	y8	5.27	90	16.0
Lamin B1	IGDTSVSYK	485.248014	2	799.383225	1	y7	5.27	90	13.0
Lamin B1	IGDTSVSYK	485.248014	2	684.356282	1	y6	5.27	90	16.0
Lamin B1	IGDTSVSYK	485.248014	2	583.308603	1	y5	5.27	90	16.0
Lamin B1	IGDTSVSYK	485.248014	2	397.208161	1	y3	5.27	90	22.0
Lamin B1	NQNSWGTGEDVK	667.802205	2	1092.495629	1	y10	5.62	130	18.7
Lamin B1	NQNSWGTGEDVK	667.802205	2	978.452701	1	y9	5.62	140	21.7

Lamin B1	NQNSWGTGEDVK	667.802205	2	891.420673	1	y8	5.62	150	21.7
Lamin B1	NQNSWGTGEDVK	667.802205	2	705.341360	1	y7	5.62	150	21.7
Lamin B1	NQNSWGTGEDVK	667.802205	2	547.272218	1	y5	5.62	150	21.7
Lamin B1	NSQGEEVAQR	559.265258	2	916.448285	1	y8	2.81	120	18.3
Lamin B1	NSQGEEVAQR	559.265258	2	788.389707	1	y7	2.81	100	21.3
Lamin B1	NSQGEEVAQR	559.265258	2	602.325650	1	y5	2.81	120	21.3
Lamin B1	NSQGEEVAQR	559.265258	2	473.283057	1	y4	2.81	100	27.3
Lamin B1	NSQGEEVAQR	559.265258	2	374.214643	1	y3	2.81	120	21.3
Lamin B2	ALELENDR	480.243263	2	888.442137	1	y7	5.99	90	15.9
Lamin B2	ALELENDR	480.243263	2	775.358073	1	y6	5.99	90	12.9
Lamin B2	ALELENDR	480.243263	2	646.315479	1	y5	5.99	90	12.9
Lamin B2	ALELENDR	480.243263	2	533.231415	1	y4	5.99	90	12.9
Lamin B2	ALELENDR	480.243263	2	404.188822	1	y3	5.99	90	15.9
Lamin B2	VLDETAR	402.216517	2	704.357344	1	y6	4.27	90	13.5
Lamin B2	VLDETAR	402.216517	2	591.273280	1	y5	4.27	90	10.5
Lamin B2	VLDETAR	402.216517	2	476.246337	1	y4	4.27	90	16.5
Lamin B2	VLDETAR	402.216517	2	347.203744	1	y3	4.27	90	16.5
Lamin B2	EGELTVAQGR	530.275095	2	744.436263	1	y7	5.49	90	17.4

Lamin B2	EGELTVAQGR	530.275095	2	631.352199	1	y6	5.49	90	17.4
Lamin B2	EGELTVAQGR	530.275095	2	530.304521	1	y5	5.49	140	8.4
Lamin B2	EGELTVAQGR	530.275095	2	431.236107	1	y4	5.49	90	14.4
Lamin B2	EGELTVAQGR	530.275095	2	360.198993	1	y3	5.49	110	14.4
Lamin B2	DLESLFHR	508.761623	2	901.489027	1	y7	8.87	110	19.8
Lamin B2	DLESLFHR	508.761623	2	788.404963	1	y6	8.87	120	16.8
Lamin B2	DLESLFHR	508.761623	2	659.362370	1	y5	8.87	120	16.8
Lamin B2	DLESLFHR	508.761623	2	572.330342	1	y4	8.87	130	16.8
Lamin B2	DLESLFHR	508.761623	2	459.246278	1	y3	8.87	120	16.8
Lamin B2	AEDGHAVAK	449.224873	2	697.362764	1	y7	1.40	120	14.9
Lamin B2	AEDGHAVAK	449.224873	2	582.335821	1	y6	1.40	130	14.9
Lamin B2	AEDGHAVAK	449.224873	2	525.314357	1	y5	1.40	130	17.9
Lamin B2	AEDGHAVAK	449.224873	2	388.255445	1	y4	1.40	120	20.9
Lamin B2	AEDGHAVAK	449.224873	2	317.218332	1	y3	1.40	120	20.9
Lamin B2	SVFEEVR	497.745639	2	907.451973	1	y7	6.75	90	16.4
Lamin B2	SVFEEVR	497.745639	2	808.383559	1	y6	6.75	90	13.4
Lamin B2	SVFEEVR	497.745639	2	661.315145	1	y5	6.75	90	16.4
Lamin B2	SVFEEVR	497.745639	2	532.272552	1	y4	6.75	90	16.4

Lamin B2	SVFEEVR	497.745639	2	403.229959	1	y3	6.75	90	22.4
Lamin B2	LVEVDSSR	452.740356	2	791.389373	1	y7	5.01	90	15.0
Lamin B2	LVEVDSSR	452.740356	2	692.320959	1	y6	5.01	90	12.0
Lamin B2	LVEVDSSR	452.740356	2	563.278366	1	y5	5.01	90	15.0
Lamin B2	LVEVDSSR	452.740356	2	464.209952	1	y4	5.01	90	12.0
Lamin B2	LVEVDSSR	452.740356	2	349.183009	1	y3	5.01	90	24.0
Lamin B2	QQEYDFK	479.219257	2	829.372660	1	y6	5.67	110	12.9
Lamin B2	QQEYDFK	479.219257	2	701.314082	1	y5	5.67	90	12.9
Lamin B2	QQEYDFK	479.219257	2	572.271489	1	y4	5.67	90	12.9
Lamin B2	QQEYDFK	479.219257	2	409.208161	1	y3	5.67	90	12.9
Lamin B2	SQHDEQVR	499.736137	2	911.432969	1	y7	3.52	150	25.5
Lamin B2	SQHDEQVR	499.736137	2	783.374391	1	y6	3.52	150	16.5
Lamin B2	SQHDEQVR	499.736137	2	646.315479	1	y5	3.52	140	19.5
Lamin B2	SQHDEQVR	499.736137	2	531.288536	1	y4	3.52	140	25.5
Lamin B2	SQHDEQVR	499.736137	2	402.245943	1	y3	3.52	140	25.5
Lamin B2	EQEMTEVR	511.234581	2	892.419293	1	y7	4.79	120	19.8
Lamin B2	EQEMTEVR	511.234581	2	764.360715	1	y6	4.79	120	16.8
Lamin B2	EQEMTEVR	511.234581	2	635.318122	1	y5	4.79	100	16.8

Lamin B2	EQEMTEVR	511.234581	2	504.277637	1	y4	4.79	100	13.8
Lamin B2	EQEMTEVR	511.234581	2	403.229959	1	y3	4.79	90	10.8
Lamin B2	DQSLGNWR	488.235772	2	860.437326	1	y7	6.80	90	19.1
Lamin B2	DQSLGNWR	488.235772	2	732.378748	1	y6	6.80	90	13.1
Lamin B2	DQSLGNWR	488.235772	2	645.346720	1	y5	6.80	90	16.1
Lamin B2	DQSLGNWR	488.235772	2	532.262656	1	y4	6.80	90	16.1
Lamin B2	DQSLGNWR	488.235772	2	475.241192	1	y3	6.80	90	16.1
Lamin C (mouse only)	VSGSR	253.140081	2	406.204472	1	y4	4.23	110	5.8
Lamin C (mouse only)	VSGSR	253.140081	2	319.172444	1	y3	4.23	110	5.8
Lamin C (mouse only)	VSGSR	253.140081	2	262.150980	1	y2	4.23	110	11.8
Lamin C (mouse only)	VSGSR	253.140081	2	175.118952	1	y1	4.23	110	14.8
All nuclear lamin isoforms	LLEGEER	487.743096	2	861.394852	1	y7	5.04	90	16.1
All nuclear lamin isoforms	LLEGEER	487.743096	2	748.310788	1	y6	5.04	90	13.1

All nuclear lamin isoforms	LLEGEEER	487.743096	2	619.268195	1	y5	5.04	90	16.1
All nuclear lamin isoforms	LLEGEEER	487.743096	2	562.246731	1	y4	5.04	90	13.1
All nuclear lamin isoforms	LLEGEEER	487.743096	2	433.204138	1	y3	5.04	100	22.1
B-type nuclear lamins	LSPSPSSR	415.721967	2	717.352593	1	y7	3.76	90	13.9
B-type nuclear lamins	LSPSPSSR	415.721967	2	630.320565	1	y6	3.76	90	10.9
B-type nuclear lamins	LSPSPSSR	415.721967	2	533.267801	1	y5	3.76	90	19.9
B-type nuclear lamins	LSPSPSSR	415.721967	2	446.235772	1	y4	3.76	90	16.9
B-type nuclear lamins	LSPSPSSR	415.721967	2	349.183009	1	y3	3.76	100	22.9

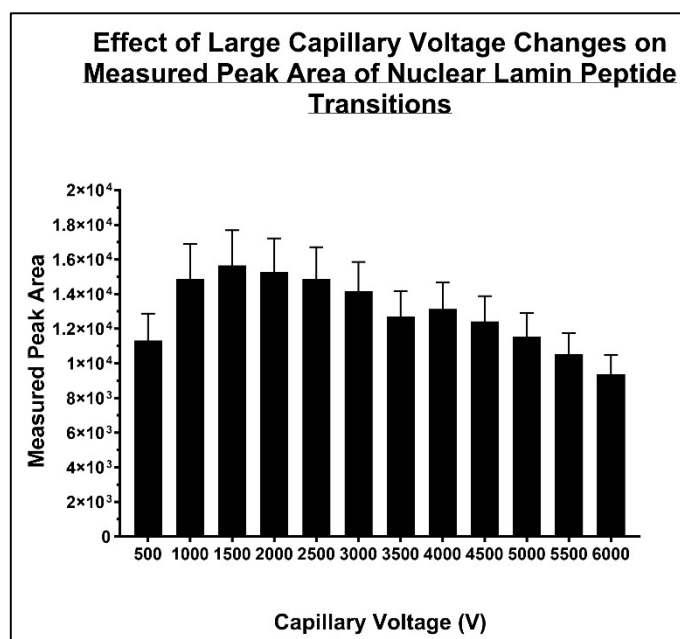


Figure 8-7: Effect of changing the capillary voltage on the mean measured peak area of 357 nuclear lamin peptide transitions, within the range of 500 V to 4000 V (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.1052$.

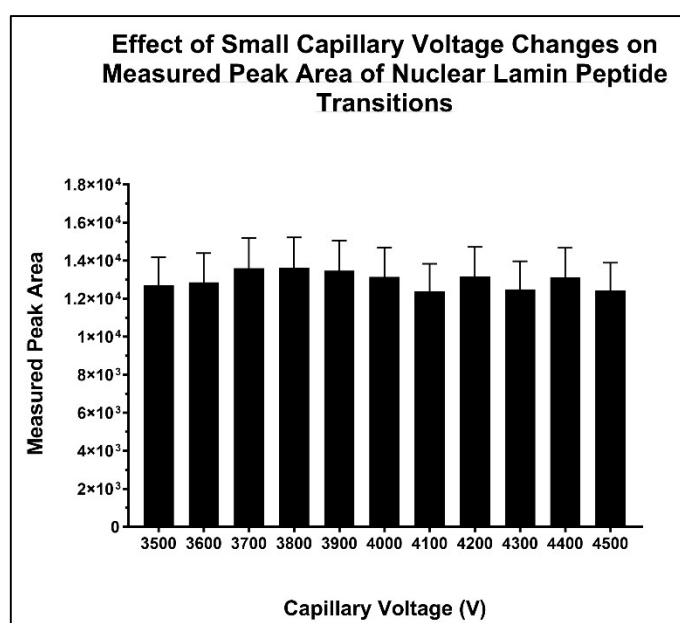


Figure 8-8: Effect of changing the capillary voltage on the mean measured peak area of 357 nuclear lamin peptide transitions, within the range of 3500 V to 4500 V (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.9999$.

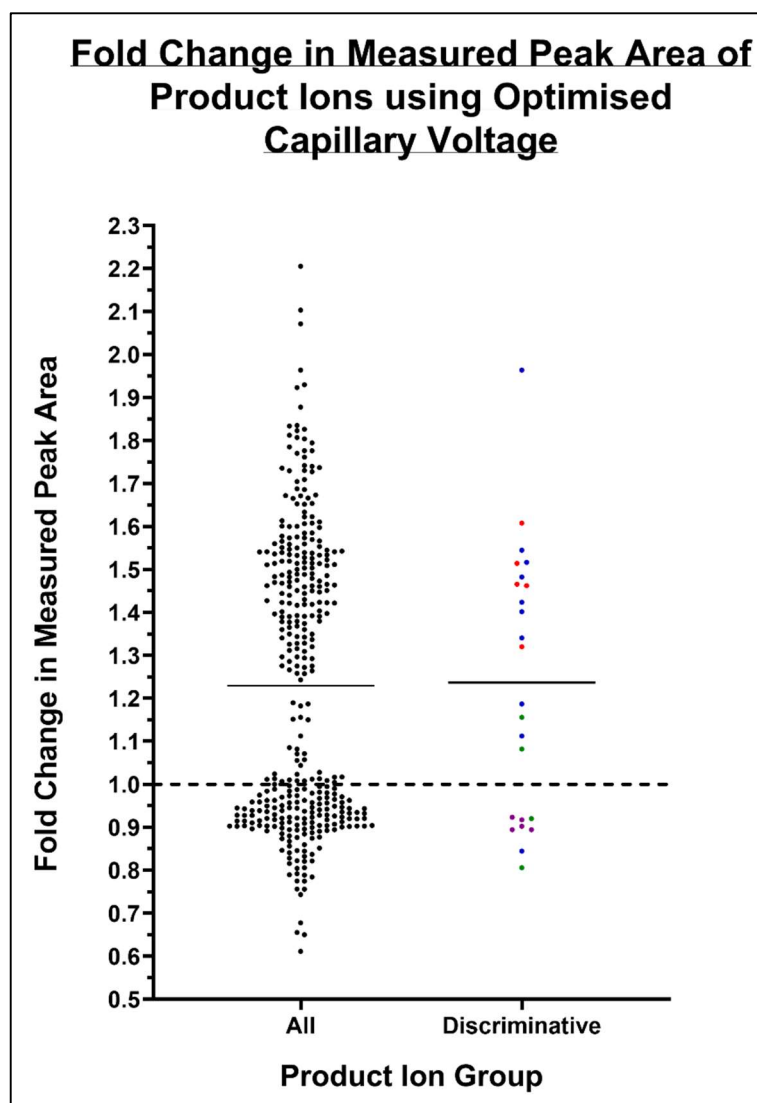


Figure 8-9: Fold change in measured peak area of product ions for 357 MRM transitions monitoring nuclear lamin peptides achieved upon changing capillary voltage from 4000 V to 1500 V (n=1). Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

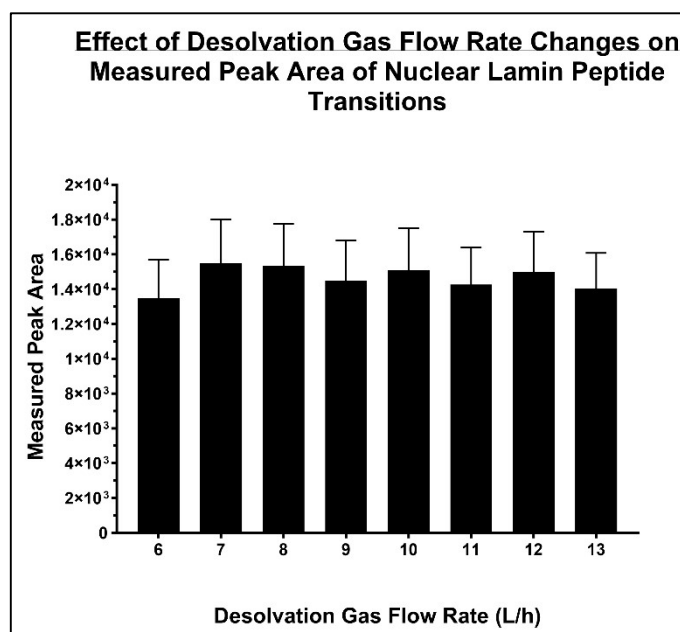


Figure 8-10: Effect of changing the desolvation gas flow rate on the mean measured peak area of 346 nuclear lamin peptide transitions, within the range of 6 L/h to 13 L/h (n=1). Error bars indicate standard error of the mean. One-way ANOVA, p=0.9987.

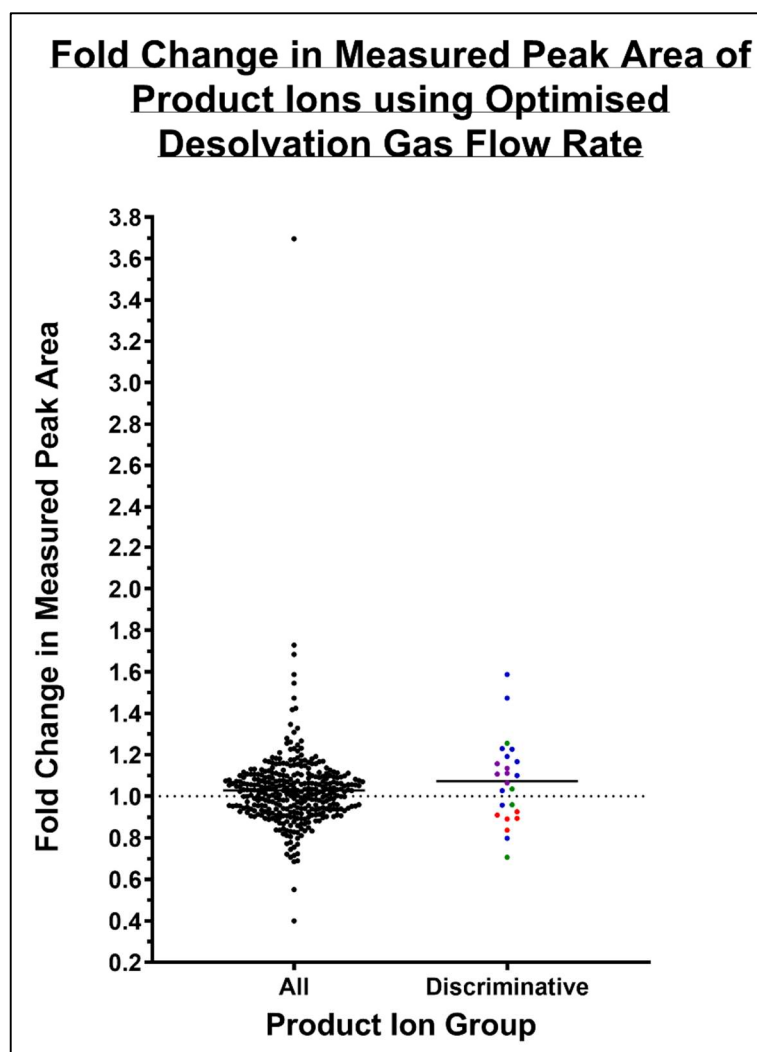


Figure 8-11: Fold change in measured peak area of 346 product ions for MRM transitions monitoring nuclear lamin peptides achieved upon changing the desolvation gas flow rate from 7 L/h to 12 L/h (n=1). Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

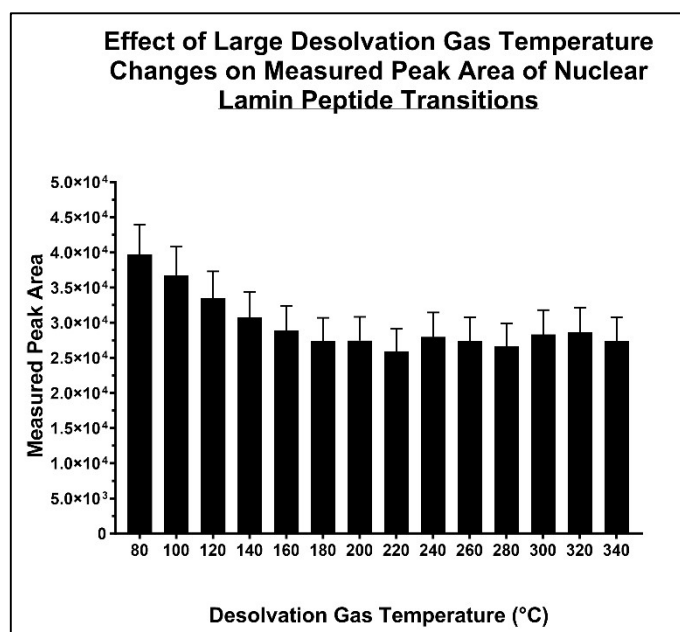


Figure 8-12: Effect of changing the desolvation gas temperature on the mean measured peak area of 339 nuclear lamin peptide transitions, within the range of 80°C to 340°C (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.1858$.

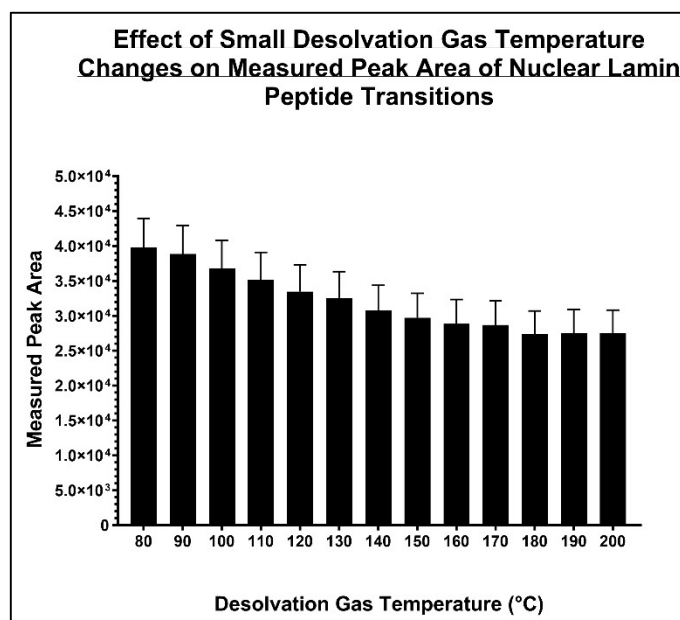


Figure 8-13: Effect of changing the desolvation gas temperature on the mean measured peak area of 339 nuclear lamin peptide transitions, within the range of 80°C to 200°C (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.1579$.

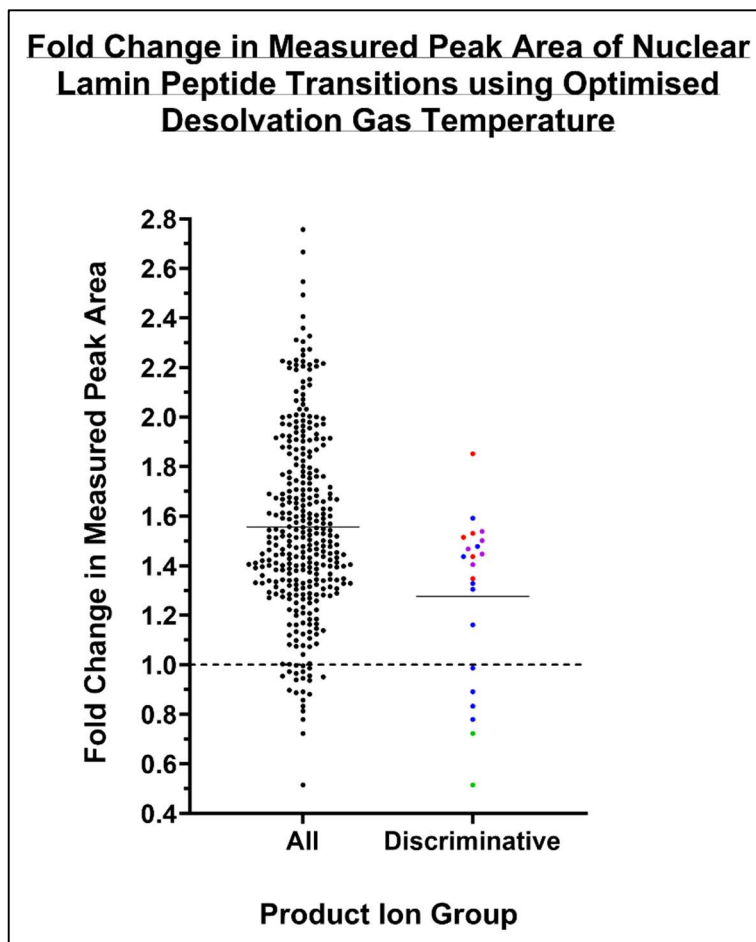


Figure 8-14: Fold change in measured peak area of 334 product ions for MRM transitions monitoring nuclear lamin peptides achieved upon changing the desolvation gas temperature from 300°C to 80°C (n=1). Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

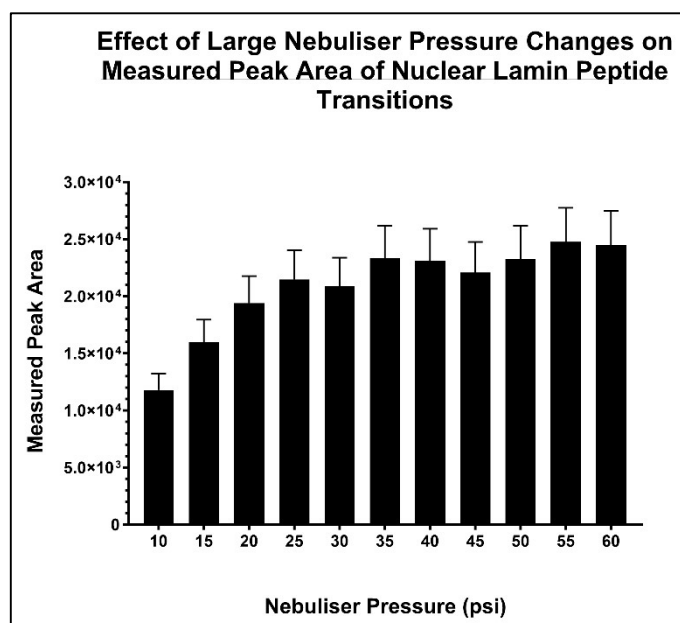


Figure 8-15: Effect of changing the nebuliser pressure on the mean measured peak area of 346 nuclear lamin peptide transitions, within the range of 10 psi to 60 psi (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.0111$, *.

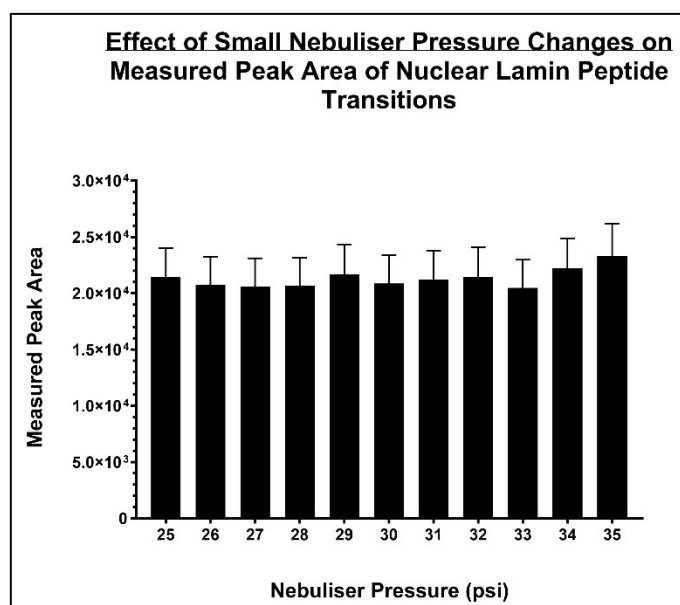


Figure 8-16: Effect of changing the nebuliser pressure on the mean measured peak area of 346 nuclear lamin peptide transitions, within the range of 25 psi to 35 psi (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.9998$.

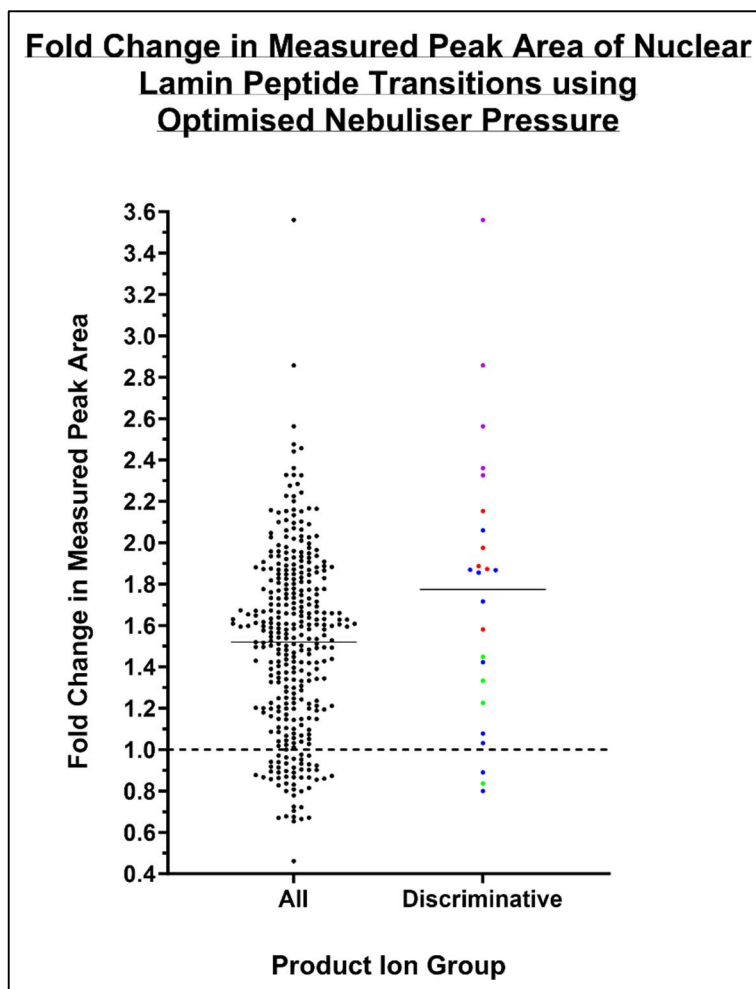


Figure 8-17: Fold change in measured peak area of 346 product ions for MRM transitions monitoring nuclear lamin peptides achieved upon changing the nebuliser pressure from 15 psi to 60 psi (n=1). Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

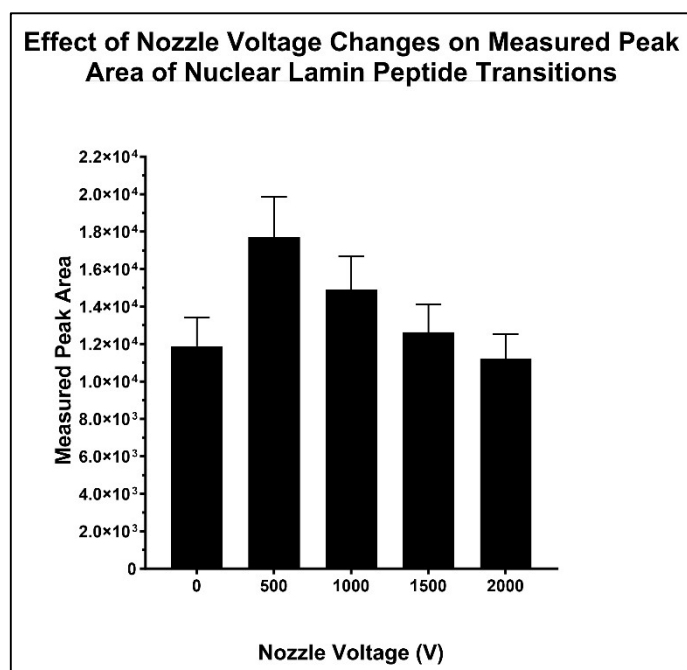


Figure 8-18: Effect of changing the nozzle voltage on the mean measured peak area of 356 nuclear lamin peptide transitions, within the range of 0 V to 2000 V (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.0424$, *.

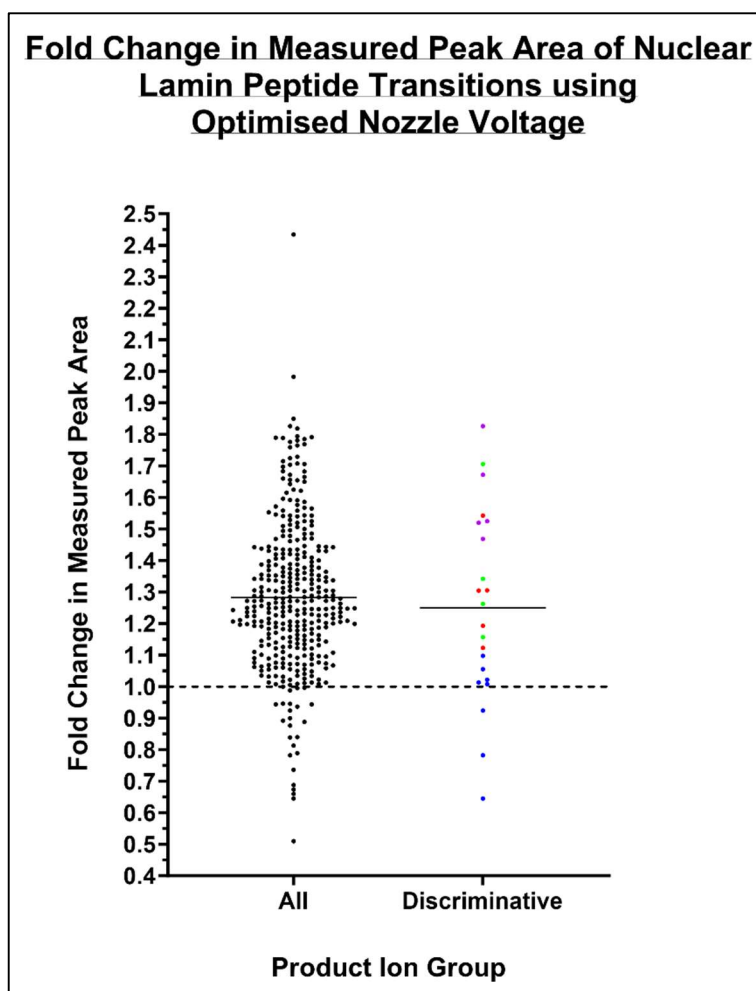


Figure 8-19: Fold change in measured peak area of 356 product ions for MRM transitions monitoring nuclear lamin peptides achieved upon changing the nozzle voltage from 1500 V to 500 V (n=1). Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

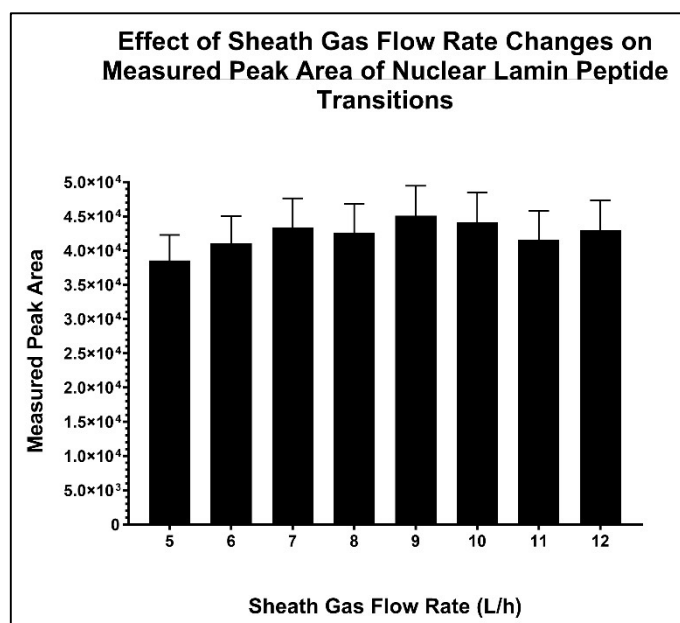


Figure 8-20: Effect of changing the sheath gas flow rate on the mean measured peak area of 362 nuclear lamin peptide transitions, within the range of 5 L/h to 12 L/h (**n=1**). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.9763$.

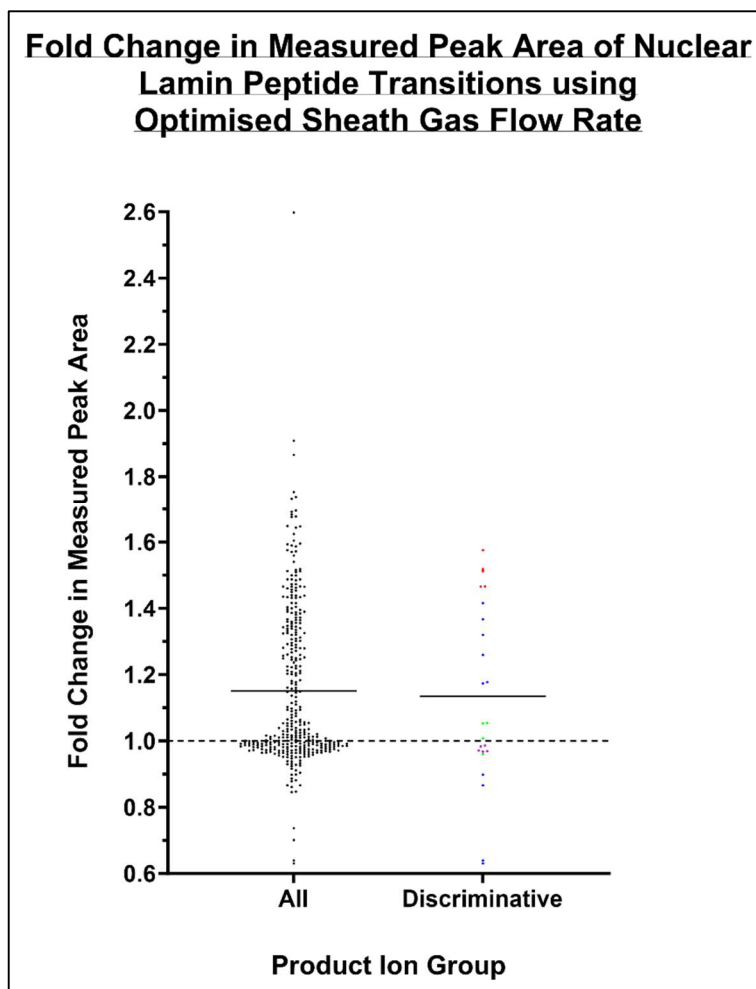


Figure 8-21: Fold change in measured peak area of 362 product ions for MRM transitions monitoring nuclear lamin peptides achieved upon changing the sheath gas flow rate from 11 L/h to 9 L/h (n=1). Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

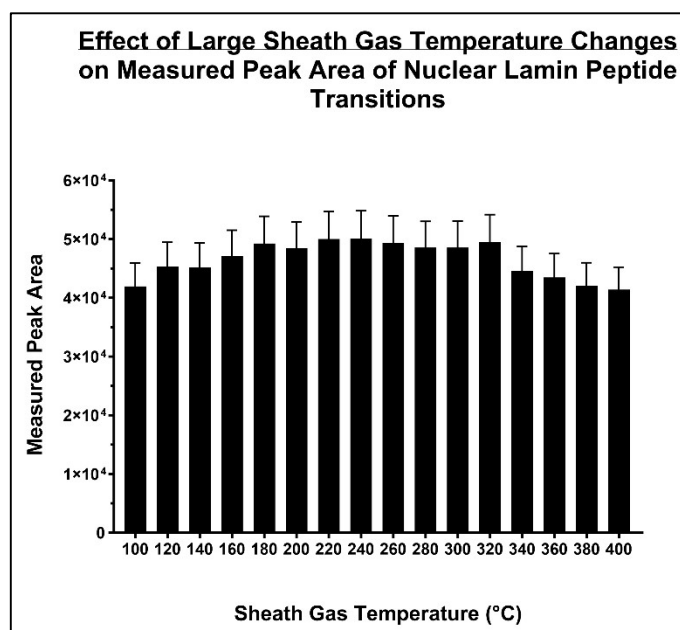


Figure 8-22: Effect of changing the sheath gas temperature on the mean measured peak area of 356 nuclear lamin peptide transitions, within the range of 100°C to 400°C (n=1). Error bars indicate standard error of the mean. One-way ANOVA, $p=0.9361$.

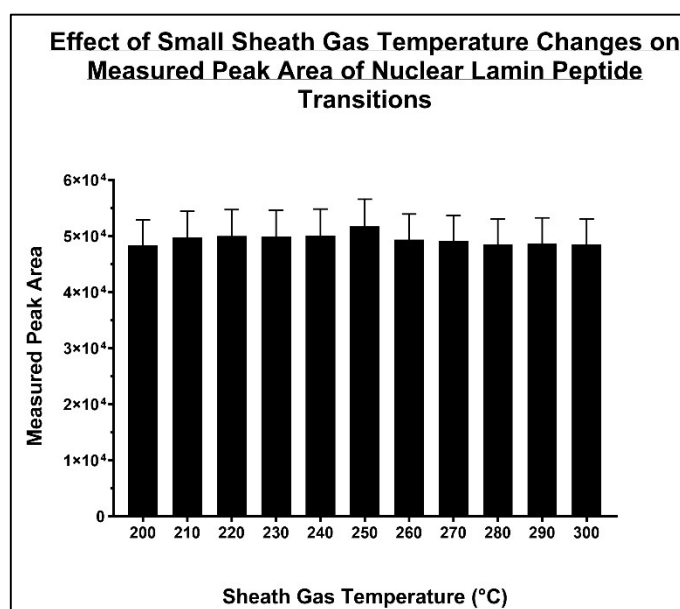


Figure 8-23: Effect of changing the sheath gas temperature on the mean measured peak area of 356 nuclear lamin peptide transitions, within the range of 200°C to 300°C (n=1). Peak area was measured in 10°C increments. Error bars indicate standard error of the mean. One-way ANOVA, $p>0.9999$.

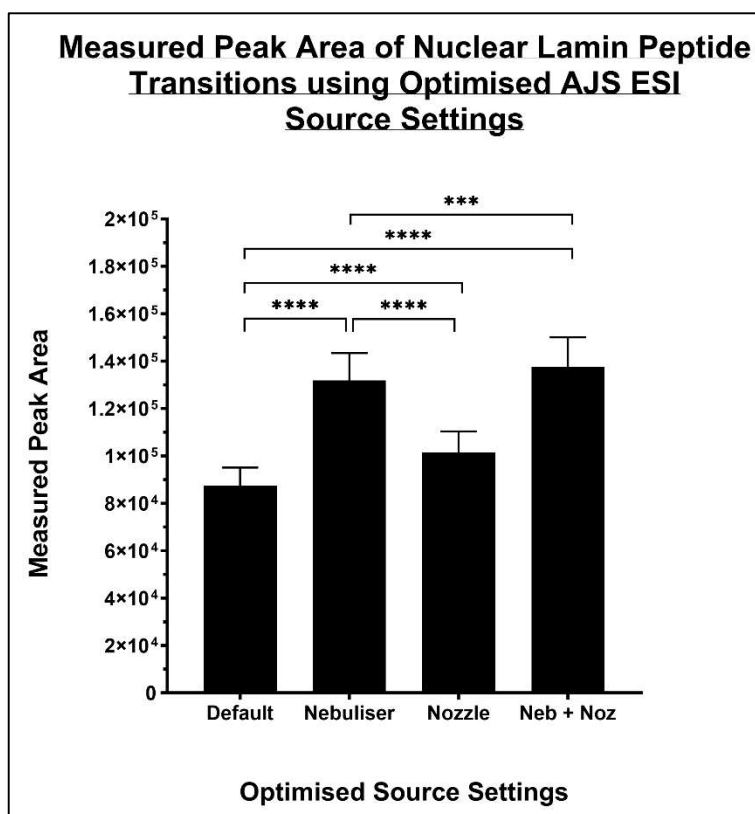


Figure 8-24: Mean measured peak area of 382 nuclear lamin peptide product ions achieved using optimised source settings (n=3). Error bars indicate standard error of the mean (SEM). “Neb + Noz” indicates the peak area achieved when both the nebuliser pressure and nozzle voltage were altered to their empirically determined optimal settings. Paired t-test (Default vs Nebuliser, $p < 0.0001$. Default vs Nozzle, $p < 0.0001$. Default vs Neb + Noz, $p < 0.0001$. Nebuliser vs Nozzle, $p < 0.0001$. Nebuliser vs Neb + Noz, $p < 0.0001$. Nozzle vs Neb + Noz, $p = 0.0003$).

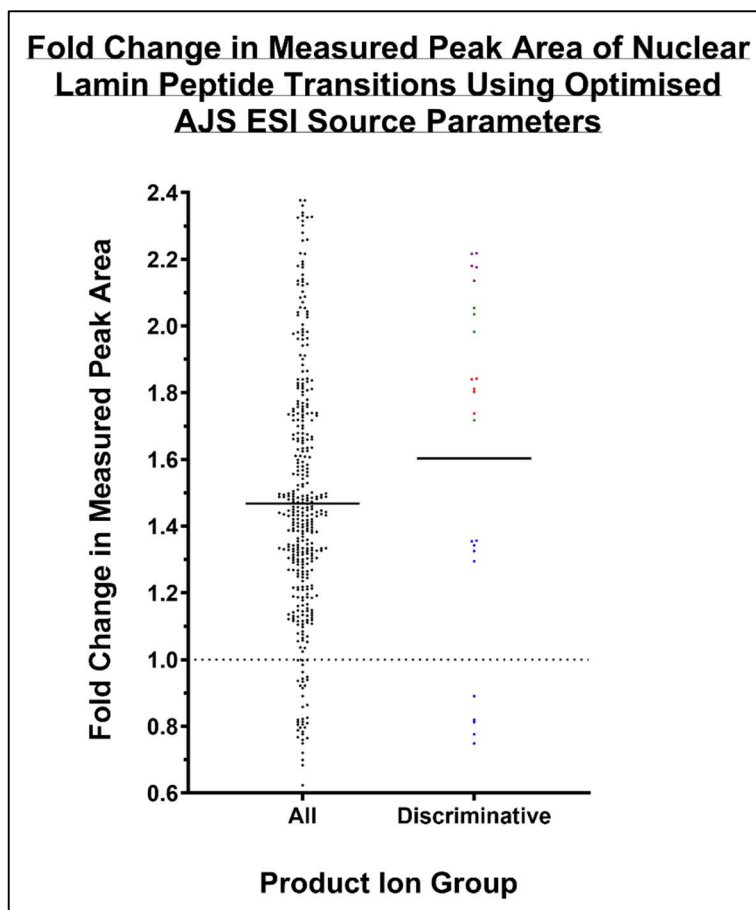


Figure 8-25: Fold change in measured peak area of 382 product ions for MRM transitions monitoring nuclear lamin peptides achieved upon optimising the AJS ESI source settings (n=3). Discriminative dataset indicates peptides of significant interest. Red = pan-lamin peptide LLEGEEER (found in all nuclear lamin isoforms). Green = lamin C-specific peptide VSGSR. Blue = lamin A-specific peptide GSHCSGSGDPAEYNLR (2+ and 3+ precursor charges measured) (mouse only). Pink = pan B-type lamin peptide LSPSPSSR. The mean is indicated by the horizontal line.

8.5. Appendix V

Table 8-VII: MRM transitions used for quantitation of lamin B1 and lamin B2 in BL-2, OPM-2 and U266 cell lines.

Target Protein	Peptide Sequence	Precursor ion m/z	Precursor ion charge	Product ion m/z	Product ion charge	Fragment	CV (%)
Lamin B1	LAVYIDK	411.242004	2	708.392667	1	y6	0.33-19.95%
				538.287139		y4	
				375.223811		y3	
	ALYETELADAR	626.314417	2	1067.50038	1	y9	
				904.437051		y8	
				775.394458		y7	
	ALDDTAR	381.193042	2	577.25763	1	y5	
				462.230687		y4	
	AEHDQLLLNYAK	707.869891	2	721.424302	1	y6	
				608.340238		y5	
	EYEAALNSK	512.750921	2	603.346051	1	y6	
				461.271824		y4	
	LVEVDSGR	437.735074	2	662.310394	1	y6	
				434.199387		y4	

				319.172444		y3	
	LAQALHEMR	534.784575	2	884.440697	1	y7	
				756.382119		y6	
	EELMESR	447.205292	2	635.318122	1	y5	
				522.234058		y4	
				391.193573		y3	
	IESLSSQLSNLQK	723.893563	2	1204.653192	1	y11	
				1004.5371		y9	
				589.330401		y5	
	Lamin B2	ALELEND R	2	646.315479	1	y5	0.22-13.38%
				533.231415		y4	
		VLDETAR	2	704.357344	1	y6	
				591.27328		y5	
				476.246337		y4	
		LVEVDSSR	2	692.320959	1	y6	
				464.209952		y4	

Table 8-VIII: MRM transitions used for quantitation of lamin B1 and lamin B2 in BL-2 AID ^{+/+} and BL-2 AID ^{-/-} cell lines.

Protein	Peptide	Precursor ion m/z	Precursor ion charge	Product ion m/z	Product ion charge	Fragment	CV range (%)
Lamin B1	LAVYIDK	411.242004	2	708.392667	1	y6	1.18-19.63
				637.355553		y5	
				375.223811		y3	
	ALYETELADAR	626.314417	2	904.437051	1	y8	
				775.394458		y7	
				432.220122		y4	
	ALDDTAR	381.193042	2	577.25763	1	y5	
				462.230687		y4	
				347.203744		y3	
	AEHDQLLLNYAK	472.249019	3	721.424302	1	y6	
				608.340238		y5	
	EYEAALNSK	512.750921	2	603.346051	1	y6	
				532.308937		y5	
				461.271824		y4	
	DAALATALGDK	523.279846	2	859.488358	1	y9	

			788.451245		y8
			675.367181		y7
QLADETLLK	515.792589	2	789.43526	1	y7
			718.398146		y6
			603.371203		y5
LVEVDSGR	437.735074	2	662.310394	1	y6
			533.267801		y5
			319.172444		y3
QIEYEEK	486.737282	2	731.324647	1	y5
			602.282054		y4
LAQALHEMR	534.784575	2	756.382119	1	y6
			685.345005		y5
			572.260941		y4
EELMESR	447.205292	2	635.318122	1	y5
			522.234058		y4
			391.193573		y3
IESLSSQLSNLQK	723.893563	2	1004.5371	1	y9
			917.505071		y8

				589.330401		y5	
		482.931467	3	702.414465	1	y6	
				589.330401		y5	
	IGDTSVSYK	485.248014	2	856.404688	1	y8	
				397.208161		y3	
	NSQGEEVAQR	559.265258	2	788.389707	1	y7	
				374.214643		y3	
	ALELENDR	480.243263	2	646.315479	1	y5	
		480.243263		533.231415		y4	
Lamin B2	VLDETAR	402.216517	2	591.27328	1	y5	2.05 - 12.69
	EGELTVAQGR	530.275095	2	631.352199	1	y6	
	SVFEEVR	497.745639	2	808.383559	1	y6	
	LVEVDSSR	452.740356	2	692.320959	1	y6	

8.6. Appendix VI

Table 8-IX: Details on animals used in Chapter 5.

Sample Name	Mouse Model	Treatment	PNA+ cell numbers	PNA- cell numbers
ACRE32	C57BL/6 Cy1-cre- <i>ATG7</i>	8 days NP-Alum immunisation	Total: 2.32×10^4 cell/mL in 1 mL Live: 2.09×10^4 cells/mL Dead: 2.32×10^3 cells/mL Viability: 90%	Total: 22.5×10^6 cells/mL in 5 mL Live: 20×10^6 cells/mL Dead: 27.5×10^5 cells/mL Viability: 88.8%
LCRE39	C57BL/6 Cy1-cre- <i>LMNB1</i>	8 days NP-Alum immunisation	Total: 2.13×10^5 cells/mL in 1 mL Live: 9.04×10^4 Dead: 1.23×10^5 Viability: 42.4%	Total: 4.47×10^6 Live: 3.53×10^6 Dead: 9.34×10^5 Viability: 79.1%
A83	ADLD (C57BL/6 with <i>LMNB1</i> -tg)	4 days OVA+LPS immunisation	N/A	N/A
A116	ADLD (C57BL/6 with <i>LMNB1</i> -tg)	4 days OVA+LPS immunisation	Total: 1.60×10^5	Total: 4.63×10^6 cells/mL in 5 mL

			Live: 7.15×10^4 Dead: 8.81×10^4 Viability: 41%	Live: 3.46×10^6 Dead: 1.17×10^6 Viability: 79.7%
A118	ADLD (C57BL/6 with <i>LMNB1</i> -tg)	4 days OVA+LPS immunisation	Total: 6.26×10^4 Live: 3.71×10^4 Dead: 2.55×10^4 Viability: 59.3%	Total: 1.33×10^6 Live: 1×10^6 Dead: 3.24×10^6 Viability: 75.4%
A121	C57BL/6 wild type	4 days OVA+LPS immunisation	Total: 5.26×10^5 Live: 3.27×10^5 Dead: 1.99×10^5 Viability: 62.1%	Total: 4.50×10^6 Live: 3.68×10^6 Dead: 8.23×10^5 Viability: 81.7%
FL06	C57BL/6 wild type	4 days OVA+LPS immunisation	N/A	N/A
FL08	C57BL/6 wild type	4 days OVA+LPS immunisation	N/A	N/A