Emerging role of clinical mass spectrometry in pathology

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ABSTRACT

Mass spectrometry-based assays have been increasingly implemented in various disciplines in clinical diagnostic laboratories for their combined advantages in multiplexing capacity and high analytical specificity and sensitivity. It is now routinely used in areas including reference methods development, therapeutic drug monitoring, toxicology, endocrinology, paediatrics, immunology and microbiology to identify and quantify biomolecules in a variety of biological specimens. As new ionisation methods, instrumentation and techniques are continuously being improved and developed, novel mass spectrometry-based clinical applications will emerge for areas such as proteomics, metabolomics, haematology and anatomical pathology. This review will summarise the general principles of mass spectrometry and specifically highlight current and future clinical applications in anatomical pathology.

INTRODUCTION

Introduction to clinical mass spectrometry

Mass spectrometry (MS) is a powerful analytical technique that measures the mass to charge ratio (m/z) of one or more molecules in a sample, where m is the molecular mass in Daltons and z is the amount of charge present on the ion. MS can be used for accurate identification of unknown analytes, quantification of known compounds, and determination of structural and chemical properties of molecules. Compared with established methods such as immunoassays, MS can detect multiple analytes of interest in a high-throughput fashion at improved speed and specificity.[1] Over the past two decades, the technology has become considerably more robust, accurate and cost-effective, leading to the steady adoption of MS in all subdisciplines of laboratory medicine.

With increasing clinical applications of MS, there are a number of reviews published on this topic, particularly focusing on clinical chemistry and more recently microbiology and haematology.[1–4] Although molecular imaging of human tissue using MS has been described for well over a decade, its application in anatomical pathology is only now at the cusp of emergence. The main objectives of this review are to outline the general principles of MS and its instrumentation, as well as to summarise the current and evolving clinical applications of MS with a spotlight on anatomical pathology.

Principles of MS

The basic principles of MS analysis are based on the production, selection, fragmentation and detection of charged ion species from analytes of interest in a sample. MS identifies and quantifies molecules by measuring their m/z. The process of MS analysis involves an initial ionisation step in an ion source, where positive or negative charged ions are produced from neutral molecules. The ions of interest are then selected in a mass analyser according to their m/z ratios, and subsequently detected on a mass detector. The specific ion of interest is plotted in a mass spectrum, where the relative abundance or intensity of the ion is plotted against m/z.

As the analytes travel through various regions of the mass spectrometer, fragmentation may occur. Since fragmentation occurs at specific bonds depending on the chemical nature and strength of the bond, each molecule produces a characteristic mass spectral fingerprint that is related to its molecular structure. Typically, low levels of ion source fragmentation are preferable for molecular mass determination, whereas higher levels of ion source fragmentation are preferable for molecular structure analysis. In tandem MS (MS/MS), two or more mass spectrometers are coupled together with various fragmentation techniques to additionally separate and identify molecular ions that have similar m/z ratios. The specific pair of m/z values of the precursor and product ions is termed a 'transition'. The peak area of the extracted ion chromatogram of an analyte of interest, identified by its m/z transition, can be integrated and calculated for quantitation using an internal standard. An internal standard is usually an isotopically labelled version of the molecule of interest that is added in constant known amounts to the samples at the beginning of the workflow. By analysing the ratio of the peak area associated with the analyte of interest and the internal standard, the exact concentration of the analyte can be calculated. When a single transition is monitored, it is termed 'selected reaction monitoring (SRM)', and when multiple transitions are monitored it is termed 'multiple reaction monitoring (MRM)'.

Instrumentation for MS

When selecting an MS instrument, mass accuracy and resolution are desirable properties in MS analysis. High resolution increases mass accuracy and reduces interference with adjacent peaks from molecules of similar mass, thereby achieving a higher specificity. There are numerous variations in mass spectrometer instrument configurations. A typical mass spectrometer can be divided into three main components: (1) ion source, (2) mass analyser and (3) mass detector. In this section, different types of each component and common MS instruments used in the clinical laboratory will be briefly discussed.

The ion source is where positive or negative ions are produced from a neutral analyte of interest, a process known as 'ionization'. The choice of ion source used in the clinical laboratory depends on the physical properties of the molecules being analysed and the desired output of ionisation. In the clinical laboratory, electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) are the two most common soft ionisation techniques used for quantitative analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) at atmospheric pressure. ESI is an efficient ionisation technique and does not produce excessively fragmented molecules, which makes it particularly useful for small molecules, proteins and peptides. APCI is considered a harsher ionisation technique compared with ESI as it may cause loss of modifications of target compounds (eg, glucuronidation); however, it is more efficient in ionising small, heat-stable molecules and removing matrix components for analytes such as steroids and some drug molecules. Another commonly used ionisation method in the clinical laboratory is the matrix-assisted laser desorption/ionisation (MALDI). MALDI is a type of soft ionisation that produces single charged species and can analyse a variety of molecules including nucleic acid, peptides and proteins.

Once ions are generated in the ion source, the mass analysers separate and fragment ions of specific *m/z* to be detected. Quadrupole mass spectrometers are the most widely used mass analysers in clinical laboratories, where four parallel electrically conductive rods form a channel for ions to pass through from one end to another. To select a specific *m/z* ion to pass through to the detector, a combination of direct current potentials with oscillating radiofrequency is applied to the quadrupole rods causing the ions within a narrow *m/z* range to stay in the channel and the ions outside the range to be ejected radially. This is analogous to monochromator filters in optical techniques, and often referred as the 'mass filters'. Many clinical qualitative and quantitative methods use quadrupole mass spectrometers, including drugs,[5–10] steroids,[11–19] small molecules and metabolites,[20–24] as well as peptides and proteins.[25–29] Another commonly used mass analyser is the time-of-flight (ToF) mass analysers. In ToF mass analysers, ions travel down the flight tube accelerated by applied potential. The flight time, or the time it takes for the ion to reach the detector, is related to the *m/z* ratio of the ion, where lighter ions travel faster and reach the detector before a heavier ion does. ToF-MS has several advantages, including higher dynamic range, mass accuracy, and acquisition speed with moderate resolution and sensitivity. Notable clinical applications of ToF-MS include identification of microbials (via MALDI-ToF)[30 31] and genotyping.[32–34]

When multiple mass analysis occurs in series, it is known as tandem mass spectrometry (MS/MS). Various configurations that include arranging identical mass analysers in tandem (eg, triple quadrupole or ToF/ToF) or hybrid combinations of different mass analysers (eg, quadrupole-orbitrap) have been designed for several applications. MS/MS methods are useful for structural analyses and quantitation of compounds that have similar structure and relative mass. LC-MS/MS is a dominant MS-based technique used in clinical laboratories for quantitative analysis due to its high selectivity, ability to detect low concentrations of analytes and ability to measure multiple analytes in a single method (multiplex).

CURRENT CLINICAL APPLICATIONS OF MS IN CLINICAL LABORATORIES

One of the first uses of MS in clinical laboratories is in the area of toxicology. Confirmatory urine drug screens for clinical or forensic purposes are commonly measured by gas chromatography-MS (GC-MS), and more recently LC-MS/MS. The predictable and consistent hard ionisation fragmentation patterns produced by electron ionisation followed with GC-MS allow for the identification of unknown compounds by matching the full mass spectrum of the sample with a library or database. An important limitation of GC-MS is the requirement of compounds to be sufficiently volatile. This requires samples with poor volatility to be derivatised during sample preparation, where the analytes are chemically altered to be more suitable for analysis.

LC-MS/MS is the most commonly used method for many clinical applications today, ranging from qualitative urine drug screens to quantitative, high-throughput and esoteric analyses including therapeutic drug monitoring (eg, immunosuppressant drugs, antiretroviral drugs, antiepileptic),[5–10] steroids (vitamins, testosterone, androstenedione, 17-hydroxyprogesterone, aldosterone, cortisol, oestrogen),[11–18] inborn errors of metabolism and newborn screening (eg, amino acids, carnitine and acylcarnitine),[23 35–39] endocrinology (eg, biogenic amines, 5-hydroxyindolacetic acid, insulin, thyroglobulin)[25 26 40 41] and immunology (eg, monoclonal immunoglobulins, serum free light chains, IgG subclasses[27–29 42]). Please see recent reviews for more details.[1 3 43] MALDI-ToF MS has also recently been in development for qualitative and quantitative peptide and protein assays. Examples include monoclonal immunoglobulins and serum free light chains,[29 42 44] haemoglobin A1c,[45] insulin-like growth factor-1,[46] multiplex biomarker panel of C reactive protein, serum amyloid A, faecal calprotectin and cystatin C.[47]

In microbiology, quantitative measurements of antibiotics and antifungal drugs are available.[10 48 49] Recent implementation of MALDI-ToF MS has revolutionised the workflow for the identification of micro-organisms. The identification is done by matching the ionised proteins and peptides that are specific to the organism and present in cultures to a validated spectral library. Compared with conventional microbiological techniques (eg, Gram stain, culture, biochemical tests and susceptibility testing), MALDI-ToF MS offers significantly shorter turnaround time in terms of species identification. A limitation of MALDI-ToF MS, however, is its relative lack of specificity in differentiating closely related strains of bacterial species. In molecular diagnostics, genotyping and mutational analysis in liquid biopsy have been in development using MALDI-ToF MS.[32–34 50] Please see recent review on other clinical and near clinical applications of MALDI-ToF MS for details.[51–53]

EMERGENCE OF MS APPLICATIONS FOR ANATOMICAL PATHOLOGY

Omics

Novel applications of MS methods are driving omics research (lipidomic, metabolomic and proteomic) and biomarker discovery in a variety of specimen types including blood, urine and biopsy tissues. Proteomic analysis using MS can be approached using two methods: *top-down proteomics* and *bottom-up proteomics*. Top-down proteomics involves the intact analysis of the entire protein. However, many proteins involved in human diseases share similar masses, making the top-down method ineffective for distinct clinical subtyping. The top-down proteomics method is also unable to accurately determine the prevalence of different protein variants if they possess similar masses. In contrast, bottom-up proteomics involves enzymatic cleavage of the whole protein through peptidase digestion to generate a mixture of peptides. A commonly used peptidase is trypsin, where it cleaves proteins at predictable sites (the carboxyl side of the amino acids lysine and arginine, except when they are followed by proline),

and the peptide mixture generated from trypsin digestion is a characteristic signature of the precursor protein. The peptide mixture can then be purified through chromatography and other preparatory methods prior to MS analysis. The separation of the digested protein through chromatography and use of MS to analyse the identity and prevalence of proteins constitute a more effective method of protein identification for clinical diagnosis.

In anatomical pathology, whole proteome profiling from tissue slides is beginning to be established to examine global protein expression patterns and elucidate the pathophysiology of diseases. For instance, routine pathological diagnosis of renal diseases requires interpretation of morphological alterations observed by light microscopy (LM), immunofluorescence (IF) and electron microscopy (EM), with correlation to clinical parameters. Immunohistochemical (IHC) staining of proteins has additionally been used to identify the protein of interest. In difficult or equivocal cases, more sensitive techniques such as immunoelectron microscopy (IEM) and laser microdissection mass spectrometry (LMD-MS) analyses may assist in the confirmation of identity and localisation of renal protein deposits.[54–56] In particular, progress has been demonstrated in the clinical utility of amyloid and kidney proteome analysis, which holds importance for diagnosing amyloidosis and characterisation of renal diseases.[55 57 58]

Amyloidosis

Amyloidosis is a group of diseases that result from build-up of insoluble protein aggregates in various tissue and organs. These proteins, known as amyloid fibrils, are abnormal in structure due to protein misfolding and accumulate extracellularly in β-pleated structures. Amyloid deposits are characterised by their ability to develop both systemically and locally in specific sites of the body, rendering increased cellular toxicity and organ damage.[59] The diagnosis of amyloidosis presents a unique challenge as the clinical presentation is often subtle and may overlap with several different conditions. Clinical management depends on the disease phenotype, which depends on the protein composition of the amyloid fibrils. For example, hereditary transthyretin amyloidosis and immunoglobulin-associated amyloidosis, both present with similar clinical syndrome, are managed differently with liver transplantation or stem cell transplantation/chemotherapy, respectively.[60] Accurate and comprehensive subtyping of amyloid diseases is critical for appropriate clinical management, since there are distinct treatment options for the different subtypes. To date, there are over 36 different subtypes of localised and systemic amyloidosis syndromes.[61 62]

For diagnosis, the presence of amyloid deposits in tissue biopsy is first confirmed. On light microscopy, amyloid deposits can be found in all compartments, are acellular and have a characteristic positive Congo red stain, where amyloid deposits show reddish-brown and apple-green birefringence under polarised light. On EM, amyloid fibril deposits are solid, non-branching and randomly arranged, with a diameter ranging from 7 to 14 nm.[63] Then, the protein composition (subtype) of the amyloid deposits is determined via a variety of techniques. For immunoglobulin-associated amyloidosis, intense staining for immunoglobulin light chains and/or heavy chains on IF (for renal and IHC for all other organs) is diagnostic for immunoglobulin light chain (AL), immunoglobulin heavy chain (AH) and immunoglobulin heavy and light chain (AHL) amyloidosis.[63] IHC has also been used for diagnosis of serum amyloid A (AA) amyloidosis and LECT2 amyloidosis.[55] These conventional amyloidosis subtyping techniques based on antibodies often have poor specificity and sensitivity due to high background staining caused by serum contamination,[60] loss of epitope recognition from formalin fixation-induced protein cross-linking, and dependency on using multiple and specific antibodies to recognise all potential epitopes for all various amyloid subtypes.[59] The inherent limitations of antibody-based techniques led to the development of novel subtyping methods such as LMD-MS. In LMD-MS, a laser is coupled to a light microscope and used to selectively dissect a subpopulation of protein aggregates of interest from the tissue on the slide. The protein mixture is denatured, digested with trypsin and analysed via LC-MS/MS. The tandem mass spectra are then searched against different search engines for protein identification and semiquantitative measure of its relative abundance.[64] LMD-MS has a major advantage over conventional amyloid subtyping where it is a single test that can simultaneously identify and semiquantify the amyloid protein composition, thereby improving the cost-effectiveness and efficiency in amyloid diagnosis.[60] The novel MS-based method has facilitated accurate identification of rare amyloid types where antibodies may be lacking, such as AGel, AApoAI, AApoAIV AH, ALect2, Alns, ALys and their genetic variants.[62 65 66] LMD-MS can identify the subtype of amyloidosis in more than 92%–95% of cases.[59 67–69] Overall, LMD-MS has higher sensitivity and specificity compared with IF and IHC, and is currently the gold standard for amyloid subtyping.[70] LMD-MS is indicated for the confirmation of amyloid subtype, when there are equivocal Congo red or heavy and/or light chains staining, and when there are inadequate tissue sample for IF studies, in difficult cases such as differentiating congophilic fibrillar glomerulonephritis (FGN) and amyloidosis, and in rare cases such as familial and hereditary amyloidosis.[55 57 66 67 70 71]

Fibrillar glomerulonephritis

FGN is a rare glomerular disease that is associated with autoimmune diseases, viral infections and malignant neoplasm.[72] The pathogenesis and therapy for FGN remain largely unknown.[72] The fibrillar protein deposits of FGN are morphologically similar to amyloid fibrils, where they are both solid, randomly arranged fibrils, with FGN having thicker fibrils with diameter ranging from 10 to 30 nm.[63 72] There is an overlap in the thickness of the fibrils, and fibril diameter should not be solely relied on as a distinguishable feature. FGN deposits are found in the mesangium along the capillary walls of the glomeruli. FGN deposits are primarily composed of polyclonal IgG, C3, and κ and λ light chains, which can be stained with antisera to immunoglobulins by IF. However, monoclonal FGN had been reported occasionally.[73] In histology, amyloid deposits are generally identified by their characteristic apple-green colour under polarised light with Congo red staining; meanwhile, FGN deposits are Congo red-negative. Congo red positivity under polarised light is thus often the differentiating factor between amyloid and non-amyloid diseases. However, certain cases of FGN have been shown to be congophilic despite histochemically and proteomically identical to traditional FGN.[74] For these cases where Congo red is unable to differentiate the diseases, EM becomes a necessity for diagnosis; however, it is often routinely inaccessible. LMD-MS has been used to extract FGN glomeruli and examine the protein composition to search for a suitable diagnostic biomarker.[75] DnaJ homologue subfamily B member 9 (DNAJB9) was identified as the first histological kidney tissue marker for FGN.[74–77] Studies showed that DNAJB9 colocalises with IgG deposition by dual IF and localisation of DNAJB9 to individual FGN fibrils by IEM. DNAJB9 IHC studies on kidney biopsy demonstrate high sensitivity (98%) and specificity (>99%) for FGN.[76 77] As a result, the DNAJB9 IHC test is currently available in some clinical laboratories to reduce reliance on Congo red staining and inaccessible EM, which significantly simplify the diagnosis of

FGN.[76] Through these LMD-MS-based studies, a new variant of FGN termed 'heavy chain fibrillary glomerulonephritis' was also identified, which is characterised by Congo red-negative, DNAJB9-negative fibrillar deposits consist of heavy chain immunoglobulins that appear to be distinct from typical FGN both pathogenetically and clinically.[72 76 78] More recently, serum levels of DNAJB9 were measured by immunoprecipitation-based MRM and demonstrated to be elevated in patients with FGN when compared with non-FGN glomerular diseases, such as immunoglobulin light chain amyloidosis or multiple myeloma, with adjustment for estimated glomerular filtration rate (eGFR) differences.[79] Serum DNAJB9 predicted FGN with a moderate sensitivity (67%) and high specificity (98%) in a discovery cohort.[79] Further studies are needed to elucidate new variants of FGN, clinical utility of serum DNAJB9 as a non-invasive FGN biomarker, pathogenetic role of DNAJB9 and possibility as a targeted therapy.[72] Overall, LMD-MS is indicated to help differentiate difficult cases, such as when there are equivocal Congo red staining, in the presence of heavy chain component to distinguish monoclonal heavy chain FGN from AH amyloidosis and heavy chain deposition disease (HCDD), and differentiating congophilic FGN from amyloidosis, FGN with concurrent renal diseases (IgA, membranous and diabetic nephropathy), and FGN from immunotactoid glomerulopathy (ITG) associated with chronic lymphocytic leukaemia (CLL).[55 72 78]

Immunotactoid glomerulopathy

ITG is another rare glomerular disease that is frequently associated with monoclonal gammopathies and hypocomplementaemia, but not cryoglobulinaemia and autoimmune diseases.[80] The pathogenesis of ITG is largely unknown, and treatment depends on the underlying haematological process.[80] In pathology, ITG is characterised by proliferative glomerulonephritis pattern by LM, monotypic IgG (>90% cases) with κ or λ light chain restriction and C3 staining by IF, and protein deposits defined by microtubular structure with distinct hollow centres measuring 10–90 nm in diameter deposited in organised, parallel arrays in the mesangium, subendothelial and subepithelial space of the glomeruli by EM.[63 81] LMD-MS confirmed the presence of monotypic immunoglobulins, and the activation of classical and terminal pathways of complement as C3 and C4 was identified in ITG cases.[80] Comparing the kidney proteome profiles of amyloidosis, FGN, ITG and cryoglobulinaemic glomerulonephritis by LMD-MS in a discovery cohort, apolipoprotein E (apo E) was hypothesised to be required for fibrillogenesis, where the size and organisation of the protein deposits (ie, fibrillar vs microtubular) are associated with the ratio of apo E to immunoglobulin/amyloidogenic protein.[57] Overall, LMD-MS for ITG is indicated to confirm the identity of protein deposits and to distinguish difficult cases. For instance, ITG associated with CLL typically has small microtubules that may not show the distinct hollow centres and may resemble FGN fibrils even by EM, and LMD-MS proteomic analysis can support the differential diagnosis.[82]

Monoclonal immunoglobulin deposition disease and proliferative glomerulonephritis with monoclonal IgG deposits

Non-organised monoclonal immunoglobulin deposits are seen in monoclonal immunoglobulin deposition disease (MIDD) and proliferative glomerulonephritis with monoclonal IgG deposits (PGNMID). MIDD is classified into three subtypes depending on the composition of deposits: light chain deposition disease, HCDD, and light and heavy chain deposition disease. The light chain deposition disease is the most common subtype.[58] On LM, MIDD most commonly shows nodular sclerosing lesions, although rare cases have membranoproliferative and mesangial proliferative pattern of injury, and a thickened glomerular and tubular basement membrane.[58] Sometimes MIDD nodules may appear similar to diabetic mesangial nodules when IF staining for immunoglobulins is ambiguous.[55] On IF, diffuse linear staining of the heavy or light chain immunoglobulins along the glomeruli, tubules and vascular myocytes is observed, depending on the subtype.[63] On EM, MIDD shows a characteristic punctate granular deposits in the mesangium, and along the glomerular and tubular basement membrane.[70]

Meanwhile, the majority of PGNMID shows a membranoproliferative or endocapillary proliferative glomerulonephritis pattern on LM. On IF, PGNMID shows restricted staining for a single light chain isotype (κ more common than λ) and a single heavy chain subtype. Monoclonal IgG3 is the most common deposit in PGNMID, although rare cases have been reported which include other IgG subtypes, IgA, IgM or light chains.[83–85] On EM, PGNMID shows non-organised granular deposits in the subendothelial and mesangial space.[63]

MIDD and PGNMID differ in their deposit localisation and composition. The deposits in MIDD can been found in glomerular, tubular and vascular regions, whereas PGNMID deposits are confined to the glomeruli.[70] PGNMID deposits are typically intact immunoglobulins, whereas the heavy chains in MIDD are typically composed of truncated IgG1 that lacks the CH1 domain.[70] Both can rarely also be IgA or IgM or light chain driven.[63 70] Patients with PGNMID with monoclonal IgG3 deposits are less likely (30%) to have detectable serum monoclonal proteins compared with IgG1 and IgG2 subclasses.[54 86]

Diagnosis for both MIDD and PGNMID depends heavily on IF staining of immunoglobulins. However, it is important to note that occasionally the degree of immunoglobulin deposition in the glomerulus may be below the detection threshold, epitopes may be masked, or antibodies unavailable due to extremely rare heavy chains such as IgD.[54 87–90] There are also reports of discrepancies between immunoglobulins identified in serum and/or urine studies (ie, serum and urine protein electrophoresis, immunofixation, and serum free light chain studies) and immunoglobulins identified on renal biopsy by IF, which presents diagnostic challenges.[54 91] Therefore, LMD-MS is particularly useful to confirm the presence of immunoglobulin heavy and light chains when IF studies are equivocal or unavailable (eg, IgG subclasses, IgD HCDD), and when immunoglobulins in serum and/or urine identified were discordant with renal deposits identified by IF.[54 87 90]

C3 glomerulopathy: C3 glomerulonephritis and dense deposit disease

C3 glomerulopathy is a rare disease that is caused by dysregulation of the alternative complement pathway and is characterised by renal deposits of complement factors from the alternative and terminal pathways with minimal or no immunoglobulins.[55 63 92] C3 glomerulopathy comprised two pathological entities: C3 glomerulonephritis (C3GN) and dense deposit disease (DDD). On LM, both C3GN and DDD are characterised by mesangial proliferative, membranoproliferative or endocapillary proliferative glomerulonephritis.[63] On IF, bright C3 staining with at least two orders of magnitude in dominance and an absence of immunoglobulin staining is expected.[92] However, there are reports of masked monotypic immunoglobulin deposits in paraffin-embedded tissues, where negative staining of immunoglobulins was observed on IF with positive staining of immunoglobulins revealed on protease digestion.[88 89] Additionally, approximately 30% of postinfectious glomerulonephritis are C3-dominant on renal biopsy, although it is characterised by codeposition of IgG and C3.[92] C3GN and DDD

are best distinguished by their ultrastructure on EM. C3GN is characterised by moderate electron dense, ill-defined granular deposits in the mesangium, subendothelial and/or subepithelial space, and DDD is characterised by highly electron dense, osmiophilic 'sausage-like' intramembranous deposits and mesangial rounded nodular deposits.[63 70] Subepithelial large 'humps' may occur in either lesions.[70 92] LMD-MS studies have enabled detailed analysis on the previously unknown protein composition of the deposits in C3 glomerulopathies.[93 94] Surprisingly, both C3GN and DDD have similar protein compositions from the alternative and terminal complement pathway, where C3 and cleavage product C3dg were present as the predominant glomerular complement proteins and lesser amounts of C9, C5, C6, C7 and C8 were also detectable.[93 94] Factor H-related proteins (FHR1–5) and fluid-phase regulators of the terminal complement complex (clusterin and vitronectin) were also identified.[93] There was a lack of complement proteins from the classical pathway, such as C1, C2 or C4.[93]

Dysregulation of the alternative complement pathway may be due to primary causes such as de novo mutations in complement activating and regulating proteins or due to secondary causes that result in overactivation of the pathway.[58] There is a high prevalence (60%–80%) of patients aged over 50 years old with C3 glomerulopathy and concurrent monoclonal gammopathy at the time of diagnosis, which far exceeds the prevalence of monoclonal gammopathy of unknown significance and C3GN for aged less than 50 years old.[89 95 96] Monoclonal immunoglobulins have been demonstrated to act as C3 nephritic factor or factor H autoantibody, which results in the overactivation of the alternative pathway of complement.[97–100] The subtype of 'C3 glomerulopathy with monoclonal gammopathy' is coined to distinguish C3 glomerulopathy deposits with little to no immunoglobulins in the setting of monoclonal gammopathy, where the monoclonal immunoglobulin contributes to the dysregulation of alternative complement pathway. The high prevalence of C3 glomerulopathy with monoclonal gammopathy and the potential of masked or codeposition of immunoglobulin can present diagnostic challenges to C3 glomerulopathies. Thus, LMD-MS studies are indicated when there are equivocal IF staining for immunoglobulins and C3, and to confirm the cause and protein deposits profile for proliferative glomerulonephritis (immune-mediated vs complement-mediated).[55]

MS imaging

Another emerging technology that has potential clinical applications in pathology is MS imaging (MSI), which employs MS to map the spatial distribution of molecules in a thin tissue sample section. The gold standard of diagnosis for tissue samples has traditionally been a combination of chemical staining and light microscopy, with the results being interpreted by a skilled anatomical pathologist. MSI, however, is remarkable for its ability to image thousands of molecules, such as lipids, proteins, peptides, drugs and metabolites in tissues. MSI also possesses practical advantages compared with current methodology, including analysing tissue directly (in situ), identifying endogenous biomolecules and exogenous compounds in a label-free and multiplex manner, and at the same time correlating molecular spatial distribution to traditional histology. The technique allows for an understanding of the molecular basis and mechanism of disease with relation to tissue morphology. Clinical applications for MSI have been proposed to include tumour typing and disease staging,[101] tumour margin assessment, intraoperative tumour excision,[102] drug localisation[103 104] and biomarker discovery.[101 105]

MSI by MALDI-ToF MS was first described by Caprioli and colleagues[106] over two decades ago. MSI can be performed on fresh/frozen[107–109] and formalin-fixed paraffin-embedded (FFPE) tissues.[110–113] While a wide range of biomolecules, such as peptides, lipids, glycans, small molecules and metabolites, can be detected on FFPE tissues, only lipids and intact proteins are detected on fresh/frozen tissues.[114–116] Fresh/frozen tissues require immediate freezing, cryosection to micrometre-thick slices, and being subsequently thaw-mounted to a slide.[117] FFPE tissues require additional sample preparation steps to remove paraffin prior to MSI analysis, and as such may introduce inter-laboratory variations as small compounds may be stripped concomitantly.[115] Nevertheless, analysis of FFPE tissues offers unique advantages, as it allows access to a large repertoire of archived tissues with well-documented clinical information and correlation to IHC and molecular data. Additional sample preparation steps can also be intentionally used to enhance signal and increase specificity for the analyte of interest, such as on-tissue chemical derivatisation or enzymatic treatment for imaging of testosterone,[118] 2-picolylamine,[119] neurotransmitters,[120] N-glycans[121] and hexosylceramides.[122]

In addition to tissue types, the method and instrumentation for MSI analysis should be considered, including factors such as image resolution, speed of analysis and the availability of clinically validated diagnostic biomarker libraries. Particularly, there is a balance and trade-off in terms of image spatial resolution and analysis speed. The higher the spatial resolution desired for imaging application, the slower the pixel-by-pixel sampling speed for enhanced analyte extraction. The availability of clinically validated diagnostic biomarker libraries can significantly impact interlaboratory variation and allowed for standardised, reproducible interpretation and reporting of pathology results. Therefore, image resolution, speed of analysis and availability of validated database directly impact clinical utility and usefulness of MSI.

One of the most common and well-studied mass spectrometers for MSI is the MALDI-ToF. Under vacuum, an ultraviolet or infrared laser irradiates the matrix cocrystallised tissue sample to desorb and ionise the analytes of interest in a raster-stepped sampling of the entire tissue surface. A mass spectrum is generated per given tissue coordinates or pixel. When regions of interest or the whole tissue is measured, a two-dimensional ion intensity map is generated pixel-by-pixel from the mass spectra obtained from the specific tissue coordinates. If serial tissue sections are analysed, advanced imaging software can generate a three-dimensional image of the ion intensity map. One of the limitations of MALDI-ToF MSI is that additional sample preparation is required, where the prepared tissue is coated with a MALDI matrix—an organic acid chemical compound that cocrystallises and extracts the analyte during the pulsed laser ionisation process. The quality of the matrix deposition and its application technique have significant effects on the analytical sensitivity (ie, signal to noise ratio) and spatial resolution of the MS image. The matrix is selected based on the physical and chemical properties of the molecule of interest. Three common matrices used for peptides, lipids and other low molecular weight molecules are α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA) and 2,5-dihydroxybenzoic acid (DHB). CHCA and SA are often used for peptides, SA and DHB are generally used for proteins, while DHB is useful for carbohydrates and lipids.[117 123] There are also new matrices developed for specific classes of molecules, such as polar lipids,[124] non-covalent bound complexes[125 126] and liquid matrices for improved homogeneity for quantitation.[127] A homogeneous coating of matrix with small, uniform matrix crystals of low-micrometre dimensions is desirable. Some techniques that employ this feature are high-throughput electrospray,[128] matrix coating assisted by an electric field[129 130] and

sublimation under vacuum to name a few.[131 132] In consideration for rapid clinical application, matrix precoated glass slides were also developed to increase throughput.[133 134] Another vacuum-based MSI technique is the secondary ionisation MS (SIMS), which uses a focused ion beam to ionise molecules in the tissue sample. Secondary ions are then generated and analysed by the mass spectrometer.[135 136] MALDI-ToF MSI typically can achieve 10–20 µm spatial resolution; meanwhile, SIMS MSI can achieve <1 µm spatial resolution.[137] However, the drawbacks of SIMS are that it is a destructive technique that obliterates all molecules that it ionises, has limitations in analysing large intact biomolecules (>2 kDa) and requires complex instrumentation.[137]

Emerging in vivo techniques

Significant caveats of the current MSI field are the need to process samples for MS analysis, increased turnaround time and limited utility of the technique to assess processed tissue specimens. To enrich the influence of MSI in the clinic, there is a desire for direct, real-time and rapid analysis of unprocessed samples. More recently, atmospheric pressure ambient ionisation MS has gained noteworthy interest for routine clinical use due to significant advantages over other MS techniques, including simpler sample preparation, simpler instrumentation, and analysis in real time in an open environment and at atmospheric pressure, which made the in vivo analysis of tissues using MSI a feasible and attractive option. Since these early experiments in the past decade, more than 40 ambient ionisation techniques have now been described in the literature.[138 139] Please refer to recent reviews for a summary of common ambient and non-ambient ionisation methods for MSI.[138 139]

Rapid evaporative ionisation MS (REIMS) is one of the first ambient ionisation MS techniques that were integrated into routine clinical surgical use.[140–142] REIMS was originally developed for the accurate identification of cancerous tissues during surgical procedures and has a typical resolution in the range of 0.5–2.0 mm.[143] The coupling of the handheld surgical tool and REIMS-MS into a single device led to the creation of the iKnife.[141] iKnife is a handheld tissue diagnostic device that can immediately analyse, in high resolution and real time, the molecular composition of the tissue it comes into contact with. To do so, REIMS is used to locally heat and disrupt the tissue during electrosurgical procedures, resulting in an aerosol made of tissue-derived gaseous ions being released. The aerosol is subsequently analysed by the mass spectrometer.[144] Through this rapid REIMS-based analysis, the surgeon is provided with continuous feedback on the composition of the tissue being excised, allowing for the precise removal of any tumour tissue. When comparing in vivo, intraoperative diagnosis made with an iKnife with traditional, postoperative histological diagnosis, the in vivo technique is accurate 92%–100% of the time.[141] Benefits to using this technique include a lower chance for misidentification of cancer cells during surgical procedures, a reduced risk of resecting healthy tissue and the increased potential to remove virtually all tumour tissues. In addition to differentiating between cancerous and non-cancerous tissues, REIMS-based technologies such as the iKnife can identify different tumour grades, as well as tissues that exhibit precancerous properties within their lipid bilayer.[144] Moreover, REIMS has been shown to accurately identify histopathological features of a poor prognosis and could potentially be used to identify cancer metastases throughout the body.[141 144] There is also potential for this technology to be used in preoperative staging of cancer.[145] The high specificity and wide applications of REIMS have ensured the conception of numerous novel applications for the technology, beyond surgical uses involved with the iKnife. iEndoscope is another novel technology that stems from REIMS, where the methodology is paired with endoscopic tools for use in diagnostic settings, such as the rapid phenotyping of tissue in rectal and colonic cancers. REIMS equipped with an endoscope allows for visible characterisation of tissue that is typical of the endoscope, while providing additional high-resolution information on tumour biology and chemistry regarding the lesion structure through MS analysis. The primary application of iEndoscope is to accurately differentiate between cancerous and non-cancerous tissues in an endoluminal setting. The endoluminal structure requires the use of an endoscopic polypectomy snare to facilitate the transfer of the aerosol generated by REIMS from the colon to the mass spectrometer. The iEndoscope has been used to identify markers associated with increasing pathogenicity within potentially cancerous tissue. One differentiating factor between REIMS-associated endoscopy and other optical spectroscopic methodologies is its ability to identify abnormal lipid metabolism within the tissue. Abnormal lipid metabolism is often associated with neoplasia, which can facilitate a more confident diagnosis of a cancerous tissue.[145] Another advantage of REIMS-associated endoscopy is its versatility, where it can be used in adjunct with many different endoscopic imaging tools to provide details related to the biochemistry of the tissue.[145] Aside from analysing human tissue, REIMS has proved useful for clinical microbiology, where it is able to directly identify bacterial cells within bacterial cell culture without sample preparation. Despite the broad applications of REIMS, be it in the form of the innovative iKnife or iEndoscope, a major disadvantage of REIMS is its destructive nature in both tissue analysis and bacterial cell culture identification, thus limiting the utility of the technique.[141]

The MasSpec Pen is a non-destructive handheld tool designed with three ports centred around the delivery and maintenance of a singular water droplet. The design involves a single water droplet being exposed to the tissue of interest. The droplet acts as the medium used to withdraw analytes, using the premise of liquid–solid chemical extraction. A pressurised vacuum system then moves the water droplet through a port to the mass spectrometer for subsequent analysis. The additional components of the MasSpec Pen include a syringe pump (to eject the water droplet), a small diameter tubing for solvent transport and a probe. The components of the MasSpec Pen must be made of biocompatible materials to ensure both safety and accuracy in in vivo analyses. Hence, polydimethylsiloxane (PDMS), polytetrafluoroethylene (PTFE) and water are the most prominent substances/materials used within the tool. Similar to the iKnife, the MasSpec Pen is highly sensitive and specific for both in vivo and ex vivo analyses and differentiation of tissue types. The MasSpec Pen succeeds in improving on the aforementioned REIMS-based tools in its ability to analyse and identify tissue samples without sample preparation and in real time, while also touting the additional benefit of being a non-destructive method of analysis.[146]

CHALLENGES AND FUTURE APPLICATIONS

MS has many applications in the clinical laboratory, including clinical chemistry, microbiology, and more recently anatomical pathology, as there are significant advantages for MS-based assays in their multiplexing capacity, high analytical specificity and sensitivity, and potential for real-time in vivo analysis. Prior to clinical implementation of these MS-based methods, a specific gap in clinical need or question needs to be addressed and the advantages and limitations of MS-based methods should be compared with traditional methods in pathology. Additionally, there are desirable and practical features to consider, including high capital costs, requirement of skilled personnel, lack of automation, lack of direct bidirectional interface between MS

instruments and laboratory or hospital information system, lack of standardisation, and regulatory requirements. Significant progress is being actively pursued by the manufacturers and clinical MS community in regard to regulatory requirements,[147 148] standardisation of methods,[149 150] automation in instrumentation and data analysis (eg, chromatographic peak and quality controls),[151 152] and flat file interface to laboratory information systems to facilitate seamless order-to-result workflows.[153] Operational factors such as standardised workflow, turnaround time, and comprehensive biocomputational data analysis and storage should also be considered. More details on the current state and further challenges of routinely implementing MSI in the clinical pathology laboratory are highlighted by experts in the field.[139 154] In summary, future innovations in technology and instrumentation will drive novel clinical applications of MS to the forefront.

Take home messages

- Mass spectrometry-based clinical applications are expanding into various disciplines of clinical diagnostic laboratories including clinical chemistry, microbiology, and more recently anatomic pathology.
- This review summarises mass spectrometry method principles, instrumentation, and current established clinical applications.
- This review highlights current and emerging mass spectrometry-based applications in anatomic pathology including omics, mass spectrometry imaging, and real-time in vivo techniques.

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