Opinion Paper


Diagnostic amyloid proteomics: experience of the UK National Amyloidosis Centre

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Abstract: Systemic amyloidosis is a serious disease which is caused when normal circulating proteins misfold and aggregate extracellularly as insoluble fibrillary deposits throughout the body. This commonly results in cardiac, renal and neurological damage. The tissue target, progression and outcome of the disease depends on the type of protein forming the fibril deposit, and its correct identification is central to determining therapy. Proteomics is now used routinely in our centre to type amyloid; over the past 7 years we have examined over 2000 clinical samples. Proteomics results are linked directly to our patient database using a simple algorithm to automatically highlight the most likely amyloidogenic protein. Whilst the approach has proved very successful, we have encountered a number of challenges, including poor sample recovery, limited enzymatic digestion, the presence of multiple amyloidogenic proteins and the identification of pathogenic variants. Our proteomics procedures and approaches to resolving difficult issues are outlined.

Keywords: amyloidosis; laser capture dissection; proteomics.

Introduction

Amyloidosis is a heterogeneous group of diseases which are caused when normal circulating proteins misfold and form insoluble fibrils in the extracellular space of tissues throughout the body [1]. There are over 30 known amyloidogenic proteins reported to cause human disease [2] and their amyloid deposits can damage the structure and function of affected organs and commonly lead to cardiac, renal and neurological disorders. Although amyloidosis is normally referred to as a rare disease, affecting 1 in 10,000 people worldwide, it is becoming clear that cardiac transthyretin (TTR) amyloid deposits caused by the wild-type isoform are very common in people over 80 years of age, and may be a significant contributor to heart failure in the elderly [3]. In some cases, the development of the disease is associated with increased circulating concentrations of the precursor protein: a raised inflammatory response can lead to serum amyloid A (AA) amyloid deposition [4] and a monoclonal gammopathy is associated with immunoglobulin kappa and lambda light chain (AL) amyloid. Other amyloidoses can develop at normal circulating protein concentrations, including TTR, fibrinogen Aα and lysozyme; in each case however, a gene mutation resulting in a variant amino acid residue in the protein will markedly affect disease development [1]. The clinical presentation and management of amyloidosis is dependent on the fibril protein from which the tissue amyloid deposits are derived, and the respective protein must be identified in order for appropriate therapy to be administered. Amyloid deposits are identified by Congo red staining and show apple-green birefringence when viewed under crossed polarised light [5]. Originally, the identification of the constituent protein(s) relied on the extraction of amyloid fibrils [6, 7] from tissue followed by their identification using classical biochemical sequencing techniques [8, 9]. This was a time consuming approach requiring milligram amounts of fresh tissue and was unsuitable as a routine clinical test, and could not be undertaken with formalin-fixed paraffin-embedded (FFPE) tissues, the routine
method for diagnostic histopathology. The development and application of immunohistochemistry (IHC) and related immunological techniques, using a panel of antibodies to identify amyloid protein(s) revolutionised the diagnosis of amyloidosis and has been the gold standard for amyloid fibril identification [10]. Unfortunately, IHC fails to determine the amyloid type in up to 30% of cases of systemic amyloidosis due to a combination of high background staining and lack of antibody specificity [11]. Proteomic analyses [12–14] of FFPE tissue, particularly when coupled with laser capture dissection (LCD) [15], offer a vastly improved method to identify amyloid and other proteins. A simple and reliable procedure to type amyloid proteomics was developed by Ahmed Dogan and colleagues at the Mayo Clinic as a routine clinical diagnostic test for amyloidosis [16–19]. The method works well on fresh tissue as well as on FFPE tissue. Identification is facilitated by the particular nature of amyloid, where the amyloidogenic protein is concentrated in a defined area of Congo red positive tissue. Laser micro-dissection of the Congo red stained amyloid material reduces the relative proportion of contaminating background proteins. Proteomics is becoming the de facto gold standard for identifying amyloid proteins, and is now used routinely in a number of centres. We report here on our experience over the past 7 years at the UK National Amyloidosis Centre of using proteomics to type amyloid, and outline some of the issues that may affect data interpretation.

## Amyloid proteomic procedure

Proteomics is now used routinely in the centre both as a clinical diagnostic test for amyloidosis and also for research into the pathogenesis of the disease, and over 2000 clinical biopsy samples from all tissue types have been examined. All samples are initially examined by Congo red staining for amyloid and by IHC as previously described [20, 21]. We then use the procedure originally developed by Dogan and colleagues [16] for extraction and digestion of samples, followed by analysis using LCMSMS on a Thermo Scientific Q Exactive Plus mass spectrometer. Each batch of clinical samples is accompanied by a number of controls which include a cytochrome C digest, a control protein (α-synuclein) and a sample of amyloidotic tissue from a patient with light chain (lambda) amyloidosis. Ideally each sample of Congo red positive material should be accompanied by a control cut from a Congo red negative area of tissue. This was routinely undertaken when our procedure was originally developed, showing that the amyloid and signature proteins were mainly associated with Congo red staining. As sample throughput increased, this was no longer a viable option and the use of this additional control was discontinued. We noted that samples containing serum amyloid A (SAA) and, to a lesser extent, TTR and other amyloid proteins, could result in some carryover into the next sample. To minimise contamination, we have included an acid, base and organic injection wash protocol sample (adapted from Fang [22]) for the autosampler fluidics and trapping cartridge. We also include a full blank run between each sample to confirm that there is no carryover from sample to sample. Although this doubles the analysis time for each sample it increases confidence in the amyloid typing. Full details of our procedures are given in the Supplementary Information.

### Amyloid identification: searches and the algorithm

The Mascot search engine (www.matrixscience.com) is used for protein identification with the results stored in a proteomics database which is linked to the centre’s clinical database, one of the largest collections of clinical amyloidosis data in the world. We use a single search engine for our work, although multiple search engines have been recommended to enhance protein identification of amyloid [17]. Whilst this can be of value when analysing large protein datasets [23, 24], it is, perhaps, of less importance for identifying a known amyloidogenic protein as a major constituent of laser captured Congo red positive tissue. Trypsin is used as the in silico search enzyme, although others recommend semi-trypsin [25, 26]. This can result in more peptide identities (including the N terminus), but as it removes one level of specificity it can increase the possibility of more false positives. We use semi trypsin as a secondary search parameter only when appropriate, for example, where small proteins such as LECT-2 or insulin are suspected or when further structural information is required. We follow the Mayo Clinic’s approach [17, 26, 27] and require the presence of at least two of three signature proteins, serum amyloid P component, apolipoprotein E and apolipoprotein A-IV with Mascot scores >20 to confirm amyloid. In samples which do not exhibit Congo red staining, an increased minimum score of 50 is applied. Other signatures such as vitronectin have been suggested [28, 29] and are under consideration for inclusion.

A simple algorithm has been developed (Figure 1A) to highlight the most likely candidate amyloid protein. The first decision point is to determine the total number...
of proteins in the sample. Here, a minimum Mascot score \( \geq 80 \) and at least two unique significant peptides (USP) are required. The ubiquitous keratins and haemoglobin are excluded, and a minimum acceptance criterion is set at five proteins. If fewer than five proteins are detected the sample is labelled as inadequate. The exception is where there are two signature proteins and one clear amyloidogenic protein present, or where the renal amyloid protein leukocyte cell-derived chemotaxin-2 (LECT-2) \[30, 31\] has been identified. LECT-2 is often associated with a low Mascot score and poor protein coverage despite exhibiting excellent specific immunostaining \[32\]. As digestion of native LECT-2 results in good protein coverage and high score, the apparently poor recovery from
clinical samples may be related to the fibril structure limiting digestion or some form of post deposition protein modification. A minimum score of 20 is set in this case. A higher minimum score (>2000) is set for apolipoprotein A-I [9, 33] and the signature protein apolipoprotein A-IV [25, 34, 35] both of which can form amyloid deposits [36–39] but can be present naturally in a range of tissues. Additionally, for ApoA-I, the tissue source can be used as a discriminator, with the liver, kidneys and heart being more commonly involved. Where fibrinogen Aα has been identified, an additional algorithm is applied as outlined below. The issue of multiple amyloidogenic proteins is considered, to include heavy chains which are also commonly observed in amyloid deposits, before the algorithm moves on to identify the highest scoring amyloidogenic protein. Where there are doubts, the result is labelled as uncertain or no identification. The output, containing links to the Mascot results web pages and to the patient’s clinical data, is shown in Figure 1B. The algorithm can also highlight potential non-amyloid deposits, particularly in Congo red negative glomeruli. Here the presence of DnaJ homolog subfamily B member 9 (DNAJB9) is indicative of fibrillary glomerulonephritis [40] whereas the presence of immunoglobulin light and heavy chains in the absence of any signature proteins may indicate a deposition disease [41, 42].

The algorithm-derived result is reviewed in weekly multidisciplinary team (MDT) meetings in which clinicians and scientists participate. Here the amyloid type is decided through consideration of the Mascot output together with morphological, IHC and genetic data and clinical details. Audit data for 2018 are shown in Supplementary Figure S1 where 433 clinical samples were analysed. Cardiac and renal tissue were the main tissue types, with TTR and the lambda and kappa light chains accounting for over 60% of typed amyloid.

Sample recovery

One of the issues we regularly face is with inadequate samples; in some cases, particularly in histology review sections from external laboratories, there is little material available. We can sometimes identify proteins from tiny amounts of tissue such as a single glomerulus, however, in other cases we see little from what appears to be relatively plentiful Congo red stained material. This it is probably down to issues with digestion and recovery. Figure 2A shows the amyloid in sample caps collected from two separate areas of amyloid (both ~0.4 mm²); there is far more material in one cap than the other, showing that there are losses on LCD capture. We have investigated the use of TEZ buffer in the cap as well as the use of an anti-static gun to improve recovery. Another explanation could include incomplete trypsinisation of different fibril types within amyloid. This is shown in Figure 2B where Congo red staining was performed on a fat aspirate both before and after trypsin digestion. The apple-green birefringence confirmed the presence of a remarkable amount of undigested amyloid in the tissue after the digestion. The amount of trypsin is not the limiting factor: in a separate study we used a 25-fold variation in the amount of trypsin (8–200 ng/sample) to digest FFPE sample from an AL (lambda) patient, but found no dose-dependent effect of trypsin in the score or number of USPs of lambda constant region. Unexpectedly we did identify a dose-dependent inhibitory effect of calcium in these samples, with 10 mM EDTA causing a six-fold increase in score compared with samples containing 2 mM calcium. If this undigested residual amyloid could be reduced then protein identification would be made possible on smaller amounts of tissue, and the number of inadequate samples reduced.

We have also introduced a pre-digestion sample treatment for FFPE tissue to improve the fibril denaturation and achieve a better recovery of the amyloid proteins. Sonicating the samples in acetonitrile:water:trifluoroacetic acid (80:20:0.2 v/v/v) leads to a better denaturation of fibrils and increases the efficacy of trypsin digestion (Supplementary Figure S2). The pre-treatment protocol has also been applied to several FFPE clinical samples showing that in the most of cases the number of identified proteins and Mascot scores of the amyloid component increased in the treated samples (Supplementary Table S1). It has not yet been validated as part of our clinical tests and is only used for research samples.

Multiple proteins

It is generally accepted that amyloid deposits are associated with a single amyloidogenic protein, although it is common to identify multiple amyloid precursor proteins by proteomics in a single sample. In some cases, the amyloid can arise from two precursor proteins [43–47]. For example, in cardiac amyloid samples from elderly patients it is common to identify light chains derived from a monoclonal gammopathy together with TTR arising through age-related deposition of wild-type protein.
Figure 2: Potential confounding issues in amyloid proteomics.
Panel (A) shows Congo red positive material collected into microscope caps by laser capture dissection from two 6 μm thick section of amyloid from the same piece of FFPE tissue. The dissected area of both samples was similar (~0.4 mm²), however the amount of captured material was clearly different. Panel (B) shows a piece of aspirated fat before (left) and after trypsin digestion (right). Both pre and post trypsin digestion samples shows the characteristic Congo red amyloid birefringence demonstrating that not all the amyloid had been digested. Panel (C) shows an example of misidentification of a variant form a renal sample containing fibrinogen α amyloid. Two variants were identified by proteomics. Only in the correctly identified sample (p.R573L) was the variant peptide ESSHPGIAEFPSLGK identified. The apparent presence of the p.G538R variant was only inferred from the newly identified C terminal tryptic fragment EFVSETESR which may have been present in the amyloid following natural truncation. The MSMS spectrum of EFVSETESR is also shown together with identified b, b₀, and y ions.
In general, the presence of more than one potentially amyloidogenic protein is more likely to be due to tissue background or contamination arising during collection. In most cases the amyloid fibril protein can be differentiated from background contaminants by score alone. This is not always possible, particularly where contamination with blood has occurred during collection. Here, trypsin digestion results in the release of both amyloid and thrombus-derived peptides, and this may confound amyloid fibril protein identification. In the case fibrinogen Aα (FibA) amyloid, we can differentiate the amyloid protein from contaminating blood by including the scores of fibrinogen Bβ and Gγ chains and the presence of an amyloidogenic variant into our algorithm [48]. In other cases, a range of proteins can be identified, and amyloid fibril protein identification is less clear. This was observed in a sample of localised amyloid obtained from nasopharyngeal tissue. Here the immunohistochemistry gave an equivocal result and proteomics indicated high scoring kappa and lambda light chains with other potential amyloid proteins such as ApoA-I and gelsolin. This was an external referral sample, and no clinical data were available; it was labelled as uncertain at the MDT meeting.

Tissue decellularisation is one approach to remove such ambiguities. Here fresh tissue, including fat aspirates, is treated with sodium deoxycholate detergent washes to remove contaminating soluble proteins leaving the extracellular amyloid deposits intact. The method was validated using an amyloidotic mouse model [49] and has subsequently been adapted and extended to the analysis of human tissue [50]. This approach is not currently available for FFPE tissues.

**Variants and variables**

Familial forms of amyloidosis arise from genetic mutations resulting in the formation of unstable variant proteins. Pathogenic variants have been identified for a number of amyloidogenic proteins including fibrinogen Aα (FibA) [51, 52], lysozyme [53] and TTR [54, 55]. A current list may be found at http://amyloidosismutations.com/ [56]. In principle, structural information is already available from the MS data and can be extracted by appending a list of variants to the Swiss-Prot database before searching. Proteomics for variant identification has been invaluable in some cases, particularly in the correct identification of fibrinogen Aα amyloid [48] where we have added an extra set of 12 variants, including the C terminal “VLITLG” base deletion variants [57, 58] to the Swiss-Prot database. The approach also works for some other variants, for example, p.S72P (S52P) and p.V50M (V30M) TTR. It is not always possible to identify a variant if it is included in a small or very large tryptic peptide and their ions fall outside the normal instrument acquisition range, or the peptides fail to generate suitable MSMS spectra, for example, with large peptides with four or more charges. This may be overcome by using other enzymes for digestion such as AspN, although this would not be undertaken routinely for clinical samples. Novel variants or modifications to other parts of a variant peptide (e.g. glycosylation) would preclude identification in a routine analysis, and isobaric changes such as I/L would not be identified. There are also a number of factors which could lead to misidentification of a variant, the most obvious being where the search engine conjoins peptides from the wild-type protein with a misidentified peptide derived from another protein. A high probability score for a protein does not necessarily equate with a high probability identity for the variant. We have observed TTR variants such as p.H110D (H90D) with high scores together with the correct genotyped variant in clinical samples. In some cases, this may be a simple misidentification based on a low scoring variant peptide, however, the possibility of mosaicism cannot be ruled out, with a low level acquired clone generating low plasma concentrations of a highly amyloidogenic variant protein which is preferentially deposited in amyloid. Similar issues were observed in a study of renal fibrinogen Aα amyloid [48]; here we observed three cases where two variants were observed by proteomics although only one was identified by gene sequencing. In the example shown in Figure 2C both p.R573L (R554L) and p.G538R (G519E) fibrinogen Aα variants were identified with high Mascot scores, however, only p.R573L was confirmed by gene sequencing. The variant peptide containing the p.S72P → L alteration was present, confirming the identification, however, the peptide containing the putative p.G538R variant arginine was absent, and was only identified from the cleavage product C-terminal to the arginine, EFVSETFSR. Whilst this peptide may have arisen through non-specific digestion during trypsinisation, a more intriguing suggestion is that the peptide was generated from a truncated protein with N-terminal FVSETE, which was already present in the amyloid and only identified when a new in silico tryptic site was included in the search parameters. Such truncations are common in amyloid, and, in the case of TTR, have pathobiological significance [59, 60].

Misidentification of the p.V142I (V122I) TTR variant, commonly present in African Americans, is a further example of a mismatch between the proteomics and genotyping. The variant was identified in over 60% of FFPE samples from patients with ATTR amyloid, although
Proteomics – a gold standard?

Proteomics is now being recommended as the new gold standard for amyloid typing. It is compound-independent and requires no prior knowledge of the amyloid type. Can it become a replacement for immunohistochemistry and related immunological techniques? Certainly, IHC has limitations – the specificity varies with type of amyloid, specificity of antibodies, local methods and experience, and a suitable antibody must be available. In an early study [66] on 142 FFPE samples comparing IHC, carried out at our centre, with proteomics undertaken at the Mayo Clinic, there was complete concordance between the IHC-identified samples and the proteomics results in 82% of cases but in 25 samples with non-immunospecific staining the amyloid identification was achieved only by proteomics. The overall superiority of proteomics has been confirmed in a more recent study from our laboratory [21] and is exemplified in data from our most recent annual audit (2018): IHC failed to identify the amyloid protein in 166/433 cases (Supplementary Figure S2c), of which proteomics identified approximately 60% of these unknowns. The audit data also highlight issues with the proteomics approach. One common reason for not identifying the amyloid was “inadequate sample”. We are a national referral centre for amyloidosis, receiving clinical samples from the UK and overseas, and cannot control the amount or quality of individual samples, and in such cases, IHC can be more effective in identifying the amyloid type. It may be that optimization of the pre-analytical stages of proteomics would increase sensitivity and could eliminate any non-amyloid contaminating proteins, thus resulting in proteomics eventually superseding IHC. However, this will only be the case in centres with the resources and expertise to offer such a service. Other centres without such resources will probably continue to use IHC.

Amyloid proteomics is currently carried out in only a relatively few centres worldwide and there is a need to agree on common standards and share best practice. The European Amyloid Proteomics Consortium was established in London (December 2017) to address these issues. The largest clinical proteomics centre at the Mayo Clinic has outlined their criteria for validating their procedures [26] and we have now received accreditation by the UK Accreditation Scheme (UKAS). Amyloid proteomics is now central to our diagnostic service. It is a remarkably robust method for amyloid typing, but there are limitations. If we begin to rely entirely on the output from increasingly complex search engine algorithms, then it will be important to be aware of these limitations, and how unanticipated effects could result in an incorrect diagnosis. Finally, proteomics is only part of our diagnostic armoury, and we have previously [61] used Hooker’s three-legged stool analogy to argue that the three legs of clinical experience, biochemistry (Congo red staining, IHC and genetic analysis) and proteomics are jointly required to make a robust diagnosis of amyloidosis.

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References


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