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Analysis and Quantification of Post-Translational Modifications in Proteomic Datasets from Tissue-Derived Amyloidosis Samples

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1. Introduction

1.1. Amyloidosis and Amyloid Fibril Formation

A group of disorders collectively known as **amyloidoses** are defined by the presence of amyloid fibres in the affected tissues, as the name suggests [1]. These present highly ordered, cross- β -sheet insoluble structures originating from misfolding and aggregation of autologous proteins [1], [2], [3]. The progressive accumulation of amyloid deposits can disrupt tissue architecture and impair organ function, potentially leading to severe clinical pathologies and organ failure causing several serious diseases, including Alzheimer's disease, type 2 diabetes, and cardiac amyloidosis [1], [2], [4], [5].

More than 40 human proteins have been identified as amyloidogenic precursors, with amyloidoses being classified as sporadic (around 25 of them) or hereditary (around 15) according to the mode of transmission [6].

Table 1.1 Amyloid fibril proteins and their precursors in human (Buxbaum et al., 2022)

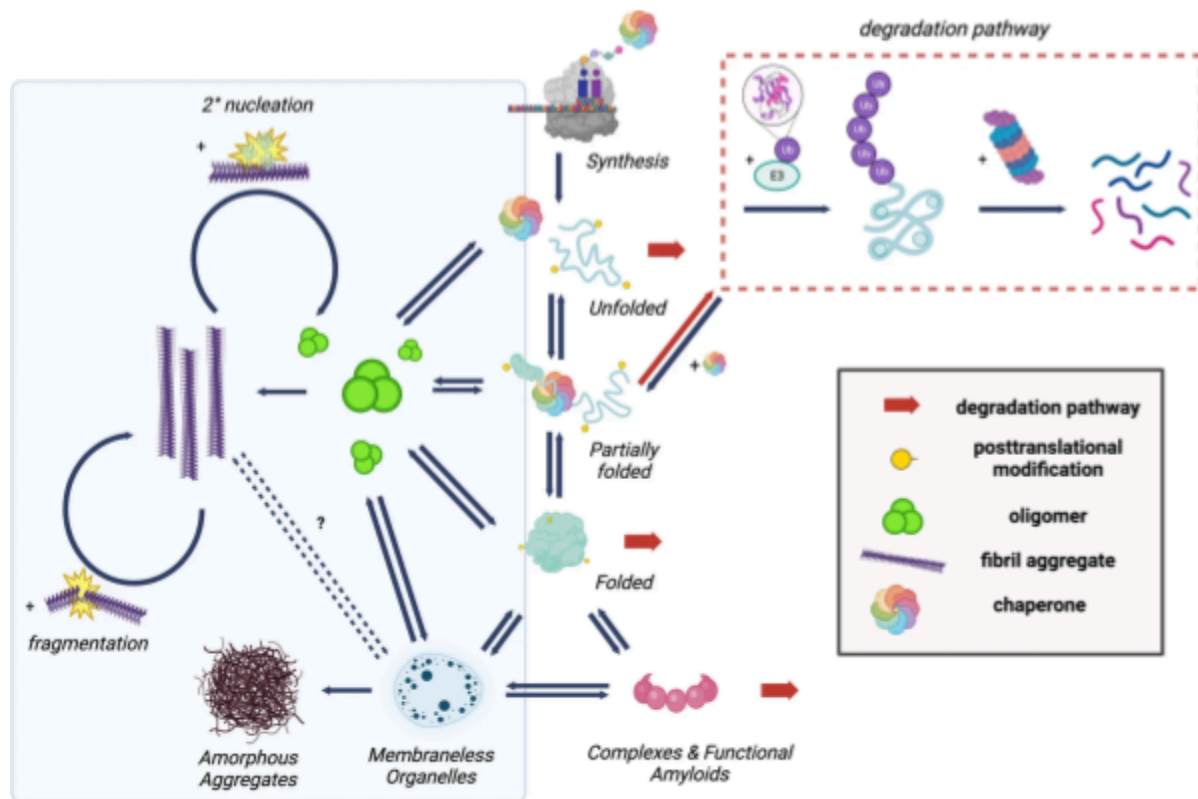
Fibril protein	Precursor protein	Systemic and/or localised	Acquired or hereditary	Fibril protein	Precursor protein	Systemic and/or localised	Acquired or hereditary
AL	Immunoglobulin light chain	S, L	A, H	ATMEM106B	Transmembrane 106B (TMEM106B)	L	A
AH	Immunoglobulin heavy chain	S, L	A	ACal	(Pro)calcitonin	L S	A A
AA	(Apo) Serum amyloid A	S	A, H	AIAPP	Islet amyloid polypeptide	L	A
ATTR	Transthyretin, wild type	S	A	AANP	Atrial natriuretic peptide	L	A
	Transthyretin, variants	S	H	APro	Prolactin	L	A
A β 2M	β 2-microglobulin, wild type	S	A	ASom	(Pro)somatostatin	L	A
	β 2-microglobulin, variants	S	H	AGluc	Glucagon	L	A

AApoAI	Apolipoprotein A I, variants	S	H	APTH	Parathyroid hormone	L	A
AApoAII	Apolipoprotein A II, variants	S	H	AIns	Insulin	L	A
AApoAIV	Apolipoprotein A IV, wild type	S	A	AEnf	Enfurvitide	L	A
AApoCII	Apolipoprotein C II, variants	S	H	AGLP1	Glucagon-like peptide 1 analog	L	I
AApoCIII	Apolipoprotein C III, variants	S	H	AIL1RAP	Interleukin-1 receptor antagonist protein	L	
AGel	Gelsolin, variants	S	H	ASPCd	Lung surfactant protein	L	A
ALys	Lysozyme, variants	S	H	ACor	Corneodesmosin	L	A
ALECT2	Leukocyte chemotactic factor-2	S	A	AMed	Lactadherin (MFG-E8)	L	A
AFib	Fibrinogen α , variants	S	H	AKer	Kerato-epithelin	L	A
ACys	Cystatin C, variants	S	H	ALac	Lactoferrin	L	A
ABri	ABriPP, variants	S	H	AOAAP	Odontogenic ameloblast-associated protein	L	A
ADanb	ADanPP, variants	L	H	ASem1	Semenogelin 1	L	A
A β	A β protein precursor, wild type	L	A	ACatKe	Cathepsin K	L	A
	A β protein precursor, variant	L	H				
A α Syn	α -Synuclein	L	A	AEFEMP1e	EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1)	L	A
ATau	Tau	L	A				
APrP	Prion protein, wild type	L	A				
	Prion protein variants	L	H				
	Prion protein variant	S	H				

Although these disorders differ in the precursor protein involved, tissue distribution, and clinical presentation, they share a common molecular feature: the formation of protein aggregates with characteristic amyloid architecture. Recent advances in cryogenic electron microscopy (cryo-EM) have enabled the determination of amyloid fibril structures at near-atomic resolution [7], [8]. These studies have revealed that amyloid fibrils contain highly ordered cores stabilized by extensive intermolecular interactions. However, despite the structural insights provided by cryo-EM, many questions remain regarding the biochemical processes that contribute to fibril maturation, persistence, and the mechanisms of organ dysfunction.

The process of protein misfolding can be triggered by genetic mutations, oxidative stress, changes in pH or ionic strength, abnormal post-translational modifications (PTMs), proteolytic cleavage, and disturbances in intracellular trafficking or protein quality-control mechanisms [9], [10]. Under physiological conditions, molecular chaperones, the ubiquitin proteasome system, and the autophagy-lysosome pathway maintain proteostasis by facilitating protein refolding or degradation; however, when these mechanisms become overwhelmed or impaired, misfolded proteins can accumulate and initiate aggregation [9], [11], [12]. Partially unfolded proteins expose hydrophobic regions that promote intermolecular interactions, leading to the formation of soluble oligomers, protofibrils, and eventually highly ordered amyloid fibrils characterized by a cross- β -sheet architecture [9], [10], [12]. Mature fibrils may additionally contribute to toxicity through extracellular deposition, disruption of tissue architecture, sequestration of functional proteins, and continued generation of toxic oligomers [12], [13], [14]. Collectively, these observations indicate that organ toxicity arises from a combination of direct cellular injury caused by misfolded protein species and the structural and functional consequences of progressive amyloid deposition, although the relative contribution of oligomers and mature fibrils varies between amyloidosis types and remains incompletely understood [12], [13], [14], [15].

Figure 1.1 Proteins different conformational states and fibrils formation in the cell. [Rinauro, D. J., 2024](#)



From a clinical point of view, disease phenotype, prognosis and management critically depend on the specific fibril precursor protein and on the involved organs [12], [13], [14]. Among the systemic forms, i.e. those in which amyloid deposition is widespread in the body, light chain (AL) and transthyretin (ATTR) amyloidoses are the most frequent types overall. In these forms, several organs can be affected, but involvement of the heart is especially dangerous and significantly affects the prognosis. Other types, however, are frequent in specific geographic regions or ethnic groups, such as those caused by apolipoprotein AI (AAPOAI) in Northern Italy and by Leukocyte Chemotactic Factor 2 (ALECT2) in Latin Americans.

In the case of cardiac targeting, amyloid accumulation can compromise cardiac function by increasing myocardial stiffness and impairing diastolic relaxation, while renal deposition can disrupt glomerular architecture and filtration [13], [14]. Other possibly affected sites are the gastrointestinal tract, liver, soft tissues and systemic/autonomic nervous systems [13], [14]. In neurodegenerative diseases, the accumulation of amyloid- β and tau is associated with neuronal

and synaptic dysfunction in Alzheimer's disease, while α -synuclein aggregation contributes to cellular dysfunction in Parkinson's disease [11], [12]. Similarly, pancreatic accumulation of misfolded islet amyloid polypeptide (amylin) can damage β -cells, promote apoptosis, and contribute to the loss of functional β -cell mass in type 2 diabetes [15].

1.2. Proteomics and Mass Spectrometry in Amyloid Research

Given the central position of protein aggregates in the pathogenesis of amyloidoses, mass spectrometry-based proteomics has become an essential approach for investigating these diseases. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) enables comprehensive identification and characterization of proteins present within complex biological samples while simultaneously providing information regarding protein abundance, sequence coverage, and post-translational modifications. Mass spectrometry can be used to analyze intact proteins (top-down analysis), but most often identification and characterization are obtained through the analysis of peptides generated by enzymatic digestion of protein samples (so called bottom-up approach), most often using trypsin [16], [17].

In relation to amyloidosis, proteomic analyses are useful for several important purposes. First, they enable identification of the amyloidogenic protein [18], [19]. Second, they provide insights into the molecular composition of amyloid deposits, including co-deposited proteins and associated biological factors [20]. Thanks to its ability to identify the deposited protein directly, without the need of antibodies, proteomics has become a mainstay of the diagnostic process in these diseases, and tens of thousands of datasets from patients' samples are already available in public or private repositories [21], [22].

In addition to qualitative identification of the amyloidogenic protein, quantitative proteomics enables the comparison of protein abundance between biological samples, providing information about differences associated with disease, tissue type, or experimental conditions [16], [23]. Differential proteomics can therefore be used to investigate changes in the protein composition of amyloid-containing tissues relative to appropriate control samples [22], [24]. Several quantitative proteomics approaches have been developed, with label-free quantitation

being the most frequently used in the case of tissue samples where no labeling procedures can be applied. Label-free quantification (LFQ) computes protein abundance based on the intensity or ion chromatogram peak area of the corresponding peptide ions. Differential proteins may provide candidates for further investigation as tissue biomarkers or as components of molecular pathways associated with amyloid deposition and proteotoxicity [22].

In addition to protein identification and quantitation, proteomics allows the investigation and site-specific localization of post-translational modifications (PTMs), which can independently influence protein structure, aggregation behavior, and disease-associated molecular changes. Therefore, the analysis of PTMs provides an additional level of information to dissect the molecular mechanisms of diseases. Although specific protocols are often applied to increase sensitivity towards low abundance modified peptides, PTM analysis can be performed also on standard proteomic datasets, including proteomics data already available from diagnostic samples. On these, multiple PTMs can be simultaneously identified. Comparison of modification patterns in different disease stages allows identifying proteins and pathways that may be affected during disease development.

1.3. Protein Post-Translational Modifications (PTMs) and Their Role in Physiology and Pathology

Post-translational modifications (PTMs) are chemical alterations that occur after protein synthesis and contribute significantly to the functional diversity of the proteome [25], [26].

More than 400 types of PTMs have been identified so far [27]. These modifications include the addition of chemical groups, such as phosphorylation, acetylation, and methylation, the attachment of complex molecules, such as glycans and lipids, the conjugation of small proteins, as in ubiquitination, and the modification of individual amino acid residues as in oxidation, deamidation or glycation. Proteolytic cleavage is also commonly considered a post-translational processing event. Some PTMs, such as phosphorylation and acetylation, are regulated by enzymes that add or remove the modifying groups, whereas other PTMs like deamidation can occur through spontaneous chemical reactions and are generally considered irreversible under physiological conditions. Although hundreds of PTM types are known, their

biological roles and proteome-wide distributions have been characterized to varying degrees. Phosphorylation, ubiquitination, acetylation, and glycosylation are among the most extensively studied modifications [27], [28].

PTMs are increasingly recognized as important contributors to both normal physiology and disease processes [29]. PTMs can affect protein folding, conformational stability, aggregation propensity, and interactions with other biomolecules by altering the physicochemical properties of proteins. Consequently, abnormal PTM patterns have been implicated in many pathological conditions, including cancer, neurodegenerative disorders, cardiovascular diseases, and protein aggregation disorders [30], [31], [32].

The identification and characterization of these modifications can provide insight into the molecular mechanisms underlying dysfunction. Table 1.2 summarizes selected PTMs, their principal target residues, the mass shift they account for, the chemical changes they introduce, and some of their established functional implications [27], [29], [32], [33], [34], [35].

Table 1.2. Selected post-translational modifications and their functional implications

PTM	Target residue(s)	Mass shift (Δ mass, Da)	Chemical change	Selected functional implications
Phosphorylation	Ser, Thr, Tyr	+79.9663	Addition of a phosphate group	Regulates enzyme activity, cell signalling, protein interactions, and cellular responses.
Acetylation	Lys, protein N-terminus	+42.0106	Addition of an acetyl group	Alters protein charge and interactions, regulates protein stability, activity, and chromatin structure.
Methylation	Lys, Arg	+14.0157 (mono), +28.0313 (di), +42.0470 (tri)	Addition of one or more methyl groups	Modulates protein interactions, gene regulation, and signalling.
Ubiquitination	Lys	+114.0429 (diGly remnant after tryptic digestion)	Covalent attachment of ubiquitin	Regulates protein degradation, trafficking, signalling, and protein quality control.
Glycosylation	Asn (N-linked), Ser, Thr (O-linked)	Variable	Attachment of carbohydrate structures	Influences protein folding, stability, trafficking, and cell recognition.

Hydroxylation	Pro	+15.9949	Addition of a hydroxyl group (–OH)	Contributes to collagen stability and extracellular matrix organization, can regulate oxygen-sensing pathways.
Deamidation	Asn, Gln	+0.984	Conversion of a side-chain amide into a carboxyl group, potentially forming Asp/isoAsp from Asn	Alters local charge and structure, may affect protein stability, interactions, and aggregation propensity.
Oxidation	Met, also Trp and other residues	15.9949 (single oxygen addition)	Addition of oxygen or other oxidative chemical changes	Can alter protein activity, conformation, stability, and redox signalling.
S-nitrosylation	Cys	+28.9902	Attachment of a nitric oxide-derived group to a thiol	Modulates redox-sensitive signalling and protein activity.
palmitoylation	Cys (S-palmitoylation)	+238.2297	Covalent attachment of a lipid group	Influences membrane association, subcellular localization, and protein interactions.
Disulfide bond formation	Cys–Cys	–2.0157 per disulfide bond	Formation of a covalent S–S bond between cysteine residues	Stabilizes protein structure and supports folding and extracellular protein integrity.

The role of PTMs in the context of amyloid diseases is scarcely characterized; their contribution could indeed be relevant at multiple levels. In the first place, PTMs may influence multiple stages of the aggregation pathway. Modifications such as oxidation, deamidation and glycosylation can alter the precursor protein stability, promote or inhibit fibril formation, affect fibril architecture, and modify interactions between amyloid deposits and surrounding tissues [30], [36], [37]. On a distinct level, modulation of the extent of specific PTMs within resident cells in target tissues could influence the cell's physiology and contribute to the still underdefined mechanisms of organ damage in these diseases. While initial information is available on a subset of PTMs affecting the amyloidogenic precursors, virtually nothing is known regarding changes in protein PTMs in affected tissues [22], [24].

1.4. Deamidation as a Modification Associated with Protein Ageing

Protein deamidation is an important non-enzymatic post-translational modification associated with molecular aging and the progressive alteration of protein structure and function. It involves the conversion of asparagine (Asn) and glutamine (Gln) residues into their corresponding acidic residues, principally aspartate (Asp) and glutamate (Glu), respectively. Asparagine deamidation can also generate isoaspartate (isoAsp), which introduces an additional methylene group into the peptide backbone and changes its geometry. Consequently, deamidation may contribute to the gradual accumulation of molecular damage in long-lived proteins and has been associated with several age-related degenerative processes [38], [39], [40]. The rate of deamidation varies considerably between individual residues and proteins. It is influenced by the amino acid sequence surrounding susceptible residue, protein conformation and structural flexibility, pH, temperature, ionic strength, and the local chemical environment. Under physiological conditions, sequence-dependent half-times for deamidation can range from approximately 1 to 500 days for asparagine residues and from 100 to 5,000 days for glutamine residues, although the actual rates in intact proteins may differ because of structural constraints [41]. The biological consequences of deamidation extend beyond its value as a marker of protein residence time [42]. By introducing additional negative charge and, in the case of isoAsp formation, altering the peptide backbone, deamidation can change local conformation, protein stability, molecular recognition, and susceptibility to degradation [39], [42]. Depending on the site and protein context, these changes may impair normal protein function, destabilize folded structures, or promote aggregation. Deamidation has consequently been investigated in relation to cataract formation and neurodegenerative disorders [39], [40], [42].

From a proteomics perspective, reliable measurement of age-associated deamidation presents an additional challenge because the modification can occur both *in vivo* and *in vitro*. Deamidation introduced during sample storage, protein extraction, or enzymatic digestion can be mistaken for an endogenous modification. This is particularly relevant to mass spectrometry-based studies, in which deamidation is commonly detected through a mass increase of approximately 0.984 Da following conversion of Asn or Gln. Although this mass shift

supports identification of modified peptides, confident characterization requires appropriate peptide-spectrum evidence, consideration of alternative assignments, and control of preparation-induced artifacts. Site-specific quantification is therefore essential when comparing samples, especially when the objective is to distinguish biological differences in modification abundance from technical variation [39].

Deamidation represents a biologically relevant age-associated modification because its non-enzymatic kinetics permit the progressive accumulation of molecular changes in susceptible proteins, particularly those with long residence times. Investigating its site-specific distribution and abundance using mass spectrometry can therefore contribute to understanding how post-translational modifications accumulate in long-lived proteins and how these changes relate to tissue aging and degenerative pathology.

1.5. Lysine Acetylation

Lysine acetylation is a PTM in which an acetyl group is added to the ϵ -amino group of a lysine residue. Although acetylation was initially studied mainly in histones and its role in chromatin regulation, subsequent proteomic studies have established that it is widespread among non-histone proteins, including enzymes involved in metabolism and cellular signaling [43], [44]. Enzymatic lysine acetylation is regulated by three functional groups of proteins. Lysine acetyltransferases (KATs) transfer acetyl groups from acetyl-coenzyme A (acetyl-CoA) to lysine residues, lysine deacetylases (KDACs) remove acetyl groups, and acetyllysine-binding proteins, including proteins containing bromodomains, recognise acetylated residues and mediate downstream interactions. However, Non-enzymatic acetylation can also occur through chemical reactions between lysine residues and acetyl-CoA, particularly in the mitochondrial environment. Through the coordinated action of these writers, erasers and readers, lysine acetylation can function as a reversible regulatory mechanism linking cellular metabolism to protein function [43], [44], [45]. The functional consequences of lysine acetylation are context-dependent. In cellular metabolism, acetylation can influence the activity of mitochondrial enzymes and proteins involved in energy production [46], [47]. In *Salmonella enterica*, demonstrated that acetylation altered the activities of key metabolic enzymes involved

in glycolysis, gluconeogenesis and the distribution of metabolic flux through central pathways, illustrating how this post-translational modification can coordinate metabolic responses to different carbon sources [48]. Lysine acetylation has also been investigated in cancer, where changes in acetylation-related enzymes and protein targets may contribute to altered cellular signaling and disease-associated processes [44].

This modification has also been found in amyloidogenic proteins [49]. For example, acetylation of the tau protein promotes its aggregation and inhibits the degradation of phosphorylated tau [50], [51]. It has also been observed that, in a mutant form of huntingtin, acetylation of the N-terminal methionine promotes aggregation, whilst acetylation of lysine at position 444 plays a role in neuronal protection by facilitating the clearance of the mutant protein [44], [52]. Analysis of other ex vivo fibres from a patient with ALECT2 also revealed the presence of acetylated forms at certain lysine residues, the authors of this study argue that masking the positive charge of lysine inhibits the formation of weak bonds that stabilise the native form [53]. Therefore, overall, the effect of acetylation on the formation of amyloid fibrils depends both on the site of modification and on the specific protein involved.

MS-based proteomics is an important approach for identifying and quantifying lysine-acetylation sites. Since acetylated peptides may represent only a small fraction of the total peptide population, enrichment using acetyllysine-specific antibodies can improve their detection [43].

2. Aim of the Study

The overall aim of this thesis was to detect and quantify specific post-translational modifications in proteomic datasets, derived from the liquid-chromatography tandem mass spectrometry (LC-MS/MS) analysis of tissue amyloid samples. Through the integration of quantitative proteomics, PTM analysis, and structural interpretation, I aimed to provide new insights into the molecular alterations associated with amyloid diseases and to improve understanding of how protein modifications contribute to amyloid formation, persistence, and tissue pathology.

More specifically:

- 1) In the first stage, I sought to characterize glutamine deamidation, as a case-study PTMs, in LC-MS/MS datasets from several types of amyloid fibrils extracted from tissues. In parallel to identification, I performed a quantitative evaluation of site-specific occupancies and location of residues exhibiting elevated levels of deamidation. Finally, I mapped the identified deamidated sites on the available structures of amyloid proteins, to get insights on the structural determinants of PTM susceptibility and on the possible contribution of this PTM in misfolding.
- 2) In the second part of my thesis, I performed a proteome-wide analysis of acetylated lysines in whole cardiac tissues from a mouse model of light chain amyloidosis. This study was not only focused on the identification of the modified proteins and peptides, but also on the quantitation of differences in the abundance of each specific modified site between affected animals and controls.

3. Methods

3.1. Sample Processing and Acquisition of LC-MS/MS Data.

For whole tissue proteomics of mice hearts, cardiac ventriculi were obtained from three groups of age and gender matched animals (three animals per group) [2] 1) induced SAI-DH mice (Amyloid-positive, indicated as TG-AMY), 2) non-induced SAI-DH mice (Amyloid-negative, TG-NOSEED), 3) non transgenic SAI mice (Non-TG). Tissues were stored unfixed and frozen until use. Tissue was minced and washed 3 times with ice-cold PBS supplemented with protease inhibitors (Complete, Roche), followed by potter homogenization in lysis buffer (8 M Urea, 0.15% Rapigest, 0.1 M DTT) and centrifugation to remove insoluble material.

For proteomics of AL-H7 fibrils, amyloid aggregates were extracted from frozen autopsied tissues [54]. Briefly, overnight tissue digestion with *C. histolyticum* collagenase (5 mg/ml in a buffer containing 20 mM Tris, 140 mM NaCl, 2 mM CaCl₂, pH 8.0) was followed by 15 cycles of homogenization in Tris EDTA buffer (20 mM Tris, 140 mM NaCl, 10 mM EDTA, pH 8.0) to remove soluble proteins, and by repeated homogenization of the remaining pellet in ultrapure water. The fibril-containing supernatants from the water homogenization cycles were retained.

Protein extracts were reduced with dithiothreitol (DTT, 0.1M) alkylated with iodoacetamide (final concentration 0.15 M) and quantified using the Bradford assay. Twenty µg of proteins per sample were digested overnight in 0.1 M Ammonium Bicarbonate / 5% Acetonitrile with 1 µg of MS-grade trypsin (Trypsin gold, Promega). Peptides were purified using ZipTips, dried and analyzed by mass spectrometry. Peptide mixtures were reconstituted into 1% acetonitrile (ACN) 0.1% TFA in water and transferred to MS vials (Waters) for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. Proteomics analysis was performed on a Thermo Scientific Exploris 120 mass spectrometer coupled to a Vanquish Neo nanoLC system. Peptides were trapped on a Thermo Scientific Acclaim PepMap C18 cartridge (0.3 mm × 5 mm, 5 µm/100 Å) and then separated on a Thermo Scientific Easy-Spray Acclaim PepMap C18 column (75 µm × 15 cm, 3 µm/ 100 Å packing) with a 40-min linear gradient of acetonitrile:water:formic acid (3:97:0.1–44:56:0.1 v/v/v) at 300 nL/ min. A full MS scan (*m/z* 350–1400) was acquired, and the 10 most intense ions were selected for fragmentation (HCD). The whole dataset consists of

samples from three amyloid-positive mice and three control ones, each analysed in three technical replicates (LC-MS/MS runs), resulting in a total of nine amyloid and nine control measurements.

3.2. Retrieval of LC-MS/MS Datasets from Public Repositories

For the analysis of glutamine deamidation of amyloid fibrils, three LC-MS/MS datasets were previously acquired from tissue-derived amyloid fibrils were retrieved from the public proteomics repositories MassIVE and PRIDE: apolipoprotein A-I (ApoA-I) fibrils extracted from the heart of a patient with hereditary ApoA-I amyloidosis carrying the L90P mutation, leukocyte cell-derived chemotaxin 2 (LECT2) fibrils extracted from the kidney of a patient with ALECT2 amyloidosis carrying the I40V variant, and AL59 fibrils extracted from the heart of a patient with light-chain amyloidosis.

The ApoA-I dataset was obtained from MassIVE under accession **MSV000098187**, corresponding to the dataset described by Nguyen et al. (2026) [55], [56]. The LECT2 dataset was obtained from MassIVE under accession **MSV000098220**, corresponding to the study reported by Afrin et al. (2026) [53], [57]. The AL59 dataset was retrieved from PRIDE under accession number **PXD049369**, associated with the study of cardiac AL amyloid fibrils reported by Schulte et al. (2024) [58], [59].

The raw LC-MS/MS files (.raw) were retrieved from the selected public datasets and re-analyzed using Proteome Discoverer version 3.1 to identify proteins, peptides, and glutamine-containing peptides, and to quantify the corresponding modified and unmodified peptide forms. The AL-H7 fibril dataset was generated and processed in the laboratory.

3.3. Identification of Proteins and Specific Post-Translational Modifications.

Raw LC-MS/MS data were processed using the Proteome Discoverer software (Thermo Fisher Scientific) version 3.1, based on the Sequest algorithm [60], using human or mouse protein databases downloaded in November 2024 from UNIPROT and augmented with the corresponding amyloidogenic protein sequences: AL59 and H7 immunoglobulin light chains,

apolipoprotein A-I (ApoA-I), and leukocyte cell-derived chemotaxin 2 (LECT2), as appropriate for the samples analysed.

Peptide and protein identification was performed using Thermo Proteome Discoverer version 3.1.0.638 and the Sequest HT search engine. Trypsin was used as the digestion enzyme with semi-specific cleavage specificity and up to three missed cleavages. Carbamidomethylation of cysteine (+57.021 Da) was set as a fixed modification, while methionine oxidation (+15.995 Da) was included as a variable modification. Depending on the specific analysis, one additional variable modification was included in the search: deamidation of asparagine and glutamine residues for the tissue-derived fibril study, or lysine acetylation (+42.011 Da) for the mouse proteomic study. The maximum number of equal modifications per peptide was set to four. Precursor and fragment mass tolerances were set to 10 ppm and 0.02 Da, respectively. Peptide-spectrum matches and peptides were validated using q-values, with strict and relaxed target false discovery rates (FDRs) of 1% and 5%, respectively. Quantification was based on precursor-ion intensity, normalized using total peptide amount, and protein abundances were calculated by summing peptide abundances. Protein-level hypothesis testing was performed using ANOVA for individual proteins.

3.4. Label-Free Calculation of Peptide and Protein Abundance

Label-free quantification of peptide and protein abundances was performed using the Precursor Ions Quantifier node in Thermo Proteome Discoverer (version 3.1.0.638). Precursor abundances were determined from ion areas, using unique and razor peptides, with shared quantitative results enabled. Abundances were normalized using the total peptide amount, without additional scaling. Protein abundances were calculated by summing peptide abundances, and protein ratios were calculated based on protein abundance. Differential protein abundance was assessed using analysis of variance (ANOVA) for individual proteins. Missing-value imputation was not applied.

3.5. Assessment and Quantification of Peptides containing glutamine deamidation in amyloid fibrils extracted from tissues.

All glutamine-containing peptides identified in the peptide groups table referred to the relevant fibril protein were filtered and examined for the presence of corresponding deamidated forms. The actual presence of the modification was confirmed by manual inspection of the MS/MS spectra, to avoid false assignments. Only spectra with sufficient MS/MS sequence coverage to exclude mis-assignments were considered reliable. For each glutamine residue, the abundance of the deamidated peptide and its corresponding unmodified one was obtained from the chromatography peak areas reported by Proteome Discoverer. Relative deamidation occupancy was estimated as:

$$Occupancy (\%) = \frac{Deamidated\ peptide\ abundance}{Deamidated\ peptide\ abundance + Unmodified\ peptide\ abundance}$$

When both modified and unmodified peptide forms were detected, occupancy was calculated directly from their measured abundances. When only the unmodified peptide was identified and no corresponding deamidated peptide was detected, the residue was considered non-deamidated and assigned an occupancy value of 0%.

3.6. Structural Mapping of Post-Translationally Modified Sites on Available Protein Structures

The identified glutamine residues were mapped onto available cryo-electron microscopy (cryo-EM) fibril structures downloaded from the PDB repository using the following accession numbers: 9NON for LECT2, 9FAA for AL59, and 9PVY for ApoA-I fibrils. Structural visualization and residue annotation were performed using UCSF ChimeraX software version 1.13 [61]. Residues were color-coded according to their deamidation occupancy as follows: Red: highly deamidated residues; Orange: low-level deamidation; Blue: no detectable deamidation. Neighboring side chains were displayed to evaluate potential steric shielding effects.

3.7. Large-Scale Analysis of Post-Translational Modifications in Mouse Cardiac Proteomes

Raw LC-MS/MS files referred to the mice heart proteome were analyzed for a proof of principle investigation of lysine acetylation of lysine. Modified peptides identified by Proteome Discoverer were extracted from the peptide-level results. As described for deamidation, only spectra with sufficient MS/MS sequence coverage to exclude mis-assignments were considered reliable and were considered for further analysis. Peptide abundances were reported by Proteome Discoverer; differences between amyloid samples and controls were statistically evaluated as described later. For each acetylated peptide, the abundance ratio between amyloid samples and controls was also calculated as follows:

$$\text{Peptide Ratio} = \frac{\text{Peptide Abundance}_{\text{Amyloid}}}{\text{Peptide Abundance}_{\text{Control}}}$$

Similarly, a protein abundance ratio was calculated:

$$\text{Protein Ratio} = \frac{\text{Protein Abundance}_{\text{Amyloid}}}{\text{Protein Abundance}_{\text{Control}}}$$

To avoid PTM quantification biases due to different abundance of corresponding proteins, peptide abundance ratios were normalized to the corresponding protein abundance ratios:

$$\text{Normalized Deamidation Ratio} = \frac{\text{Peptide Ratio}}{\text{Protein Ratio}}$$

This normalization was used to identify PTM changes that exceeded those expected from differences in protein abundance alone.

3.8. Statistical Analysis

Differences in protein abundance were statistically evaluated in Proteome Discoverer using ANOVA for individual proteins. Multiple-testing-adjusted p-values were used to assess statistical significance, with adjusted p-values < 0.05 considered significant.

Differences in the abundance of specific modified peptides were evaluated separately using a two-tailed Welch's two-sample t-test. For each biological replicate, abundance values from the three technical replicates were averaged before comparison between groups. A raw p-value < 0.05 was considered statistically significant.

4. Results

4.1. Analysis of Glutamine Deamidation on Extracted Amyloid Fibrils

In the first part of my thesis, I investigated the presence of Q deamidation in amyloid proteins extracted from tissues of patients with systemic amyloidosis. Some proteomic datasets were retrieved from public repositories (PRIDE and MASSive) (AApoAI, AL-59, ALECT2), whereas AL-H7 was processed in our laboratory. The amyloid proteins showed different patterns of deamidation. While some fibrils had negligible levels, others contained highly Q-deamidated residues. Also, on the same fibril, some glutamine residues possessed high occupancy and others showed no detectable deamidation. This variable distribution suggested dependency on protein sequence and structural context. To investigate this possibility, I mapped modifications on the fibril structure and examined whether solvent accessibility, residue burial within the fibril core, or steric interactions with neighboring residues could contribute to the observed differences in deamidation occupancy.

4.1.1. ApoA-I fibrils contain prominent deamidation hotspots

The ApoA-I fibrils analyzed in this study were extracted from the heart of a patient affected by hereditary ApoA-I amyloidosis carrying the L90P mutation. The fibrils were previously characterized by cryo-electron microscopy and mass spectrometry by Nguyen et al. (2026) [56]. Raw LC-MS/MS data were obtained from the associated dataset, MassIVE database (a member of ProteomeXchange) under accession code MSV000098187, and re-analyzed using Proteome Discoverer [55], [60]. A total of 865 master proteins were identified in the fibril analysis. ApoA-I was identified as the most abundant protein in the sample. The protein was detected with 106 unique peptides and 1,289 PSMs, resulting in 79% sequence coverage (Figure 4.1.).

ApoA-I fibrils displayed multiple deamidated glutamine residues. Several positions exhibited measurable occupancy, including Q5 (6.57%), Q84 (9.66%), Q98 (22.91%), Q109 (11.86%), and Q127 (9.31%). Two residues showed high occupancies: Q32 (44.30%), Q216 (64.16%) (Table 4.1.).

Figure 4.1. Sequence coverage of ApoA-I identified in the fibril analysis.

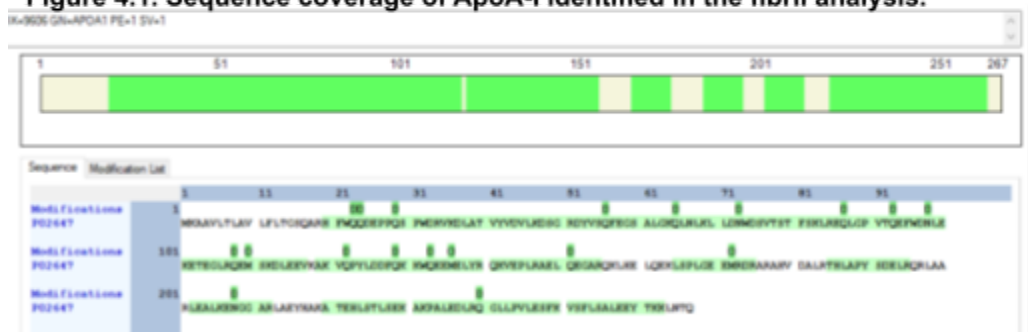


Table 4.1. ApoA-I Glutamine Deamidation Occupancy Rate

Glutamine Site*	Total Modified Abundance	Total Unmodified Abundance	Occupancy %
5	1.37E+09	1.94E+10	6.57%
32	8.96E+10	1.13E+11	44.30%
41	0.00E+00	2.66E+08	0.00%
63	8.61E+07	6.99E+10	0.12%
69	4.01E+07	7.00E+10	0.06%
84	1.24E+09	1.16E+10	9.66%
98	2.92E+08	9.83E+08	22.91%
105	2.29E+07	1.25E+09	1.79%
109	1.10E+08	8.18E+08	11.86%
117	0.00E+00	1.84E+07	0.00%
127	5.08E+07	4.95E+08	9.31%
216	7.81E+08	4.36E+08	64.16%

*Sites are reported after removing the precursor of 24 amino acids

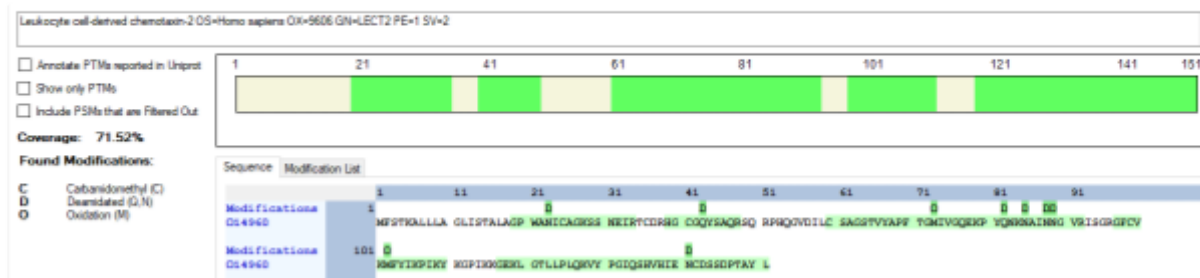
These values indicate that a substantial fraction of ApoA-I fibril molecules carry deamidation, and the different distribution of modification across the protein sequence suggests that specific regions of the fibril structure are more susceptible to chemical modification.

4.1.2. Limited deamidation in LECT2 fibrils

The LECT2 fibrils were extracted from the kidney of a patient affected by ALECT2 amyloidosis carrying the I40V variant, as reported in Afrin et al. (2026) [53]. Raw LC-MS/MS data were obtained from the associated MassIVE dataset under accession code MSV000098220 [57]. A

total of 1,268 master proteins were identified in the fibril preparation. LECT2 was identified as the most abundant one. The protein was detected with 40 unique peptides and 305 peptide-spectrum matches (PSMs), resulting in 72% sequence coverage (Figure 4.2).

Figure 4.2. Sequence coverage of ApoA-I identified in the fibril analysis.



LECT2 fibrils showed low overall modification levels. The most frequently modified residue was Q43 (4.83%) (Table 4.2.).

Glutamine Site*	Total Modified Abundance	Total Unmodified Abundance	Occupancy %
25	1.62E+08	3.20E+09	4.83%
29	0.00E+00	3.36E+09	0.00%
59	0.00E+00	2.04E+09	0.00%
64	2.33E+07	2.02E+09	1.14%
109	0.00E+00	1.87E+10	0.00%
116	0.00E+00	1.39E+10	0.00%

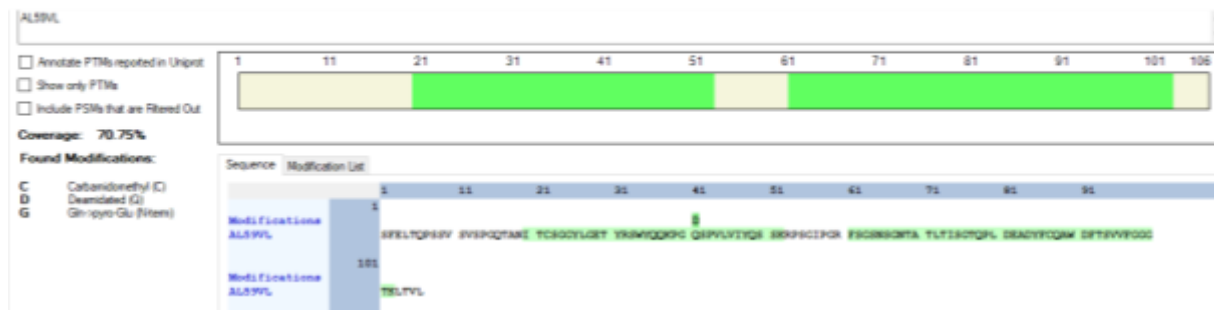
*Sites are reported after removing the precursor of 18 amino acids

4.1.3. AL59 fibrils have negligible deamidation

The AL-59 fibrils were extracted from the heart of a patient affected by light chain amyloidosis and are composed of a monoclonal immunoglobulin light chain. The fibrils were extracted and characterized as reported by Schulte et al [58]. The mass spectrometry dataset was downloaded from the associated data on PRIDE with the accession code PXD049301 [59]. A total of 368 master proteins were identified in the AL59 fibril preparation. The amyloidogenic protein AL59 was the most abundant protein in the dataset. The protein was detected with 49 unique

peptides and 177 peptide spectrum matches (PSMs), resulting in 71% sequence coverage (Figure 4.3.).

Figure 4.3. Sequence coverage of AL59VL identified in the AL59 fibril preparation.

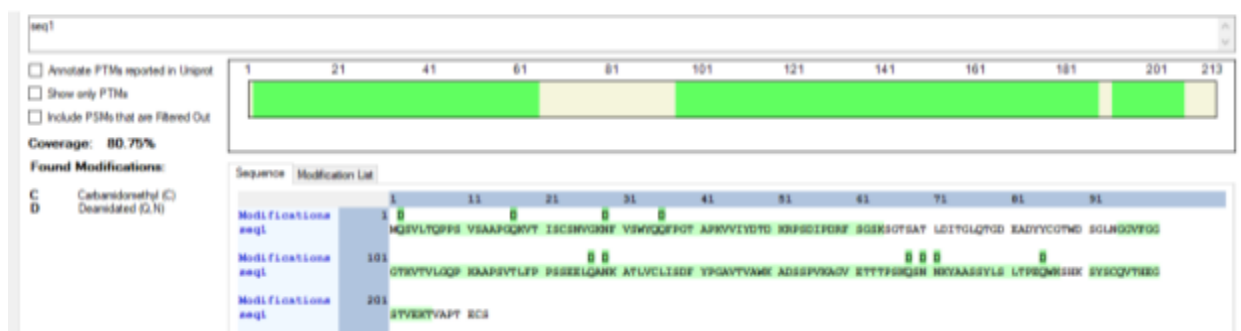


Analysis of AL59 fibrils revealed no detectable deamidation across all identified glutamine residues. Residues Q36, Q37, Q49, Q78 and Q88 displayed no measurable modified signal, while Q41 showed no quantifiable evidence of substantial deamidation. Occupancy values were effectively zero across all examined positions.

4.1.4. Low-level deamidation in H7 fibrils

A total of 236 master proteins were identified in the H7 fibril analysis. The amyloidogenic H7 protein was the most abundant protein in the sample. The portion was detected with 91 unique peptides and 1,192 PSMs, resulting in 81% sequence coverage.

Figure 4.4. Sequence coverage of the H7 amyloid protein identified in the fibril analysis



Similarly to AL-59, H7 fibrils showed limited evidence of deamidation (Table 4.3.). Most glutamine residues exhibited no detectable modification. The highest occupancy was observed at residue Q1 (3.09%), while Q126 showed approximately 1% occupancy. All remaining detected glutamine residues displayed negligible modification levels.

Table 4.3. H7 Glutamine Deamidation Occupancy Rate			
Glutamine Site	Total Modified Abundance	Total Unmodified Abundance	Occupancy %
1	0.00E+00	3.34E+10	0.00%
6	6.24E+07	6.25E+09	0.99%
16	0.00E+00	9.39E+08	0.00%
34	0.00E+00	3.53E+09	0.00%
35	0.00E+00	1.61E+09	0.00%
108	0.00E+00	2.61E+09	0.00%
126	2.07E+07	6.47E+08	3.09%
167	0.00E+00	5.33E+09	0.00%
184	0.00E+00	5.33E+09	0.00%
194	0.00E+00	3.52E+09	0.00%

4.2. Mapping of Deamidation Sites onto Available Amyloid Fibril Structures

4.2.1. ApoA-I fibrils.

The ApoA-I fibril structure displayed the most significant deamidation hotspots among the analyzed amyloid proteins. In particular, Q32 deamidation had an occupancy of approximately 44.3%, whereas Q41 and Q69 showed essentially no detectable deamidation. Q63 exhibited only minimal modification (~0.1%). Structural mapping of these residues (Figure 4.5.) aligns with their deamidation occupancies. As shown in figure 4.5., Q32 is positioned on a curved surface region of the fibril allowing good solvent exposure with limited interactions with the neighboring side chains, while Q63 although its side chain appears outwards, looks partially shielded by surrounding residues. The other blue positions shown in Figure 4.5., Q41 and Q69 appeared to be located inside the fibril structure and surrounded by neighboring side chains. Together, these observations suggest that both solvent accessibility and local steric environment may influence the susceptibility of individual residues to deamidation.

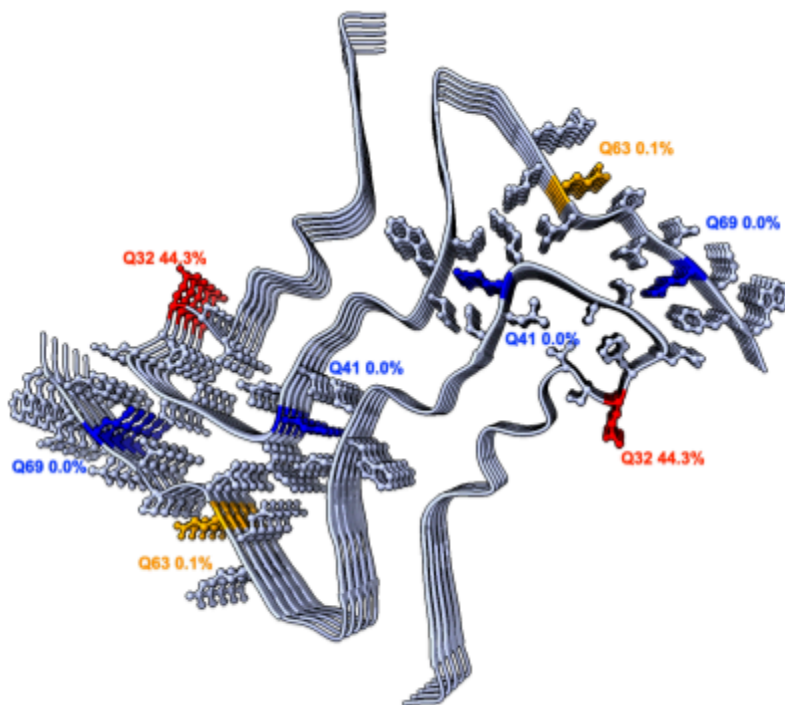


Figure 4.5. Mapping of glutamine deamidation sites onto the ApoA-I cryo-EM fibril structure. Glutamine residues identified in the proteomic analysis were mapped onto the ApoA-I fibril structure using UCSF ChimeraX. Residues are colored according to their deamidation occupancy: red, high occupancy (Q32, 44.3%); orange, low occupancy (Q63, 0.1%); and blue, no detectable deamidation (Q41 and Q69, 0.0%). Occupancy values are indicated adjacent to the corresponding residues. The sequence was excluded from residue numbering as described in Table 4.1.

4.2.2. LECT2 fibrils

Structural mapping of LECT2 showed a similar trend as for AAPOA1. The most highly deamidated residue, Q25 (4.8%), is located on the surface of the fibril with its side chain oriented outward and limited interaction with the neighboring residues (Figure 4.6). Additionally, the mildly modified position Q64 (1.1%) is also located on the fibril surface, however, its side chain appears partially surrounded by neighboring residues, suggesting a degree of steric hindrance that may reduce accessibility relative to Q25. Several locations Q59, Q116, and Q109 showing no detectable deamidation are positioned within more protected regions of the fibril structure with limited exposure to the solvent. Interestingly, Q29 also showed no detectable deamidation suggests that neighboring side chains may partially restrict access to this residue or that this residue is protected by interaction with surrounding environment molecules, indicating that local steric constraints may contribute to the absence of detectable modification.

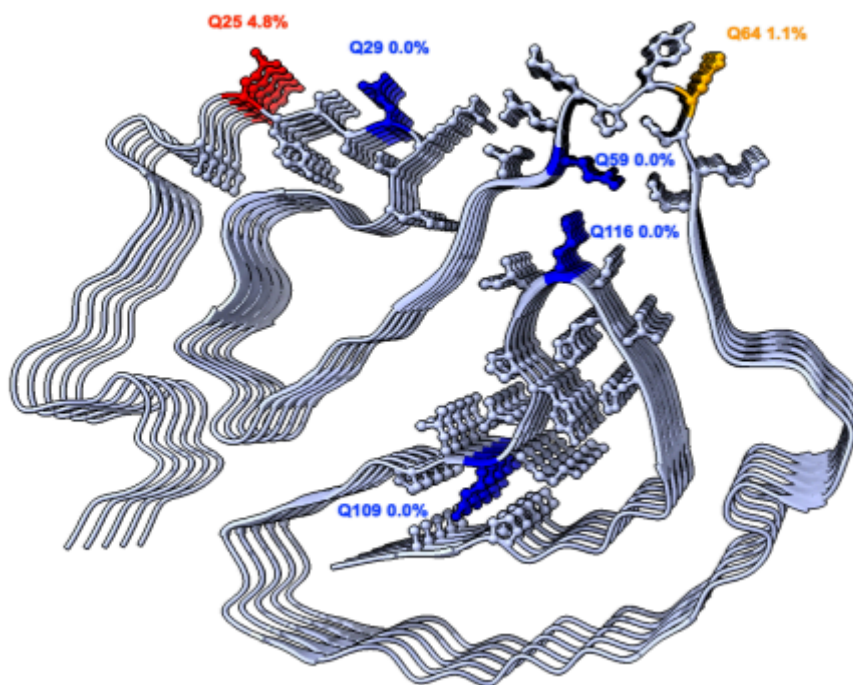


Figure 4.6. Mapping of glutamine deamidation sites onto the LECT2 cryo-EM fibril structure. Glutamine residues identified in the proteomic analysis were mapped onto the LECT2 fibril structure using UCSF ChimeraX. Residues are colored according to their deamidation occupancy: **red**, highest occupancy (Q25, 4.8%); **orange**, low occupancy (Q64, 1.1%); and **blue**, no detectable deamidation (Q29, Q59, Q109, and Q116, 0.0%). Occupancy values are indicated adjacent to the corresponding residues. Residue numbering corresponds to the LECT2 sequence following removal of the 18-amino-acid signal peptide, as described in Table 4.2.

4.2.3. AL59 fibrils

AL59 showed negligible deamidation across all identified glutamine residues. Mapping of identified glutamine residues revealed that all analyzed positions except Q37 are concentrated within the structured fibril core, where extensive intermolecular packing is observed (Figure 4.7). The apparent burial of these residues within the fibril structure is consistent with the absence of detectable deamidation observed in the proteomic analysis. The side chain of Q37 is oriented outwards, yet it is shielded by neighboring side chains.

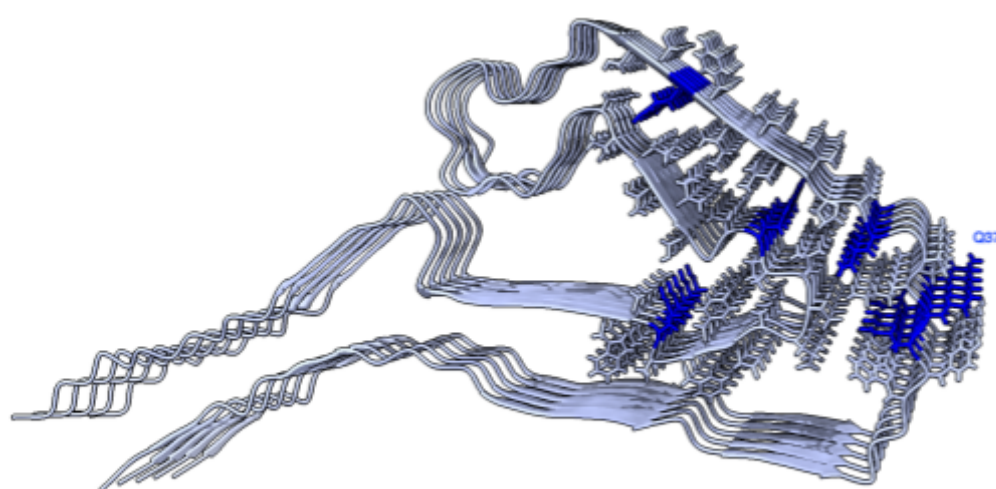


Figure 4.7.
Mapping of
glutamine
residues onto the
AL59 cryo-EM
fibril structure.
Glutamine residues identified in the proteomic analysis were mapped onto the AL59 fibril structure using UCSF ChimeraX. All identified glutamine residues exhibited 0% deamidation occupancy and are therefore shown in **blue**.

Comparison of AL59, ApoA-I, and LECT2 demonstrates that deamidation susceptibility is strongly site-dependent. Residues exhibiting the highest occupancies were generally located in solvent-accessible regions with limited steric constraints, whereas residues displaying little or no deamidation were either buried within the fibril core or partially shielded by neighboring side chains. These observations suggest that the local structural microenvironment plays a major role in determining deamidation propensity within amyloid fibrils.

4.3. Large-Scale Analysis of Differentially abundant PTMs in Whole Tissue Proteome

The second part of my thesis project was dedicated to the development of an approach to quantitatively investigate PTMs in whole tissue proteomes. To this aim, I exploited the availability of an established mouse model of AL amyloidosis, in which affected animals can be compared with well-defined controls. The analysis was performed on raw MS data referring to two mice groups, corresponding to a total of 18 raw data files (three technical replicates for each of three biological replicates in each group).

As a PTM case study, I focused on Lysine acetylation. The raw data were processed using Thermo Proteome Discoverer, allowing protein identification and quantification, followed by the identification and quantitative analysis of acetylated peptides. Overall, Proteome Discoverer identified 79 acetylation sites across 61 acetylated proteins. These numbers represent the initial identification results.

As a first step, protein abundances were compared between amyloid-positive and control samples to assess differences in the underlying proteome. Subsequently, the analysis focused on lysine-acetylated peptides to investigate differences in protein-normalized acetylation signals. Statistical analysis identified four protein-site combinations with nominal *p*-values below 0.05. The corresponding MS/MS spectra were then examined to assess the fragment-ion evidence supporting the assignment and localisation of acetylation at these sites. The spectra are presented in Figures 4.8–4.11.

Figures 4.8–4.11 show the MS/MS spectra for the acetylated peptides assigned to K5 of L-lactate dehydrogenase A chain (P06151), K26 of mitochondrial malate dehydrogenase (P08249), K10 of ADP/ATP translocase 1 (P48962), and K299 of mitochondrial isocitrate dehydrogenase [NADP] (P54071), respectively.

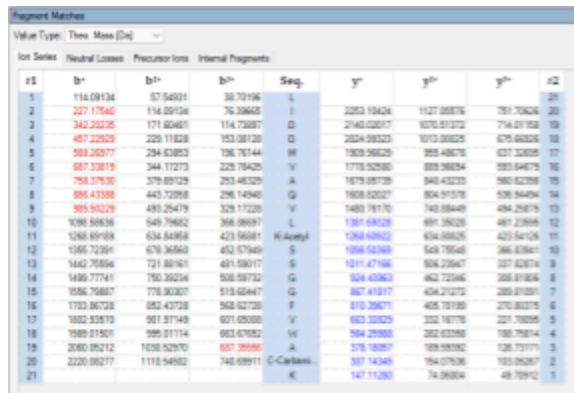


Figure 4.8. MS/MS spectrum of the acetylated peptide LIDDMVAQVLKSSGGFWACK from mitochondrial isocitrate dehydrogenase [NADP] (P54071). Acetylation was localised at K299, corresponding to the 11th residue (K11) in the displayed peptide sequence. The annotated fragment ions include m/z 1268.60922 (y^+), supporting peptide identification and modification site localisation.

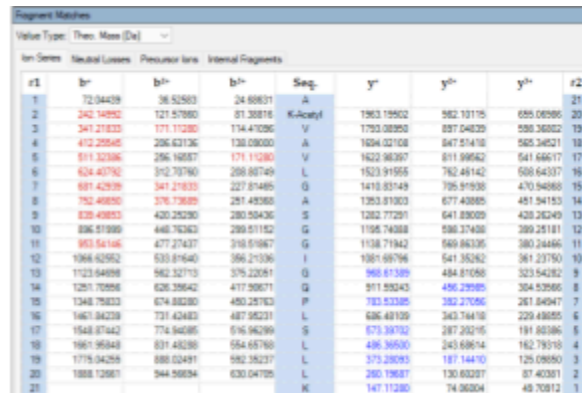


Figure 4.9. MS/MS spectrum of the acetylated peptide AKVAVLGASGGIGQPLSLLK from mitochondrial malate dehydrogenase (P08249), with acetylation localised at K26 corresponding to the 11th residue (K11) in the displayed peptide sequence. The annotated fragment-ion spectrum includes a detected ion at m/z 242.1492 (b^+), supporting the fragmentation pattern used for peptide identification and modification site assignment.



Figure 4.10. MS/MS spectrum of the acetylated peptide GDQALSFLKDLFLAGIAAVSK from ADP/ATP translocase 1 (P48962). Acetylation was localised at K10, corresponding to the 9th residue (K9) in the displayed peptide sequence. Annotated fragment ions include m/z 1002.5247 (b^+) supporting peptide identification and modification site localisation.



Figure 4.11. MS/MS spectrum of the acetylated peptide ATLKDQLVNLK from L-lactate dehydrogenase A chain (P06151). Acetylation was localised at K5, corresponding to the 4th residue (K4) in the displayed peptide sequence. The annotated fragment ions include m/z 456.28166 (b^+) supporting peptide identification and modification site localisation.

Table 4.4 summarises the four protein-site combinations showing statistically significant differences in protein-normalized lysine acetylation signals between amyloid-positive and control mouse samples ($p < 0.05$). All four sites exhibited higher normalized signals in the amyloid-positive group. The largest increase was observed for mitochondrial malate dehydrogenase at K26 (ratio = 3.09), followed by mitochondrial isocitrate dehydrogenase [NADP] at K299 (ratio = 2.06). These findings highlight four acetylation sites for which the quantitative analysis indicated differences between the two groups and for which the corresponding MS/MS spectra supported modification assignment.

Table 4.4. Statistically significant differences in protein-normalized lysine acetylation between amyloid-positive vs control mouse samples.				
Protein Description	Protein Accession	Mod Site	Normalized PTM Shift (Ratio)	P-Value
L-lactate dehydrogenase A chain (LDHA)	P06151	K5	1.38	3.23E-03
Malate dehydrogenase, mitochondrial (MDH2)	P08249	K26	3.09	7.48E-03
ADP/ATP translocase 1 (SLC25A4/ANT1)	P48962	K10	2.1	3.57E-02
Isocitrate dehydrogenase [NADP], mitochondrial (IDH2)	P54071	K299	2.06	8.85E-03

The four proteins identified in the differential acetylation analysis have annotated roles in cellular metabolism, with three directly associated with mitochondrial function. LDHA catalyses the interconversion of pyruvate and lactate, whereas MDH2 and IDH2 participate in mitochondrial metabolic pathways. SLC25A4 mediates adenine nucleotide exchange across the inner mitochondrial membrane, supporting the distribution of ATP produced by mitochondria.

The increased protein-normalised acetylation signals observed at the identified lysine residues indicate site-specific differences that warrant further investigation. However, the functional consequences of these modifications remain undetermined. Further studies would be required to establish the functional significance of these site-specific differences.

5. Discussion and Conclusions

In this thesis project, I worked on developing approaches for the assignment and quantification of protein post-translational modifications (PTMs) in complex proteomic samples analyzed via mass spectrometry. This work was primarily computational, involving the evaluation of high-resolution mass spectrometry data. Some of these data were acquired in the laboratory where I conducted my research and some obtained from public databases.

The first part focused on the quantitative analysis of PTMs, aiming to identify and quantify the extent of specific PTMs at defined amino acid positions and to map these modifications onto protein structures. This approach was used to assess, as a case study, glutamine deamidation, a non-enzymatic modification associated with protein aging [40], in amyloid proteins extracted from patient tissues. By combining peptide-level quantification with structural information from cryo-electron microscopy-derived fibril structures, this analysis aimed to investigate whether the extent of deamidation was associated with the structural environment of individual glutamine residues.

In the second part, I analysed proteomic datasets from cardiac samples of a mouse model of AL amyloidosis, evaluating differences in lysine acetylation between affected animals and non-transgenic controls [2]. This analysis investigated whether the relative abundance of acetylated peptide forms differed between the two groups after accounting for the abundance of the corresponding proteins.

The results obtained in both sections allow for several observations. Regarding glutamine deamidation in amyloid fibrils, the analyses revealed substantial differences between samples and individual residues. While deamidation was negligible or detected only at low levels in the AL59 and H7 fibrils, selected glutamine residues in the ApoA-I and LECT2 fibrils exhibited measurable modification. This is consistent with the possibility that non-enzymatic modifications accumulate during the prolonged persistence of amyloid deposits in tissues. However, the observed differences indicate that deamidation is not uniformly distributed across amyloid fibrils.

Quantification of occupancy, defined as the fraction of the measured modified and unmodified peptide signal represented by the modified form, revealed that deamidation levels varied considerably between glutamine residues. Structural mapping, made possible by the availability of amyloid fibril structures obtained via cryo-electron microscopy and accessible through the PDB database, suggested that solvent accessibility and the orientation of the side chain may contribute to this variation. The proteomic data were therefore broadly consistent with the structural information, providing mutual confirmation. In parallel, the occurrence of deamidation on the fibril surface rather than in its interior supports the hypothesis that deamidation occurs largely after and not prior to fibril formation and is therefore not a significant predisposing factor for fibrillar aggregation.

A more detailed analysis allows for further insights. Generally, deamination at any given position affects only a fraction of the total population, with the non-deaminated form remaining dominant. An exception to this trend is observed in the APOAI sample obtained from MassIVE under accession MSV000098187 [55]. The highest estimated occupancy was observed at Q216, at 64.16%, followed by Q32, at 44.30%. This may indicate either especially prolonged fibril persistence within the patient or differences in the stringency of the sample storage conditions prior to analysis.

Furthermore, regarding surface residues, deamination levels vary significantly among glutamine residues: levels are higher where the side chain projects fully outward and lower where the side chain is partially shielded by the steric hindrance of neighboring residues. Although deamination levels are also influenced by sequence, this observation indicates that a residue's chemical environment modulates its behavior. Furthermore, we cannot rule out the possibility that exposed, minimally deamidated glutamines (e.g., Q63) are also protected through interactions with other molecules, such as proteins known to associate ubiquitously with amyloid fibrils (e.g., apolipoproteins, serum amyloid P, chaperones, etc.).

In conclusion, analyzing the presence, structural location, and abundance of non-enzymatic post-translational modifications can serve as a method to assess sample quality and the consistency of structural analysis results, as well as to provide indirect information about the surrounding molecular environment surrounding amyloid fibrils.

In the second part of my thesis, I analyzed proteomics datasets from two groups of mice, transgenic with cardiac AL amyloidosis and non-transgenic controls. The analysis adopted an unconventional perspective, aiming to identify not only differentially abundant proteins, but also differentially abundant PTMs. The rationale of this study stands in the fact that protein function is well known to be modulated not only by changes in abundance, but also through post-translational modifications. In particular, cells possess a complex apparatus of enzymes deputed to control a large spectrum of PTMs, such as kinases, acetylases, phosphatase etc. Given the fact that PTMs usually affect only a fraction of the whole pool of a given protein, the comprehensive proteomic profiling of specific PTMs typically requires enrichment strategies before mass spectrometry. However, it is also possible to investigate the most abundant PTMs in standard proteomic datasets, thus extracting an additional level of information that is usually not accounted for.

As a case study, I assessed and compared the levels of lysine acetylation across the two groups amyloid and control, each represented by three biological replicates and three technical replicates per biological sample, giving a total of 18 raw mass spectrometry files. This PTM is controlled by the family of protein acetylases and deacetylases and can significantly affect the function of the corresponding target by converting the positively charged lysine residue into the non-charged acetyl lysine. Although the acetylation events that I detected are likely only a fraction of all those present in the tissue, due to the lack of enrichment, four protein-site combinations showed statistically significant differences in protein-normalized acetylation signals. This provides proof of principle of the fact that the cardiac proteome in amyloid mice is not only quantitatively but also in its PTM-associated characteristics.

The four sites identified in Table 4.4 were more acetylated in the amyloid-positive mouse. Three of the four proteins are associated with mitochondrial function, including metabolic reactions and adenine nucleotide transport, while LDHA participates in pyruvate-lactate metabolism.

Protein acetylation has been associated with nutritional status and oxygen availability, with important consequences on metabolism [62]. A substantial proportion of mitochondrial proteins contain acetylation sites, particularly proteins involved in fatty acid metabolism, the

tricarboxylic acid (TCA) cycle, the electron transport chain and oxidative phosphorylation. Mitochondrial protein acetylation can occur through non-enzymatic mechanisms in the presence of acetyl-CoA in the mitochondrial matrix, while deacetylases also contribute to the regulation of protein acetylation.

MDH2 participates in the TCA cycle by catalysing the interconversion of malate and oxaloacetate, whereas IDH2 catalyses the conversion of isocitrate to α -ketoglutarate and contributes to mitochondrial redox metabolism. The increased normalized acetylation signals at MDH2 K26 and IDH2 K299 may therefore be relevant to the investigation of metabolic changes associated with cardiac amyloidosis.

In the present analysis, a lysine acetylation site in ADP/ATP translocase 1 showed statistically significant differences between amyloid-positive and control mouse samples. K10 had a protein-normalized ratio of 2.10 ($p = 0.036$). These findings suggest that the protein-normalized acetylation signals at site differed between the two groups, although the magnitude of the observed effect varied substantially between sites. The potential functional relevance of these findings is supported by Mielke et al. (2014), who identified acetylation at K10, K23 and K92 of ANT1 in human skeletal muscle [63]. Using molecular dynamics simulations, molecular docking and computational modelling, the authors predicted that acetylation at K23 could substantially reduce the affinity of ANT1 for ADP, potentially decreasing adenine nucleotide exchange [63], [64]. Their findings suggest that acetylation at specific ANT1 residues may influence transporter function.

This thesis demonstrates how mass spectrometry-based proteomic datasets can be used to investigate PTMs beyond conventional protein identification and quantification. The analysis of glutamine deamidation in human amyloid fibrils revealed substantial site-specific variation, partly associated with the structural environment of individual residues. In the mouse model of cardiac AL amyloidosis, four lysine acetylation sites showed increased protein-normalized signals in amyloid-positive samples, highlighting potential differences in metabolic proteins between the two groups.

Although these findings provide additional insights into the molecular characteristics of amyloid deposits and associated proteomic changes, further studies are needed to establish their biological significance. Future work could validate the identified modifications in larger sample sets, investigate their functional consequences, and extend the analysis to other PTMs.

Overall, this work illustrates the value of re-examining existing mass spectrometry datasets to obtain additional information about protein modifications and complement conventional proteomic analyses.

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