



## CKJ REVIEW

# Proteomics and mass spectrometry in the diagnosis of renal amyloidosis

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## Abstract

The amyloidoses are a ‘group’ of disorders, all of which are associated with deposits that display similar staining and ultrastructural features and are toxic to tissues. Many proteins—currently 31 protein types and many more variants—have been shown to undergo such transformations. Among the various currently known amyloidoses, there are marked differences with regard to their pathogenesis and incidence, while the associated clinical picture is frequently overlapping. However, the therapies that are currently available are amyloid-type specific. The diagnosis of amyloidosis thus involves two steps: (i) a generic diagnosis, followed by (ii) an amyloid type-specific diagnosis or ‘amyloid typing’. Immunofluorescence in frozen sections or immunohistochemistry (IHC) in paraffin sections has traditionally been used in the typing of amyloid. However, IHC of amyloid differs significantly from IHC in other areas of surgical pathology; both caution and experience are necessary for its interpretation. The rationale for the application of proteomic methods to amyloid typing lies in the relative abundance of amyloid proteins in tissue where, frequently, it is the ‘dominant’ protein. Proteomic techniques include the following steps: sample preparation, protein extraction and digestion into peptide fragments, followed by their subsequent separation and measurement by mass spectrometry (MS) and protein identification by informatics. The advantages as well as the limitations of both methods—immunohistochemistry and MS-based proteomics—are discussed. The current recommendations for the application of proteomics in renal amyloidosis are summarized.

**Key words:** amyloid typing, amyloidosis, immunohistochemistry, laser microdissection, mass spectrometry-based proteomics, onconephrology

## Introduction

The amyloidoses are protein folding disorders in which certain proteins undergo a conformational change leading to the formation of a  $\beta$ -pleated sheet secondary structure [1–3]. As a consequence of this process, the protein acquires an affinity for the dye Congo red, with green birefringence when viewed under polarized light, and has a fibrillar morphology at the electron microscopic level. Many proteins—currently 31 protein types and many more variants—have been shown to undergo such transformations. Thus, the amyloidoses are a ‘group’ of disorders, all

of which are associated with deposits that display similar staining and ultrastructural features and are toxic to tissues. In this review, a summary of currently available options for amyloid typing in renal pathology and the role of mass spectrometry (MS)-based proteomics is provided.

Several mechanisms have been proposed to explain why these amyloid deposits form, and it is possible that more than one mechanism may be involved concurrently [4, 5]. Thus a sustained increase in the concentration of the precursor protein, proteolytic remodeling or intrinsic protein instability and instability due to a mutation are all known mechanisms. These

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are accompanied by failure or overload of the protein quality control system responsible for *in vivo* clearance and recycling of abnormally folded proteins. The latter may also explain why the amyloidoses primarily affect older patients whose chaperone systems have been weakened by prior insults. During this process, serum amyloid P component (SAP), apolipoprotein E, glycosaminoglycans (GAGs) and possibly other extracellular components are recruited. These compounds are not only involved in the formation of amyloid fibrils but also in their persistence, by protecting them from cellular degradation mechanisms [4, 5]. Of particular note is the fact that SAP and apolipoprotein E are universally associated with all types of amyloid fibrils thus far studied, such that these components are known as ‘amyloid signatures’. Thus, colocalization of SAP and amyloid protein is seen by immunohistochemistry (IHC) and is also detectable by proteomic methods; clinically, SAP scintigraphy is used to assess the amyloid load in patients.

The pathogenicity of amyloid is not simply the consequence of tissue displacement or replacement by deposits. In recent years, it has become apparent that amyloid protein oligomers or protofibrils exert a greater direct toxic effect than the mature fibrils [4, 5]. Importantly, this may explain why patients who respond to therapy demonstrate clinical improvement despite their seemingly unreduced load of amyloid deposits.

Among the various currently known amyloidoses, there are marked differences with regard to their pathogenesis and incidence, while the associated clinical picture is frequently overlapping. However, the therapies that are currently available are amyloid type specific [1–3, 6–24]. The principal goal of diagnosis is the distinction between treatable versus nontreatable diseases; moreover, amyloidoses with a genetic component also require genetic counseling. Although relatively few amyloid types are found in the majority of patients, clinicians must also consider rare amyloidoses before a specific therapy can be implemented. While some amyloidoses are localized, systemic or systemic and/or localized, others are known to affect specific organs (i.e. cerebral, endocrine organs, etc.) or are seen in defined geographic areas (i.e. Icelandic).

### Amyloidoses in renal pathology

The most prevalent type of amyloidosis is that derived from the immunoglobulin light chain, AL. Among systemic amyloidoses, it affects 85% of patients. Its primary target organs are the heart and kidney. AL is always associated with an underlying clonal plasma cell proliferation. However, a true B-cell/plasma cell neoplasia is diagnosed in only 10–15% of AL patients. These patients typically have clinical manifestations that are derived from both the large clone (bone lesions, hypercalcemia and infections) and the M-protein itself (light chain cast nephropathy and hyperviscosity). However, in 85% of patients with AL, there are no clinical symptoms resulting from the clone itself, which is small and, at times, difficult to detect. These patients, however, have symptoms derived from the M-component-associated disease, such as AL, light chain deposition disease or other renal diseases with non-organized deposits [25]. Most importantly, these ‘small dangerous clones’ producing AL are ultimately lethal and must be eradicated by methods similar to those applicable to a large tumor burden. These methods involve intense chemotherapy (myeloablative melphalan with autologous stem cell transplantation) and, more recently, therapies targeting proteasomes and aggresomes.

The second most prevalent type of amyloidosis is that derived from the serum amyloid A precursor, SAA. This precursor protein is produced by the liver in response to chronic inflammatory

conditions. Familial cases, associated with a mutation in genes for nonamyloid fibril proteins that play a permissive role in the development of amyloid, have also been increasingly recognized. Regardless of the etiology, treatment, with a resultant reduction of SAA levels, is associated with prolonged survival.

While AL and AA together affect ~90% of patients with systemic amyloidoses, the remaining 10% of patients have other types. Thus a diagnosis of AL, although admittedly the most likely based on statistics, cannot be assumed in any individual patient, and a precise diagnosis of the amyloid type is required. The non-AL, non-AA amyloidoses comprise diverse conditions, including hereditary, nonhereditary and even iatrogenic diseases; in addition, wild-type transthyretin can also be amyloidogenic and can cause a systemic amyloidosis that primarily affects the heart. This latter type of amyloidosis is almost certainly underdiagnosed and therefore its true incidence is difficult to estimate [1].

Among the hereditary amyloidoses, amyloidosis derived from a transthyretin mutant, ATTR, is the most prevalent type. Although there are three known geographic hot spots (Portugal, Sweden and Japan), the disease is known to occur worldwide and it is the most common hereditary amyloidosis in the USA. It is estimated that 3.9% of African Americans carry a TTR V122I mutation. Several other hereditary amyloidoses have also been discovered (AFib, AApolipoAI and AII, among others) [11–24]. Since in several of these hereditary amyloidoses the liver is either the exclusive or predominant source of the abnormal protein, liver transplantation has been offered to affected patients as a form of ‘surgical gene therapy’ [19, 20]. Currently, pharmacologic therapies are being tested in clinical trials for ATTR, both hereditary and wild-type. In other amyloidoses, most notably systemic amyloidosis derived from the leukocyte chemotactic factor 2 (ALLECT2), no therapy is currently available [13–17]. However, it is important not to misdiagnose these amyloidoses as AL since therapy for AL can have grave consequences for the patient (Figure. 1).

### Diagnosis of the amyloid type: antibody-based versus proteomics

The diagnosis of amyloidosis involves two steps: (i) a generic diagnosis of amyloidosis, followed by (ii) amyloid typing. In renal pathology, in particular in the USA/North America, frozen section immunofluorescence has been used very effectively for the detection of proteins derived from serum. It continues to be the first step in amyloid typing in renal pathology [26–29]. With experience, and the use of an antibody panel, it can successfully type ~85% of amyloidoses, but not 100%. It must be stressed that cases yielding negative or equivocal results must be reported as undetermined and other means of amyloid typing must be considered. The amyloid type cannot be determined based on the distribution of deposits or on clinical grounds. Amyloid-type determination must be based on the tissue deposits alone. Collateral studies (serum-free light chain assays, etc.) are performed to support the diagnosis of the amyloid type but NOT to make it. Also, patients can have both monoclonal gammopathies and hereditary amyloid proteins. Precise identification of the amyloid protein is critical since treatment depends on the type of amyloid-forming protein.

IHC in paraffin sections has traditionally been used in the typing of amyloid, in particular for nonrenal biopsies [7, 8, 10, 28]. However, IHC of amyloid differs significantly from IHC in other areas of surgical pathology; both caution and experience are necessary for its interpretation [29–31]. The challenges of amyloid

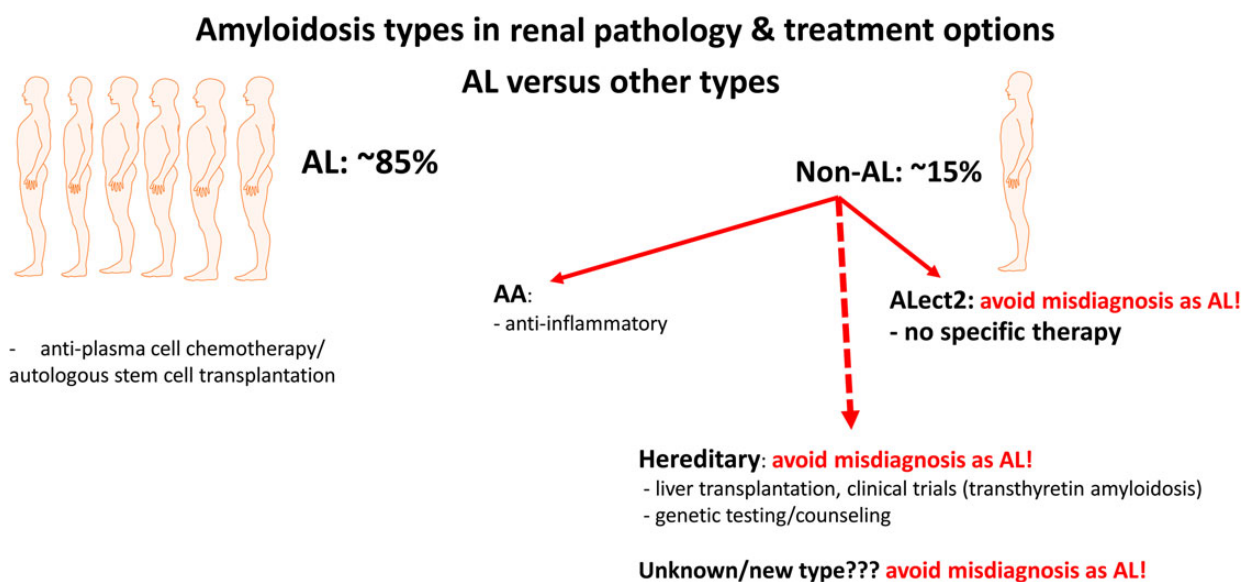


Fig. 1. Amyloidosis types in renal pathology and amyloidosis type-dependent treatment options.

IHC include (i) a lack of commercially available amyloid and amyloid-type-specific antibodies, (ii) amyloid heterogeneity, (iii) serum contamination and (iv) a lack of availability of adequate controls [29–32].

The main differential diagnosis includes AL versus all other types. Note that commercially available antibodies are typically raised against the native protein and not the amyloid protein. Furthermore, in AL in particular, amyloid fibrils may be derived from the light chain fragment rather than the intact light chain molecule. The truncated light chain may predominantly contain the variable region, which may or may not be detectable by commercial antibodies that are typically raised against the constant region.

'Comparative immunohistochemistry', which utilizes testing with a panel of antibodies rather than a single antibody, helps to avoid some of these obstacles [28]. More recently, the use in immunoperoxidase stains of antibodies raised against free light chains has been reported [33]. These latter antibodies do not react with light chains present in the complete antibody, which contains both light and heavy chains. In contrast, most antibodies raised against light chains react with whole immunoglobulins as well as free light chains, and there are generally many more of the former than the latter. Antibodies that detect only free light chains produce cleaner backgrounds, with no 'contamination' by circulating immunoglobulins. While further improvements in IHC procedures can be expected, the availability of other methods for amyloid typing is both needed and welcome.

### Application of proteomics to amyloid typing

The rationale for the application of proteomic methods to amyloid typing lies in the relative abundance of amyloid protein in tissue where, frequently, it is the 'dominant' protein [34–38]. Moreover, amyloid proteins, being relatively small, are particularly suited to evaluation by MS. The diagnosis of amyloid by proteomic methods is based on the presence of large numbers of peptides from the amyloidogenic protein in conjunction with apolipoprotein E and serum amyloid P component (also known as the amyloid signature).

### History

The word proteome is derived from a combination of the words 'prote' in and gen'ome' and was coined by Marc Wilkins in 1994. The term proteomics was first coined in 1997 as an analogy with genomics, the study of the genome. The proteome is the entire set of proteins produced by an organism or system. However, each tissue or bodily fluid has its own proteome. Thus the plasma proteome consists of >1000 proteins. However, 20 of the most abundant proteins make up almost all of the protein by weight, whereas all other proteins are present in only trace amounts. Similarly, cellular proteomes have more proteins than plasma, but here again only a small number of proteins make up the bulk of the proteome. For example, the glomerular proteome comprises 1817 proteins, of which 401 are derived from contamination with plasma.

Several major developments preceded the application of proteomics to pathology, including electrophoretic separation techniques, liquid chromatography, MS, laser capture microdissection, methods for protein retrieval from formalin-fixed paraffin-embedded tissues and bioinformatics (software and databases). Proteomics is a relatively new field, and its rapid evolution is largely a consequence of the advances in MS that have occurred during the past several years. A more thorough analysis of the different techniques used to identify proteins in general, and amyloid from tissue in particular, which is beyond the scope of this review, can be found in several recent reviews [34–44].

### Mass spectrometry

The principal application of MS is substance identification in a sample by accurate determination of its molecular mass. However, MS can measure only the mass of charged molecules in a gas; the proteins in living organisms are in liquid and are usually not charged. Thus, the application of MS to protein studies requires the ability to gently apply a charge to proteins without destroying them. This is a relatively recent development, for which John Fenn and Koichi Tanaka received a Nobel Prize in 2002. The application of a charge to proteins can be achieved by either electrospray ionization (ESI) or matrix-assisted laser desorption/

ionization (MALDI). ESI and MALDI can be considered complementary techniques. They deal with the analyte in two different physical states—liquid and solid, respectively. The solution-based technique of ESI was the easiest to couple to MS for the analysis of both proteins and peptides. MALDI is still most often used for the analysis of single analytes or digests of a single protein. MALDI is also considered to be more ‘high throughput’ than ESI. A charged molecule moves (‘flies’) through an electrical or magnetic field in a precise way determined by its mass. MS analyzes ions by their mass:charge ratio ( $m/z$ ), detects the separated ions and collects the data.

Proteomic techniques include the following steps: sample preparation, protein extraction and digestion into peptide fragments, followed by their subsequent separation and measurement by MS and protein identification by informatics.

Sample preparation for MS aims to increase the concentration of the protein of interest in the tissue under study. In studies involving amyloid deposits, areas containing a deposit are identified in paraffin sections by staining with Congo red and visualization under fluorescent light. This form of amyloid detection (i.e. using fluorescent illumination) is more sensitive than conventional polarization, largely due to visualization of the entire area containing amyloid deposits, without the presence of the polarization shadow encountered in the conventional polarization technique. The latter phenomenon, as the name implies, is associated with the tendency for only a fraction of the amyloid deposits to be birefringent at any particular orientation of the specimen and, in this case, only by rotating the slide table can the adjacent amyloid deposits be seen. Amyloid deposits, identified by this means, are subsequently dissected from paraffin sections using a laser; typically 10- $\mu\text{m}$ -thick paraffin sections are

used. The dissected area thus contains predominantly, but not exclusively, amyloid deposits. A combination of laser microdissection (LMD) or laser capture microdissection (LCM) and tandem MS (MS/MS) has typically been used for amyloid typing in paraffin sections, and this technique is frequently abbreviated as LDMS, LMDMS or LMD-MS/MS [37].

Subsequently the proteins, including amyloid proteins, must be extracted from the paraffin section. Protein retrieval from formalin-fixed paraffin-embedded tissue is done using a process similar to antigen retrieval in IHC. Thereafter the tissue is subjected to trypsin digestion, which results in a mixture of peptides. Typically, high-performance liquid chromatography (HPLC) is then used for separation of the peptides. Another method that has been used is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). However, this approach is more labor intensive and less amenable to automation than the gel-free HPLC approach and, for clinical purposes, has generally been abandoned. Thus, MS analyzes the peptides, not the protein, since proteins are too large. It is difficult to make large proteins ‘fly’; hence, proteins are fragmented into peptides (twice in MS/MS).

The mixture of peptides is subjected to ESI and the peptides are sprayed into MS1, which measures the parent mass of the peptide. A precursor ion is mass-selected by MS1 and typically fragmented by collision-induced dissociation (CID), followed by mass analysis of the resulting fragments, derived from the parent peptide mass, by MS2. These measurements are used to predict the amino acid sequence (Figure. 2). MS/MS provides structural information by establishing the relationship between precursor peptides and their fragmentation products. The fragmentation of proteins can also be done in cyberspace, using a program that predicts how peptides will fragment.

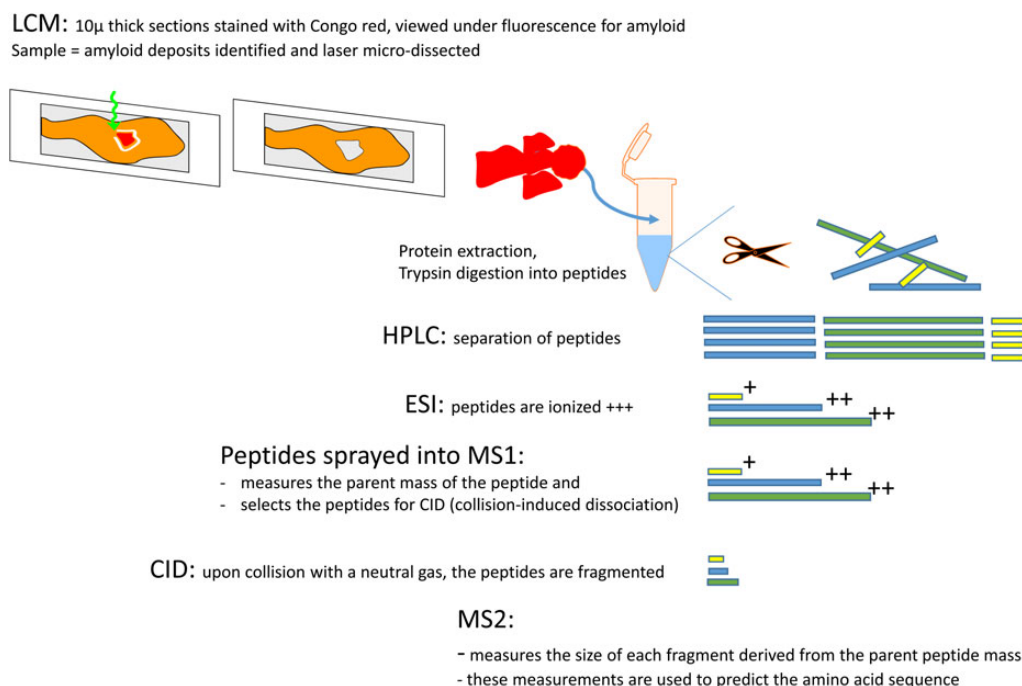


Fig. 2. Flow chart for LMD-MS/MS-based proteomic diagnosis of amyloidosis in formalin-fixed and paraffin-embedded biopsy specimens. Paraffin sections are stained with Congo red and amyloid deposits are visualized under fluorescent light and laser microdissected. The proteins are extracted and digested into peptides with trypsin. Peptides are separated by HPLC, ionized by ESI and sprayed into the first mass spectrometer (MS1). MS1 measures the parent mass of the peptide and selects the peptides for CID. Upon CID fragmentation, the size of each fragment derived from the parent peptide mass is measured by MS2. These measurements are used to predict the peptide amino acid sequence, and the data are presented as a list ranked according to the relative abundance of each protein identified. Figure drawn with Motifolio kit.

There are two basic approaches to liquid chromatography-MS/MS-based proteomics: shotgun proteomics and targeted proteomics. While the former, commonly employed in amyloid typing, aims at global identification of proteins in the mixture, the latter tries to identify specific proteins [38, 40, 41].

Results are displayed as spectra of the relative abundance of detected peptides, and in this respect, shotgun proteomics is only semiquantitative [38]. Molecules in the sample can be identified by correlating known masses to the identified masses or through a characteristic fragmentation pattern, analogous to using fingerprints to identify a person. Protein identification is achieved by matching the identified peptides to a database of proteins, such as UniProtKB/Swiss-Prot or NCBI nr [40, 41]. The findings are more convincing if they are based on matching mass spectra derived from several peptides (Figure. 3). Different algorithms may be used for data analysis. Although this may be variable between laboratories, the most commonly used algorithms include Mascot, Sequest and X!Tandem [37, 40, 41]. Thus a list of peptides identified by MS is generated, their amino acid sequence is predicted based on the fragmentation pattern and protein matching is attempted based on the amino acid sequences that are available in the database. A scaffold, with proteins and the probability scores of their positive identification, is generated. For clinical samples, a probability score >90% is desired. The list is ranked according to the relative abundance of each protein identified. Spectra values represent the total number of mass spectra collected by MS and matched to the protein using proteomics software. A higher number of mass spectra indicate a greater abundance of the protein. Similarly, the greater the extent of amino acid sequence coverage, the higher the level of confidence in the protein identification. For clinical applications, several samples are run in parallel and the minimum number of spectra in all samples is determined during the validation process [37].

Although MS-based proteomics is applicable to amyloid typing using a small amount of tissue, such as a kidney biopsy, there is a definite requirement for a minimum amount of amyloid in the sample. In general, it has been reported that amyloid deposits as small as a single glomerulus (60 000 μ<sup>2</sup>) dissected from a 10-μm-thick section may be sufficient [45]. However, one glomerulus, only partially obliterated by amyloid, may not be sufficient. Thus other factors include the distribution of amyloid deposits within the specimen and whether it is amenable to laser microdissection. Hence, delicate, ‘chicken-wire’-type deposits will be too difficult to dissect, while in the nondissected sample, such deposits will be overshadowed by other proteins. This situation has certainly been encountered in fat biopsies [46–48]. In the latter, amyloid typing using MS-based proteomics is possible in specimens with a Congo red score ≥3+ (on a scale of 0–4), whereas samples with a Congo red score of 1 or 2 are not informative [47]. While the diagnosis of amyloidosis still requires examination of solid tissue samples, MS techniques have also been applied to the study of amyloidogenic precursors in body fluids. Most efforts have focused on the analysis of serum transthyretin in patients with potentially amyloidogenic mutations [49–51]. However, more recently, detailed proteomic studies of serum-free light chains have been reported [52].

Despite the progress made in the clinical application of MS-based proteomics thus far, and its vast potential, this technology is currently only available in large referral centers. The nontrivial factors limiting its accessibility include the significant upfront cost of equipment, standardization and the requirement for highly specialized personnel (at the PhD level) to do this type of analysis and regulatory and reimbursement requirements of such tests in the clinical setting [45].

In summary, MS-based proteomics has several limitations: (i) low-abundance proteins/peptides may be difficult to detect since their signals may be overshadowed by data from more abundant

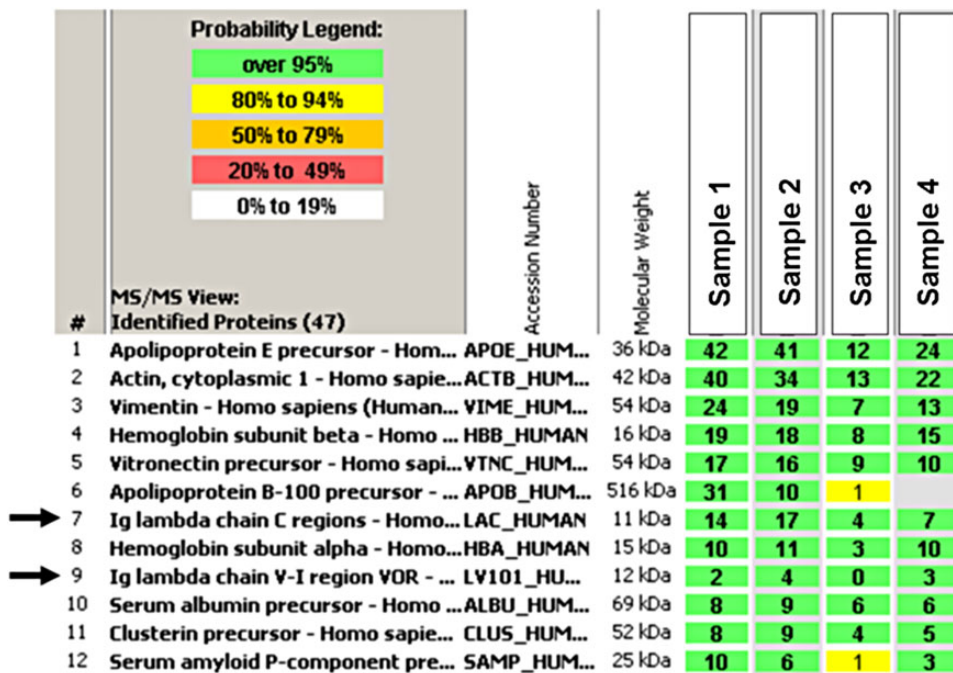


Fig. 3. LMD-MS/MS-based proteomic analysis identifies immunoglobulin lambda light-chain constant and variable regions as the main component of the amyloid deposits (arrows). The amyloid P component (SAP) and apolipoprotein E (APOE), known as ‘amyloid signatures’, are also present, as well as stromal components of the glomerulus. Reprinted with permission from Dogan [38].

proteins, (ii) peptide fragments obtained after enzymatic digestion must be of a size that is appropriate for MS and (iii) there is reliance on computational predictive algorithms to a reference human genome obtained from publicly available databases. In contrast, the main advantages of MS-based proteomic analysis include (i) the global identification of proteins and (ii) the serendipitous discovery of unsuspected proteins/biomarkers.

### IHC versus MS-based proteomics

To understand the relative strengths and weaknesses of IHC and MS-based proteomics, it is helpful to compare these techniques with the differences that exist between *in situ* hybridization and conventional cytogenetics. In the former, paraffin sections are typically used, no extraction is required and the information gained can be very precise, providing that one knows what to look for and that the corresponding probe is available. In conventional cytogenetics, tissue must be harvested fresh and the cells grown in order to be subsequently spread for chromosomal evaluation. The final evaluation material is a picture of the entire (global) set of chromosomes, which may show expected as well as unexpected abnormalities, leading to the discovery of new data. However, an important limiting factor is the size of such abnormalities: while chromosomal deletions/translocations can be detected, gene abnormalities will not be seen in a conventional karyotype.

Traditionally, antibodies have been used to identify proteins, and today they are still the gold standard for protein identification and quantification. Antibodies are also routinely used to confirm MS proteomics-based protein identification. However, the interpretation of antibody reactivity with altered/nonnative proteins such as amyloid proteins represents a unique challenge, and caution and experience are necessary. In the case of antibodies, no sample preparation, including protein extraction and separation, is needed, while in MS-based proteomics, these steps are necessary. Moreover, the identification of proteins by MS depends on enzyme cutting sites [38]. For a given protein to be analyzed by MS, fragments of a size appropriate for such analysis must be generated by enzymatic digestion, typically by using trypsin. However, some human proteins may contain large fragments that have no internal cutting sites for trypsin and are thus too large for MS to handle. While antibody availability may present a limiting factor for the identification of certain amyloid deposits by IHC, this latter method is sensitive, fast and cheap, whereas MS requires sophisticated equipment, is not widely accessible and has a longer turnaround time. The differences between IHC and MS-based proteomics are summarized in Table 1.

Limited data have thus far been published where IHC results were directly compared with MS-based proteomics results. In a study by Gilbertson *et al.* [53], 142 biopsies from 38 different tissue types, most commonly kidney, were studied by IHC; routinely a panel of 11 commercially available antibodies was used in paraffin sections. In 108 consecutive biopsies, the amyloid type was successfully determined by IHC and these results were subsequently confirmed by LDMS in all cases (100% concordance). However, in the same study, in 34/142 specimens (24%), IHC was negative or inconclusive. In these latter cases, LDMS allowed amyloid typing in 25 additional cases, whereas in 9 cases (6.3%) the results were inconclusive. Among the 34 cases yielding inconclusive results by IHC, there were 17 cases of AL (10 kappa and 7 lambda). A further eight cases comprised those for which no corresponding antibody was included in the IHC panel of antibodies: amyloid derived from immunoglobulin heavy chain (AH, three cases), amyloid derived from apolipoprotein AIV (AApoAIV,

**Table 1.** Comparison between IHC and MS-based proteomics

Immunohistochemistry	MS-based proteomics
Routinely used to identify proteins	Newer technology
Need to have an antibody for each protein to identify	Global protein identification—entire proteome
Must know which protein to look for to get the correct antibody	Can identify unknown proteins and discover new biomarkers
Antibodies are not available for most proteins	Not dependent on antibody for diagnosis
Antibody reactivity dependent on fixation, truncation, etc., which may affect specificity	Identification dependent on enzyme cutting sites and informatics
No separation needed	Separation needed
Sensitive	Less sensitive for low-abundance proteins
Cheap	Expensive
Fast	Longer turnaround time
Readily accessible	Not readily accessible

three cases) and amyloid derive from atrial natriuretic factor (AANF, two cases). The failure to identify amyloid protein by LDMS in nine cases was due to the presence of an insufficient quantity of amyloid in the specimens (two cases), technical failure (one case) and inconclusive interpretation (six cases). The above authors, and others, concluded that, at the present time, proteomics should be considered to be complementary to IHC but, as yet, cannot be considered the new gold standard for the typing of amyloid [53]. Further bioinformatics development and standardization will also be required before it can be widely and reliably applied in the clinic.

### Conclusions

Based on the published literature [9, 10, 53], thus far, in renal pathology, the current applications of LD/MS can be summarized as follows:

- typing of amyloid deposits where routine immunofluorescence/IHC is equivocal or negative
- confirmation of the amyloid type
- detection of less common/unusual amyloid types: AFib, ALECT2, AGel, AApoAI, etc.
- cases where there is an inadequate sample for immunofluorescence typing.

### Conflict of interest statement

None declared.

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