Mass Spectrometry Based Proteomics in the Diagnosis of Kidney disease

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Abstract

Purpose of review: Laser microdissection (LMD) and mass spectrometry (MS) is a new technique that consists of dissection of glomeruli, tryptic digestion of dissected material, analysis by mass spectrometry, and generation of a protein profile using different algorithms. The review focuses on the use of this methodology as an ancillary technique in a clinical laboratory for the diagnosis of kidney diseases.

Recent Finding: LMD/MS is used in the diagnosis and typing of kidney diseases with organized deposits such as amyloidosis. Uncommon and familial forms of renal amyloidosis are diagnosed and typed based on the presence of specific amyloidogenic proteins. LMD/MS is used to confirm and identify immunoglobulins (Ig) and complement factors in immune complex-mediated and complement-mediated proliferative glomerulonephritis, respectively. In particular, LMD/MS can detect monoclonal Ig in cases of equivocal immunofluorescence studies in monoclonal Ig-associated glomerulonephritis. LMD/MS can detect specific complement factors of the alternative pathway and terminal pathway in complement-mediated glomerulonephritis.

Summary: LMD/MS is currently used for diagnosis and typing of amyloidosis. In addition, LMD/MS is useful in determining the type of Ig and complement factors in immune-complex and complement-mediated glomerulonephritis, respectively.
**Introduction**

The pathology and diagnosis of kidney disease is based on examination of the kidney biopsy material by light microscopy, immunofluorescence microscopy and electron microscopy.[1] In addition, immunohistochemical staining of proteins of interest have been used to identify a protein of interest. These techniques have resulted in unraveling the pathogenesis of many kidney diseases. However, given the inherent limitations of these targeted techniques, newer methods that could examine global protein expression to diagnose and understand the pathophysiology of the kidney disease in a clinical setting are required. Laser microdissection (LMD) combined with mass spectrometry (MS)-based proteomics is a new diagnostic tool which enables analysis of global protein expression patterns in areas of interest, such as a glomerulus, interstitium and the tubules. Using this technique, the protein profile of diseased glomeruli can be compared with the protein profile of a normal glomeruli. In this review, we discuss the role and usefulness of LMD/MS in the diagnosis of various renal diseases.

**Basic methodology**

The methodology is recently published.[2-5] Briefly, glomeruli are identified on 6 or 10 μm-thick sections of formalin-fixed paraffin-embedded material. The sections are stained either with hematoxylin and eosin for easy identification of renal microanatomy such as the glomeruli or with Congo red for identification of amyloid deposits. In our setting, the glomeruli are laser microdissected using the Leica dissector (Leica DM 600 B). Each microdissection is called a ‘sample’ and each microdissection contains an area of 60000-100000 μm² (60,000 collected for 10μm thick section; 100,000 collected for 6μm thick section) and this may involve 4-6 glomeruli depending on size (Figure 1). Typically 2-4 samples are analyzed for each case. The microdissected material is collected into 0.5ml micro centrifuge tube caps containing 35 μL Tris/EDTA/0.002% Zwittergent buffer. Microdissected fragments are digested into tryptic peptides overnight and analyzed by liquid chromatography electrospray tandem MS. MS raw data files are queried using three different algorithms (Sequest, Mascot and X!Tandem), the results are combined and assigned peptide and protein probability scores and displayed in Scaffold (Proteome Software Inc., Portland, OR). For each sample, a list of proteins based on peptides identified by MS is generated. Peptide identifications are accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm. [6-8] The mass spectrometry data shows spectra that match to a particular protein based on the amino acid sequence available in the database. Some of the peptides from different proteins can be common and be shared depending upon the homology of their amino acid sequence. On the other hand,
unique peptides and spectra are distinctive to the particular protein. The ‘Spectra’ value indicates the total number of mass spectra collected on by MS and matched to the protein using the proteomics software. A higher number of mass spectra is indicative of greater abundance and will typically yield greater amino acid sequence coverage. A higher mass spectra value also indicates a higher confidence in the protein identification. Our clinical amyloid testing requires a minimum number of four spectra in all samples before the protein identification will be deemed clinically valid.

**Glomerular diseases with organized deposits**

LMD/MS has been useful in the diagnosis and understanding of glomerular diseases associated with organized deposits including amyloidosis, fibrillary glomerulonephritis and immunotactoid glomerulopathy.

**Amyloidosis:**

Amyloidosis is a disease characterized by extracellular accumulation of protein fibrils in various organs; the kidney is one of the most common organs involved.[9] The characteristic feature is a positive Congo-red stain where the amyloid deposits appear reddish-brown and show an apple-green birefringence under polarized light. The two common types of amyloidosis include AL amyloidosis caused by deposition of immunoglobulin (Ig) light chains and AA amyloidosis caused by deposition of acute phase reactant serum amyloid A protein. However, more than 25 types of amyloidosis are recognized including amyloidosis derived from a variety serum proteins such as apolipoprotein I, II and IV, gelsolin, leukocyte cell derived chemotaxin-2 (LECT2) and fibrinogen-α.[10] Accurate diagnosis and typing of amyloidosis is critical for the treatment and prognosis.[11] Conventional light microscopy and immunofluorescence microscopy is generally adequate for the diagnosis of most cases of AL amyloidosis. Most laboratories use immunohistochemical methods for the diagnosis of AA amyloidosis, and increasingly for LECT2 amyloidosis. However, for the remainder of cases most institutes use clinical surrogate markers such as serum studies and genetic tests which have low specificity and/or sensitivity to establish the etiology. It is for this group of amyloidosis that LMD/MS has proved to be extremely useful.

The diagnosis of amyloidosis at the proteomic level using LMD/MS is based on the presence of large spectra for the amyloidogenic protein, in addition to Apolipoprotein E and serum amyloid P component (SAP).[4] Thus, in AL amyloidosis large spectra numbers of Ig-light chain constant regions along with apolipoprotein E and SAP are present. On the other hand,
in LECT2 and fibrinogen-α amyloidosis large spectra numbers of LECT2 and fibrinogen-α chain are present, respectively, along with apolipoprotein E and SAP.

The major advantage of LMD/MS over conventional methods of amyloid typing is that LMD/MS is a single test that can identify the amyloid protein in question versus testing the renal biopsy for individual amyloid proteins via immunohistochemistry or other ancillary studies. In addition, LMD/MS is performed on paraffin block and has no special tissue requirements. It is often possible to identify the genetic variants of amyloidogenic proteins, such as variants of SAA or fibrinogen-α protein.[13] The common indications for LMD/MS studies in renal amyloidosis include confirmation of amyloid type, inadequate sample for immunofluorescence studies, difficult cases on routine renal biopsy studies such as heavy chain amyloidosis, and familial and hereditary amyloidosis.[3,10] An example of LMD/MS results of a representative case of AA, apolipoprotein AIV, fibrinogen-α, gelsolin, heavy chain (AH) amyloid, AL-kappa light chain, AL-lambda light chain, LECT2, and transthyretin amyloidosis are shown in Figure 2.

**Fibrillary Glomerulonephritis and Immunotactoid Glomerulopathy**

In addition to amyloidosis, fibrillary deposits are also present in fibrillary glomerulonephritis.[14] The fibrils in fibrillary glomerulonephritis are usually polyclonal and stain for IgG, kappa and lambda light chains. The fibrils are similar to amyloidosis in that they are randomly arranged but are thicker and measure 10-30 nm in thickness. The most important differentiating feature is that the fibrils in fibrillary glomerulonephritis are Congo-red negative. In spite of these differences, the presence of a heavy chain component, equivocal Congo-red staining, or the presence of a monoclonal gammopathy sometimes causes a diagnostic dilemma between amyloidosis and fibrillary glomerulonephritis. In such cases, LMD/MS is helpful in differentiating the two conditions. In fibrillary glomerulonephritis, large spectra of IgG1 chain C-region and apolipoprotein E are present, with no or very small spectra for SAP. On the other hand, amyloidosis shows large spectra of Ig light chain constant (C)-region, SAP and apolipoprotein E. Typically, in amyloidosis the spectra ratio of apolipoprotein E to light chain is 2-3:1, while in fibrillary glomerulonephritis, the ratio is often close to 1:1.

In immunotactoid glomerulopathy, the deposits have a microtubular structure that are often arranged in parallel arrays and measure 10-90 nm in diameter. The deposits are typically monoclonal and stain for IgG and kappa or lambda light chains, and are often associated with an underlying paraproteinemia. Although the pathologic findings are quite typical in immunotactoid glomerulopathy with the diagnosis resting on identification of the microtubular deposits, recent studies using LMD/MS have given insights into the development of fibrillary deposits in
amyloidosis and fibrillary glomerulonephritis, and microtubular deposits in immunotactoid glomerulopathy. LMD/MS of the glomeruli suggests that it is the ratio of apolipoprotein E to the amyloidogenic protein/Ig that results in fibrillar versus microtubular deposits. Thus, in amyloidosis very high spectra numbers of apolipoprotein E and moderate to high spectra number of light chains or other amyloidogenic proteins is associated with amyloid fibrils. In fibrillary glomerulonephritis, apolipoprotein E is present in moderate to low spectra numbers, and the ratio compared to Ig gamma-1 constant region is typically 1:1. On the other hand, in immunotactoid glomerulonephritis, apolipoprotein E is present in low spectra numbers while the Ig gamma-1 constant region is present in high spectra numbers. Thus, the ratio of Ig and apolipoprotein E is reversed to 3:1. From these studies, it appears that apolipoprotein E (and the ratio to the amyloidogenic/Ig protein) contributes to fibrillogenesis: high spectra numbers are associated with the thinner fibrils in amyloidosis, and smaller spectra numbers are associated with the thicker fibrils present in fibrillary glomerulonephritis (Figure 3).[15] The absence of apolipoprotein E in deposits of cryoglobulinemic glomerulonephritis (that lack substructure) further suggests that apolipoprotein E is required for fibrillogenesis.

Membranoproliferative Glomerulonephritis

Membranoproliferative glomerulonephritis results from an inflammatory response to glomerular capillary wall deposition of immune-complexes and complement factors or deposition complement factors alone.[16] Immune-complex deposition (immune-complex mediated MPGN) results in the activation of the classical and terminal pathways of complement. On the other hand, deposition of complement factors (complement-mediated MPGN) results from dysfunction of the alternative pathway of complement with accumulation of complement factors of alternative and terminal pathways, with minimal or no immune-complex deposition. LMD/MS studies have been critical not only in confirming the type of deposits in these conditions, but has been instrumental in elucidating the pathogenesis of these diseases.

Membranoproliferative Glomerulonephritis, immune complex-mediated

The cause of immune-complex mediated MPGN is varied. The most common causes include chronic infections, such as hepatitis B or C, autoimmune diseases as Lupus or Sjogren’s syndrome, or paraproteinemia in the setting of monoclonal gammopathy of unknown significance (MGUS), B-cell lymphoproliferative and plasma cell disorders. Cryoglobulins may or may not be present in these diseases. LMD/MS of immune complex-mediated membranoproliferative glomerulonephritis confirmed the presence of the immunoglobulins although the type of
immunoglobulin (IgG, IgA, IgM, Ig kappa light chains, Ig lambda light chains, Ig heavy chains) varied reflecting the different etiologies of immune-complex mediated MPGN.[2] High spectra number of C3 was present in all cases, while low spectra numbers of C4 was noted in 80-90% of the cases, indicating activation of the classical pathway of complement. Importantly, complement factors of the terminal complement complex were rarely indentified. Factor H related protein-1 (FHR1) was often present. Interestingly, apolipoprotein E was absent in cryoglobulin-mediated MPGN.

Immunofluorescence microscopy studies have variable sensitivity and specificity in identifying immune-complex deposits and thereby can be inconclusive. Monoclonal gammopathy was noted in 41% of the patients with MPGN in one study.[17] Thus accurate identification of monoclonal Ig is of paramount importance. The detection of monoclonal Ig can sometimes be difficult on routine immunofluorescence studies. In such cases, LMD/MS has been particularly useful in identifying the monoclonal Ig.

A case is highlighted to illustrate this point: A 58-year old asymptomatic woman with a history of longstanding hypertension presented with a preserved renal function, microscopic hematuria and 5 grams/24hours of proteinuria. Renal biopsy was consistent with membranoproliferative pattern of injury. Immunofluorescence studies showed only staining for segmental IgM and C3, likely representing entrapment in segmentally scarred capillary tufts. However, electron microscopy showed many subendothelial, subepithelial and mesangial electron dense deposits. The evaluation was negative for any infectious causes or autoimmune disease; however an IgG kappa monoclonal protein was identified in the serum at 0.4 mg/dL. Furthermore, serum Ig free light chain kappa to lambda ratio was elevated at 2.96. Bone marrow biopsy revealed 8% plasma cells, and there was no evidence of anemia, hypercalcemia or any lytic lesions. Thus, she was labeled as having an MGUS. Due to the discordance between serum monoclonal protein (IgG kappa) and IF findings (segmental IgM, and no light chain restriction), it was unclear if the patient's MPGN was related to the monoclonal gammopathy. LMD/MS studies were then performed which identified the deposits as IgG kappa, thus confirming that the MPGN was indeed due to deposition of IgG kappa (Figure 4).[18]

LMD/MS might be also useful in cases of light or heavy chain deposition disease where immunofluorescence microscopy results are sometimes equivocal. In such cases, there is marked mesangial expansion resulting in Periodic acid Schiff (PAS) and silver positive mesangial nodules that appear similar to diabetic mesangial nodules. The distinguishing feature is that the nodules of light/heavy chain deposition are positive for the respective Ig light/heavy chain on immunofluorescence studies while the diabetic nodules are negative. However, the
immunofluorescence studies are equivocal in some cases of Ig light or heavy chain deposition disease and LMD/MS can confirm the presence of the Ig light/heavy chains.

2. Membranoproliferative Glomerulonephritis, complement-mediated: Dense Deposit Disease and C3 glomerulonephritis

Dense deposit disease (DDD) and C3 glomerulonephritis are both complement debris diseases resulting from dysfunction of the alternative pathway of complement. They fall under the umbrella term ‘C3 glomerulopathy’ to reflect a common pathophysiology.[19,20] Both DDD and C3GN often show a proliferative glomerulonephritis on light microscopy and bright C3 staining on immunofluorescence microscopy.[21,22] They are differentiated from each other based on electron microscopy findings that shows dense osmiophilic deposits along the glomerular basement membranes and in the mesangium in DDD and distinct subendothelial and mesangial deposits (and occasional subepithelial and intramembranous deposits) in C3 glomerulonephritis. Although both conditions show bright C3 staining on immunofluorescence microscopy, the composition of the deposits are not known, and are a cause of speculation, particularly in DDD.[2]

LMD/MS in both conditions showed accumulation of complement factors of the alternative pathway and terminal complement pathway (Figure 5). Thus, large spectra numbers of C3 and C9 were found in all cases of DDD and C3 glomerulonephritis. Smaller spectra numbers of C5, C6, C7, and C8 were also present. Complement regulating proteins such as vitronectin and clusterin were also present in large spectra numbers. In addition, large spectra number of complement FHR-1 and FHR-5 were present. There was little or no significant accumulation of complement factors of the classical complement pathway, such as C1, C2 or C4. In addition, there was little or no Ig present. There was also no Factor B present, indicating absence of C3 and C5 convertase in the glomeruli, suggesting that activation of alternative and terminal pathway occurs in the fluid phase rather than resulting from local disturbance of the alternative pathway. The presence of the terminal complement complex (TCC)/ soluble membrane attack complex in the deposits of DDD and C3 glomerulonephritis is an important factor that determines the effectiveness of the new anti-complement drug, eculizumab, in these conditions.[23-25] Eculizumab acts on the terminal pathway via inhibiting C5a. Thus, mass spectrometry of glomeruli of biopsy specimens of DDD and C3 glomerulonephritis that shows large spectra number of the terminal complement pathway proteins are the cases that will likely respond to treatment with eculizumab.
To summarize, LMD/MS is useful in confirming the etiology of proliferative GN, i.e., immune complex-mediated versus complement-mediated. With regards to immune-complex MPGN, LMD/MS can detect the immune-complex deposits that were negative or equivocal on immunofluorescence studies. With regards to complement-mediated glomerulonephritis, LMD/MS can detect the components of the alternative and terminal complement pathways. Thus, therapy can be optimized based on the immune-complex or complement proteins deposited in the glomeruli.

**Necrotizing and Crescentic Glomerulonephritis**

Necrotizing and crescentic glomerulonephritis is the most severe form of renal injury. In the majority of cases, the pathologic process is due to injury resulting from circulating anti-glomerular basement membrane (anti-GBM) antibodies, immune-complex deposition, or pauci-immune causes. These are often classified as type I, type II and type III, respectively.[26] Immune-complex mediated glomerulonephritis with crescents include entities such as lupus nephritis, IgA nephropathy, etc. Pauci-immune necrotizing and crescentic glomerulonephritis is often associated with anti-neutrophil cytoplasmic antibodies (ANCA).[27] However, a small proportion of pauci-immune necrotizing and crescentic glomerulonephritis are ANCA-negative. In a recent study of one such case, LMD/MS showed large spectra for fibrinogen, C3 and terminal complement complex factors in the glomeruli along with the absence of any immunoglobulin deposition. The findings suggested activation of the alternative pathway of complement.[28] Indeed evaluation of the alternative pathway revealed a complement factor H mutation that was not previously reported. Based on this report, it was suggested that the ANCA-negative pauci-immune crescentic and necrotizing glomerulonephritis result from abnormalities in the alternative pathway of complement. LMD/MS studies of ANCA-negative and ANCA-positive pauci-immune crescentic and necrotizing glomerulonephritis are underway to determine whether a role of the alternative pathway can be identified in the ANCA-negative pauci-immune crescentic and necrotizing glomerulonephritis.

**Future studies**

There is a potential for use of LMD/MS in the study of many glomerular and tubulointerstitial diseases. For example, in the setting of nephrotic syndrome, LMD/MS can be used to identify the proteins in minimal change disease and focal segmental glomerulosclerosis to determine whether unique protein profiles are present in these states. In the setting of infection-associated
tubulointerstitial nephritis, LMD/MS can be used to identify specific viral, fungal, or bacterial proteins.

**Conclusion**
LMD/MS is a new technique that shows great promise for the diagnosis and understanding of kidney diseases. It is currently routinely used as an ancillary test for the diagnosis and typing of amyloidosis. It is also used as an ancillary test for the diagnosis of glomerulonephritis resulting from immune-complex deposition and/or complement deposition.

**Key Points**
1. LMD/MS is used in the diagnosis and typing of amyloidosis, particularly where routine immunofluorescence and immunohistochemistry are equivocal.
2. LMD/MS is used for diagnosis of the less common types of amyloidosis, such as LECT-2, Fibrinogen-α, Gelsolin, apolipoprotein A-I, II and IV, and transthyretin amyloidosis.
3. LMD/MS is used to confirm the presence of immune-complexes, particularly monoclonal deposits, in the setting of immune-complex mediated glomerulonephritis.
4. LMD/MS is used to confirm the presence of complement factors of the alternative pathway and terminal pathway, in the setting of complement-mediated glomerulonephritis.
Acknowledgements: We would like to thank Julie A. Vrana, Jason D. Theis, and Patrick Quint for the effort and enthusiasm in running the laser microdissection and mass spectrometry laboratory. Part of the methodology for LMD/MS analysis on paraffin tissue is performed under a license from Expression Pathology Inc.

Financial Disclosure: None

Conflict of Interest: None
FIGURE LEGENDS

Figure 1. Laser microdissection. Hematoxylin and eosin stained section showing (A) Glomerulus to be microdissected (top), (B) Vacant space on slide following microdissection (bottom).
**Figure 2.** Representative mass spectrometry data of amyloidosis. Representative mass spectrometry data by spectral analyses from a case of AA amyloidosis (AA), apolipoprotein A-IV amyloidosis (ApoA-IV), Fibrinogen-α amyloidosis (Afib), Gelsolin amyloidosis (Agel), Heavy chain amyloidosis (AH), AL kappa light chain amyloidosis (AL-kappa), AL lambda light chain amyloidosis (AL-lambda), LECT-2 amyloidosis (Alect2), and transthyretin amyloidosis (ATTR). The probability number (>95% is highlighted by green, 80-94% by yellow) indicates essentially the percent homology between peptides detected in the specimens and the published amino acid sequences of their corresponding proteins.
Figure 3. Comparison of LMD/MS data of organized deposits in amyloidosis, fibrillary glomerulonephritis and immunotactoid glomerulopathy. Average spectra numbers of apolipoprotein E, serum amyloid-P component (SAP), and Ig heavy (gamma-1) or light chain-C region detected in amyloidosis, fibrillary glomerulonephritis and immunotactoid glomerulopathy.
Figure 4. Representative mass spectrometry data of a case of membranoproliferative glomerulonephritis, showing spectra for Ig-kappa chain C-region and Ig gamma-1 C region. Both C3 and C4 are present indicating activation of classical pathway. Spectra for Ig-lambda light chain C was absent.
Figure 5. Representative mass spectrometry data of 3 cases of C3 glomerulonephritis (C3GN) and 3 cases of Dense Deposit Disease (DDD). Mass spectrometry shows accumulation of complement factors of the alternative pathway and terminal complement pathway, in particular C3 and C9 in all cases.
References


