Summary: Atypical Hemolytic uremic syndrome (aHUS) is an ultra-rare renal disease that affects two per 1,000,000 persons in the United States. It is characterized by microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure. Both sporadic (80% of cases) and familial (20% of cases) forms are recognized. The study of familial aHUS has implicated genetic variation in multiple genes in the complement system in disease pathogenesis thus defining the mechanisms by which complement dysregulation at the cell surface level leads to both sporadic and familial disease. This talk reviews the complement cascade, focusing on the alternative pathway and terminal complement cascade and their role in aHUS. Causes of aHUS are described, including genetic mutations in complement genes and autoantibodies to complement proteins. Therapy using eculizumab, a humanized anti-C5 monoclonal antibody, has changed the prognosis of aHUS from a disease that was associated with high mortality only 2-3 years ago to a disease that can be treated today. Nevertheless, pressing questions remain. We will touch on recent advances in our genetic understanding of aHUS and how a more detailed picture of this disease is essential to understand disease penetrance, which is highly variable, and response to therapy, both in the short and long term.
Recent Insights into C3 Glomerulopathies and MPGN

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Introduction
C3 Glomerulopathy is a recently introduced term (1) that encompasses glomerular disease characterized by the accumulation in glomeruli of C3 or its metabolites without significant deposition of immunoglobulins or of the early components of the classical pathway of complement activation, C1q and C4. On electron microscopy there are electron dense deposits corresponding to the C3 deposits seen on immunohistochemistry. The deposition of C3 in the absence of immunoglobulin and classical pathway complement activation implies uncontrolled activation of the alternative pathway of complement. C3 glomerulopathy, thus defined, is distinct from forms of thrombotic microangiopathy that may also be associated with alternative pathway activation since in those cases complement activation is on the renal endothelium and is not associated with well-defined deposits on electron microscopy. The morphology seen by light microscopy is variable and includes mesangial proliferation, a membranoproliferative pattern, endocapillary proliferation and crescentic glomerulonephritis.

It is possible to subclassify C3 glomerulopathy based on differences of morphology as seen by light microscopy and electron microscopy or by aetiology as, in some cases, an underlying gene mutation has been identified. Perhaps the simplest distinction in terms of morphology is on the basis of the appearance of the electron dense deposits seen on electron microscopy. In some cases these have a very dense osmiophilic appearance leading to the designation as dense deposit disease whereas in other cases the deposits do not show this appearance and this has been referred to as C3 glomerulonephritis. However, the distinction is not always clear-cut and that there may be overlap of pathogenesis.

Dense deposit disease (DDD)
Dense deposit disease is defined by the presence of dense osmiophilic transformation of the glomerular basement membrane on electron microscopy; on light microscopy
the morphology is variable. Previous reports have emphasized the membranoproliferative form of the disease, designating this as MPGN type II. However, while it is clear that a membranoproliferative pattern of glomerular injury is common, a range of other patterns of glomerular involvement also occurs. At one end of the spectrum the glomeruli may show only mesangial expansion and hypercellularity. In some cases the glomeruli show prominent endocapillary hypercellularity with segmental neutrophil infiltration (sometimes called exudative pattern). In others large numbers of crescents may be present warranting the diagnosis of crescentic glomerulonephritis. Walker et al. (2) collected 69 cases of dense deposit disease from centers in North America, Europe and Japan. They identified 4 distinct histological patterns on light microscopy: Membranoproliferative (25%), mesangial proliferative (45%), crescentic (18%), and acute proliferative and exudative (12%). Dense deposit disease is associated with complement dysregulation. The majority of individuals with DDD have hypocomplementaemia at some stage during their illness and it is now evident that DDD is particularly associated with dysregulation of the complement alternative pathway. The condition is strongly associated with the presence of C3 nephritic factor (C3NeF) (3). C3NeF is an IgG autoantibody that binds to the C3 cleaving enzyme complex (termed a C3 convertase) of the complement alternative pathway and potentiates its C3 cleaving function. The result is secondary C3 depletion in plasma. Furthermore, other distinct causes of excessive C3 convertase activity, all of which result in secondary C3 activation, have been associated with MPGN resembling DDD. These include dysfunctional C3 molecules, anti-factor H autoantibody, a dysfunctional factor H molecule, and factor H deficiency. Despite these strong associations between C3 dysregulation and DDD, the evidence in humans is predominantly circumstantial. Furthermore, C3NeF and C3 depletion can be found among individuals without renal disease. Whether or not C3 nephritic factors are directly pathogenic in DDD or epiphenomena has consequently been hotly debated.

Our understanding of the role of factor H in glomerulonephritis has been greatly increased by the use of gene targeted mice lacking factor H developed in our laboratory by Matthew Pickering. Similar to homozygous factor H-deficient humans, these animals have extremely low levels of plasma C3 as a result of uncontrolled alternative pathway activation (4).
At 8 months all of the homozygous factor H-deficient mice had developed light microscopic features of MPGN with 23% mortality. Novel mechanistic insights derived from the factor H-deficient mouse model include the very important observation that MPGN in this animal is totally dependent on C3 activation. Hence, preventing C3 activation (achieved by crossing factor H-deficient mice with factor B-deficient animals) completely prevented the development of MPGN (4). Furthermore, preventing C5 activation (achieved by crossing factor H-deficient mice with C5-deficient animals) did not prevent the development of the glomerular basement membrane deposits (5). However the inability to activate C5 was associated with a reduction in glomerular cellularity, serum creatinine levels and mortality. This suggests that whilst it would not be expected to prevent the development of glomerular basement membrane deposits, chronic inhibition of C5 activation in humans with MPGN associated with factor H dysfunction may be beneficial by reducing the glomerular inflammatory response. Recently reports have appeared suggesting some clinical success in treating C3 glomerulopathy patients with the anti-C5 monoclonal antibody eculizumab (6,7).

A novel observation has recently come from studies of animals with combined deficiency of both factor I and factor H (8). Factor I-deficient mice develop uncontrolled C3 activation through the alternative pathway together with renal abnormalities characterised by mesangial C3 deposits with nodular mesangial expansion, but importantly no evidence of GBM abnormalities. Remarkably, factor H-deficient mice with deficiency of factor I do not develop MPGN. In these animals the glomerular lesion is identical to that seen in mice deficient in factor I alone. Administration of a source of factor I to mice with combined deficiency of H and I resulted in the appearance of glomerular C3 staining in a pattern identical to that seen in mice with deficiency of factor H alone. These data show that in the setting of factor H deficiency, factor I is required for the deposition of GBM C3 to develop with morphological changes of MPGN. This suggests that plasma C3b targets the mesangium whilst plasma C3b metabolites target the GBM. The implication then is that in situations where GBM C3 is present, strategies that sequester C3b metabolites in the circulation will be therapeutically beneficial.
C3 glomerulonephritis

C3 glomerulonephritis (C3GN) is the term used for the form of C3 glomerulopathy without dense deposits. Morphologically, most cases show either a mesangial proliferative or membranoproliferative pattern. Many cases with the morphological appearance of MPGN III of the Strife and Anders type are forms of C3 glomerulonephritis. C3 glomerulonephritis represents a heterogeneous group in terms of pathogenesis and clinical course. There is a range of mutations that have been found in these patients in genes that code for proteins involved with the alternative pathway of complement. There are two examples of familial forms of the disease where specific co-segregating mutations have been demonstrated – CFHR5 nephropathy (9), an autosomal dominant form of glomerulonephritis endemic in Cyprus, and a familial MPGN from Ireland (10).

Servais et al. have reported the clinical features in 56 patients with C3GN and compared them with 29 patients with DDD and 49 patients with immune complex MPGN type 1 (11). The mean age at diagnosis for C3GN was 30 which was significantly higher than for DDD; 25% of patients were below 16 years of age. 27% of patients with C3GN had nephrotic syndrome at presentation as compared with 38% of patients with DDD and 65% of patients with MPGN type 1. At presentation 40% of the C3GN patients had low C3 levels in the circulation. C3NeF was found in 45% of patients which was significantly fewer than in dense deposit disease (86%).

Recent studies have drawn attention to cases of C3 glomerulopathy where the initial clinical presentation and histological features, including sub-epithelial hump-like deposits, were suggestive of post-infectious glomerulonephritis. However, the haematuria and proteinuria did not resolve leading to the subsequent diagnosis of C3GN (12,13).

In contrast to DDD where plasma C3 depletion is commonly seen, in many cases of C3GN plasma C3 depletion is not present and it must be concluded that the accumulation of C3 within glomeruli in these circumstances derives from abnormal activation or clearance within the kidney. Important clues come from the characterization of the genetic basis of two examples of familial C3GN (9,10). In both these reports abnormal structural variation was seen within the complement factor H related (CFHR) locus. This is a group of five genes adjacent to the gene for factor H that encode structurally related proteins and are known as CFHR1-5. In familial C3GN endemic in Cypriot individuals (CFHR5 nephropathy) the mutation was an
internal duplication affecting the CFHR5 gene (9). From recent structural insights into CFHR5 it is evident that this mutation results in duplication of the dimerization motif. This led to the hypothesis that the resultant abnormally large protein would, through enhanced avidity, potently de-regulate CFH (14). In the second family of Irish ancestry (10) the mutation resulted in a hybrid CFHR3-1 gene that was present in addition the normal copies of the CFHR3 and CFHR1 genes. Enhanced CFH de-regulation has also been shown in these individuals, a finding thought to be a consequence of increased CFHR protein number in those affected (14). Therefore, it appears that in both these familial diseases the effect of different CFHR mutations is to enhance the ability of CFHRs to de-regulate the activity of CFH within the glomerulus leading to enhanced alternative pathway activation and glomerular C3 deposition. It remains to be seen whether a similar mechanism is present in other forms of C3GN

Conclusions
Recent work has defined a group of diseases characterized by glomerular C3 deposition due to dysregulation of the alternative pathway of complement activation and which are collectively termed C3 glomerulopathies. In many cases the morphology is of a membranoproliferative pattern of GN and it is important to distinguish these from cases of MPGN with immune complex deposition. Recent work with experimental models and genetic analysis has begun to elucidate the pathogenesis of the C3 glomerulopathies and it is likely that rational therapies for targeting the alternative pathway will become available in the future.

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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. The common indications for LMD/MS studies in renal amyloidosis include confirmation of amyloid type, inadequate sample for immunofluorescence studies, and familial and hereditary amyloidosis. 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. 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 of 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.
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**Conflict of Interest:** None
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 were 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

General considerations: A wide array of viruses can infect the kidney. This includes two important RNA viruses, namely, human immunodeficiency virus (HIV) and hepatitis C virus (HCV) and DNA viruses such as polyomavirus BKV (BKV), cytomegalovirus (CMV), adenovirus (ADV) Epstein-Barr virus (EBV) and other viruses of the Herpesvirus group. While tissue tropism is species-dependent, podocytes, parietal capsular epithelium, capillary endothelium, tubular epithelium and interstitial cells can all support viral replication.

Some viruses produce distinctive cytopathic effect, but diagnosis of others may require in-situ hybridization, immunohistochemistry, immunoassays, culture or PCR. Electron microscopy, if combined with measurement of virion diameter, can be used to assign broad viral families, but not individual species. Ultrastructural recognition of viruses is based on size, shape, presence of an envelope, and arrangement in crystalline arrays. The electron microscopist must be cognizant of viral look-alikes such as intranuclear chromatin filaments, nuclear pores cut enface, annulate lamellae, spherical microvesicles, glycocalyxal bodies, and glycogen crystals.

Clinical presentation of viral nephropathies can be quite variable and includes asymptomatic derangements in renal function tests, proteinuria, acute or chronic renal failure, and mass lesions. A brief overview of the principal viral infections encountered in clinical practice follows. Parvovirus (an occasional cause of collapsing glomerulopathy and thrombotic microangiopathy) and rare viral agents such as Ebola virus and Hantavirus will only be mentioned in passing (1, 2).

HIV associated nephropathy (HIVAN): This is the best known viral syndrome associated with HIV infection. The classical description is an African American man with low CD4 counts, acute onset proteinuria without much edema or hypertension, and nephromegaly (3). Microscopic examination reveals collapsing glomerulopathy, tubular microcysts with pale, PAS negative, fuchsin positive protein precipitates, and a lymphocytic interstitial nephritis with a CD4/CD8 ratio of 0.2-0.8. Ultrastructural
examination reveals cylindrical confronting cisternae, nuclear bodies, granulofibrillar transformation of nuclei, and tubulo-reticular inclusions. Atypical forms of HIVAN are being recognized such as those characterized by mild disease, non-collapsing focal segmental glomerulosclerosis, mesangial sclerosis, or podocyte hypertrophy. Patients with HIVAN are surviving longer and some have shown stable renal function for up to 24 months (4). It has been discovered that G1 and G2 variants of the APOL-1/MYH9 increase the risk of HIVAN by 50%. This provides an explanation for the high incidence of HIVAN in sub-Saharan Africa and its rarity in Caucasians. It also opens the way for implementing screening programs that may allow earlier diagnosis of HIVAN and a further improvement in prognosis (5).

The differential diagnosis of HIVAN should include other syndromes directly linked to HIV infection such as IgA nephropathy with p24 or gp41/120 immune complexes, HIV associated thrombotic microangiopathy, and interstitial nephritis not attributable to any other etiology. It is also necessary to keep in mind opportunistic infections, renal toxicity due to highly active anti-retroviral therapy (crystalline nephropathy, mitochondrial tubulopathies, myoglobinuria), and the usual gamut of renal diseases which may occur in HIV infected patients by coincidence.

**Hepatitis C virus infection:** Systemic symptoms of hepatitis C virus infection usually (but not always) precede kidney involvement. These include liver disease, gastrointestinal tract symptoms, porphyria cutanea tarda, and autoimmune disorders such as Sjogren syndrome, hypothyroidism, and diabetes mellitus (6). The commonest lesion associated with hepatitis C virus in the kidney is cryoglobulinemic glomerulonephritis(7, 8). Less often, biopsies may show non-cryoglobulinemic membranoproliferative glomerulonephritis, membranous nephropathy, leukocytoclastic vasculitis, fibrillary/immunotactoid glomerulonephritis, post-infectious glomerulonephritis or incidental disease. Chronic transplant glomerulopathy, TMA and interferon-α therapy precipitated acute rejection have been described in the allograft kidney. Epidemiologic associations between hepatitis C virus infection and
proteinuria in large scale population studies suggest that the spectrum of hepatitis C virus associated disease in the kidney is wider than currently appreciated (9). Hepatitis B infection can produce the same spectrum of pathology in the kidney as hepatitis C virus, although the most common finding in biopsies is membranous nephropathy, rather than membranoproliferative glomerulonephritis. Laboratory investigations have enhanced our understanding of the pathogenesis of hepatitis C virus associated clinical syndromes. It has been shown that the virus binds to and activates B-cells by interaction with the CD81 or TLR4 receptors. Activated B-cells secrete antibodies which cross link with rheumatoid factor and form viral RNA containing immune complexes with the property of cryoglobulins. Hepatitis C virus antigens processed by dendritic cells generate Th1 cells which produce interferon-γ, and activated macrophages, which produce a variety of cytokines (IL-1β, tumor necrosis factor-α), chemokines, proteolytic matrix metalloproteases, and reactive oxygen species that damage the endothelium and produce vasculitis. Production of autoantibodies and cryoglobulins has been attributed to molecular mimicry with host matrix proteins and immunoglobulin molecules.

Infections with viruses of the Herpesvirus family: Cytomegalovirus nephritis is primarily described in the allograft kidney, and has become extremely uncommon in this era of intense viral monitoring and prophylactic or pre-emptive ganciclovir therapy (10). Most often biopsies performed in the context of CMV viremia show occasional virus infected cells in the peritubular capillary lumen or tubular epithelium. Some of the intra-capillary cells may simply reflect circulating neutrophils rather than a true nephritis.

Epstein-Barr virus (EBV) is occasionally encountered in kidney transplant biopsies. It may cause a banal interstitial nephritis or florid post-transplant lymphoproliferative disease. Clinical presentation includes a rejection like graft dysfunction, mass lesion, hydronephrosis, or lymphocele. Histologic clues to distinguish post-transplant lymphoproliferative disease from acute rejection include an expansile nodular
infiltrate, serpiginous necrosis, and lymphocyte atypia, although some forms of the disease show only mature lymphocytes and plasma cells (11). The large majority of cases will consist of CD20 positive B-cell rice infiltrates that express Epstein-Barr virus encoded EBER RNA, although EBV positive T-cell lesions, and EBV negative lymphomas also need to be kept in the differential diagnosis. Herpesvirus 6 can infect the kidney and produce rejection-like infiltrates, but the extent to which it produces kidney dysfunction is not clear. Progress in the field is hampered by non-availability of antibodies that can reliably detect viral antigens in formalin fixed tissue. Herpesvirus 8 can result in Kaposi’s sarcoma lesions within the kidney. Infection of the kidney with Herpes simplex or Varicella is exceedingly uncommon.

**Adenoviral nephritis:** This disease occurs more commonly after hematopoietic stem cell transplantation rather than kidney transplantation, presumably because of more severe immune depletion. The spectrum of disease includes asymptomatic viruria, acute tubular necrosis, granulomatous and necrotizing interstitial nephritis, and pyelonephritis. While using immunohistochemistry or in-situ hybridization to establish the diagnosis (12). It is important to keep in mind that commercially available reagents may not detect the more than 50 viral genotypes that have now been recognized. Ancillary testing such as urine culture or PCR may be necessary to establish the diagnosis. Specific anti-viral therapy is not available. Cidofovir is probably the most effective anti-viral agent available today, although the therapeutic index is quite low.

**Polyomavirus BKV:** Polyomavirus BK (BKV) is linked to polyomavirus-associated nephropathy (PyVAN) in 1%-10% of kidney transplant patients. The definitive diagnosis of PyVAN requires a biopsy demonstrating cytopathic changes and a positive immunohistochemistry or in-situ-hybridization test. There is considerable inter-laboratory variation in staining intensity and assessment of percentage of infected cells, but the binary classification of biopsies into virus positive and negative
is fairly reliable. A minimum of 2 biopsy cores should be taken, given the focal nature of PyVAN and the possibility of sampling error in up to 37% of cases (13). The histological findings PyVAN should be semi-quantitatively assessed. Extent of fibrosis and tubular atrophy may be the most important predictor of a poor outcome. The 2009 Banff conference formulated a working proposal in which primary emphasis was placed on the extent of viral cytopathic effect. Application of this system can result in an identical stage being assigned to biopsies which differ markedly in the degree of inflammation and associated unfavorable outcome (14).

The diagnosis of acute rejection concurrent with PyVAN should only be made in the presence of endarteritis, fibrinoid vascular necrosis, glomerulitis, or C4d deposits along peritubular capillaries . Trying to assess occurrence of tubulitis away from areas of obvious viral cytopathic effect is not helpful, since tubules damaged by virus can be expected to release cytokines which would cause inflammation and tubulitis in a much larger surrounding area. It is our experience that in the setting of persistent viruria (even without viremia or nephropathy) biopsies with putative episodes of acute rejection that satisfy Banff criteria for diagnosis do not always respond well to steroids (15). This suggests that the spectrum of virus associated disease in the kidney is wider than currently appreciated. In PyVAN, C4d or immune complex deposits have been observed in the tubular basement membranes, but not peritubular capillaries. MHC class II up-regulation by the tubular epithelium has been proposed as a marker of rejection but this idea is not supported by all workers in the field(16).

The American Society of Transplantation recommends that screening for BKV replication should be performed at least every 3 months during the first 2 years post-transplant, and then annually until the fifth year post-transplant. Using this strategy, at least 80-90% patients at risk for PyVAN can be identified before significant functional impairment of the renal allograft occurs. Acceptable techniques for screening include urine cytology for ‘decoy cells’ and urine or plasma PCR. Testing
for BKV viruria allows a high negative predictive value to rule out BKV nephropathy of 6 to 12 weeks before viremia and nephropathy. It can also identify a subgroup of patients with persistent viruria and increased risk for recurrent episodes of rejection-like graft dysfunction (17). Testing for BKV viremia has a higher positive predictive value than urine but with a shorter window period of 2 – 6 weeks. Plasma screening is preferred over urine in many centers as it is felt to detect clinically more significant replication. In patients with sustained plasma BKV DNA and loads of >4 log_{10} cp/mL, a diagnosis of “presumptive PyVAN” should be made in absence of demonstrable BKV replication in biopsies. An important caveat to remember in interpreting PCR tests is that no international standard is available as a reference calibrator. In addition, quantitation of viral load can be seriously compromised in the presence of uncommon mutant strains. Detection of three-dimensional viral aggregates in urine (so-called Haufen) by electron microscopy has been reported to have high positive and negative predictive values for biopsy proven BKV nephropathy (18). However, histologic disease is no longer considered the desired end point for therapeutic intervention, since nephropathy diagnosed by a biopsy performed in the setting of a rising serum creatinine has worse outcome. This situation is analogous to cytomegalovirus infection where viremia alone justifies anti-viral therapy, and it is not required to demonstrate tissue invasion.

Other polyomaviruses: While BKV accounts for the vast majority of nephropathies polyomavirus JC (JCV) or SV40 should be considered in cases where biopsies shown obvious viral inclusions, but PCR testing for BKV is negative. Immunohistochemistry for polyomavirus antigens is usually positive in these instances since the antibodies are not species specific and cross react with all three viruses. A negative PCR test for BKV may also be negative if we are dealing with a mutant virus: this issue can be resolved by using an alternate set of PCR primers directed against a different part of the viral genome. A new human polyomavirus
designated as HPyV9 has recently been amplified from the plasma of kidney transplant patients. It is not yet know whether it can cause renal dysfunction.

References