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The Clinical Impact of Proteomics in Amyloid Typing

Michelle M. Hill, PhD; Surendra Dasari, PhD; Peter Mollee, MBBS, MMedSci; Giampaolo Merlini, MD; Catherine E. Costello, PhD; Bouke P.C. Hazenberg, MD, PhD; Martha Grogan, MD; Angela Dispenzieri, MD; Morie A. Gertz, MD; Taxiarchis Kourelis, MD; and Ellen D. McPhail, MD

From the Department of Cell and Molecular Biology, QIMR Berghofer Medical Research Institute, Brisbane, Australia (M.M.H.); Faculty of Medicine, The University of Queensland, Brisbane, Australia (M.M.H., P.M.); Department of Haematology, Princess Alexandra Hospital, Brisbane, Australia (P.M.); Foundation IRRCS Policlinico San Matteo, Department of Molecular Medicine, University of Pavia, Italy (G.M.); Center for Biomedical Mass Spectrometry, Boston University School of Medicine, Boston, MA (C.E.C.); Amyloidosis Center of Expertise, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands (B.P.C.H); Department of Cardiovascular Diseases (M.G.), Division of Hematology (A.D., M.A.G., T.K.), Department of Health Sciences Research (S.D.), Department of Laboratory Medicine and Pathology (E.D.M.), Mayo Clinic, Rochester, MN.

Systemic amyloidosis, a serious and often life-threatening disease, is characterized by extracellular deposition of abnormal protein aggregates in blood-vessel walls and tissues, often leading to organ failure. Presenting symptoms are frequently vague, and pathognomonic findings are uncommon, which can result in a delay in diagnosis. However, once the possibility of amyloidosis is raised, the diagnosis can usually be established by tissue biopsy. Typically, biopsy is performed on a clinically involved organ, although sometimes tissue from a more easily accessible site, such as fat pad or bone-marrow biopsy, is sufficient.[1] Amyloid fibrils of all types share several unifying features, including an eosinophilic amorphous appearance by light microscopy and Congo red (CR)-positivity with characteristic yellow-green birefringence under cross-polarized light. By transmission electron microscopy, these fibrils are nonbranching, randomly ordered, and 10 nm in diameter. However, the amyloid type is defined by its constituent amyloidogenic precursor protein, and each type has unique clinicopathologic features and specific therapeutic regimens. There are 36 currently recognized canonical amyloid types, at least 17 of which can be systemic.[2] Historically, patients with amyloidosis were treated with supportive care, but, over time, tailored therapies have been developed for specific amyloid types. For example, immunoglobulin light-chain (AL) amyloidosis therapy is predicated on suppression of the underlying plasma-cell dyscrasia to eliminate the amyloidogenic monoclonal light chains, whereas wild-type transthyretin amyloidosis (ATTRwt) can now be treated using a variety of recently developed pharmacologic agents. Other types, such as leukocyte chemotactic factor 2 amyloidosis (ALECT2), do not currently have specific therapy but are the subject of ongoing research. Even for amyloid types for which there is no specific therapy, an accurate diagnosis is critical to avoid treatment for other types of amyloidosis.

Correct typing of the amyloid precursor protein is of paramount importance for appropriate patient management. The utility of antibody-based typing methods—such as immunohistochemistry, immunofluorescence, and immuno-gold with electron microscopy—is variable. Immunofluorescence and immuno-gold may not be practical for routine clinical use, as the former requires frozen tissue, and the latter requires special fixation and specialized electron microscopy. Immunohistochemistry on formalin-fixed, paraffin-embedded (FFPE) tissue is widely available, but its specificity for amyloid typing is suboptimal, in part because of cross-reactivity with deposited immunoglobulins.[3] The other antibody-based methods are also affected by this problem, albeit to a lesser extent.[4] In all cases, the range of amyloid diseases that is likely to be detected by antibody-based methods is limited by bias toward suspected amyloid types (ie, one finds only what one looks for). For example, immunohistochemistry for amyloid typing is usually done for only 3 amyloid types (AA [serum amyloid A], ATTR, and AL), thus not allowing for detection of more rare amyloid types. The

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limitations of antibody-based typing methods can thus result in assigning an incorrect amyloid type to a specimen, with potentially devastating effects on the patient. Furthermore, immunohistochemistry and immunofluorescence require different tissue sections for each antibody tested. This can deplete biopsy tissue that is often small to begin with, such as from heart and kidney, the sites most commonly involved by amyloidosis.

APPLYING PROTEOMICS TO AMYLOIDOSIS TYPING

As amyloid protein is the molecular culprit in systemic amyloidosis, shotgun proteomics technology—which directly identifies the proteins present in the deposit—is well suited to this diagnostic need. The proteins are digested into peptides, which are analyzed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Sophisticated software and reference protein sequence databases are used to process the LC-MS/MS data and generate a list of proteins present in the sample.

Approximately 20 years ago, matrix-assisted laser desorption/ionization (MALDI)-MS and LC-MS/MS methods for analysis of purified amyloidogenic proteins in plasma, urine, and fibrillar deposits were introduced,[5] demonstrating the ability to detect mutant/variant proteins. Only a few centers at that time had the instrumentation and expertise necessary for the application of these approaches. Thanks to multidisciplinary research on LC-MS/MS amyloid typing over the past 15 years, and a significant increase in the availability of high-performance user-friendly instrumentation in clinical laboratories, amyloidosis diagnostic proteomics workflows from 2 tissues types have been established in several centers and validated globally, positioning proteomics to become the new gold standard for amyloid typing.

The LC-MS/MS amyloid typing for clinical use was developed initially for subcutaneous adipose aspirates.[6] The first cohort study for this method was reported by Brambilla et al in 2012, using 26 cases from Pavia, Italy,[7] and independently validated by Vrana et al at the Mayo Clinic in 2014,[8] in a validation cohort of 43 CR-positive and 26 CR-negative subcutaneous fat aspirates. Vrana et al[8] also reported 90% sensitivity in a cohort of 366 CR-positive cases. The 4-year clinical study was performed on whole fat aspirate specimens without a minimum required amount of CR-positive material, and thus the less-than-perfect sensitivity could be attributed to sampling differences.

A different approach for clinical amyloid typing involves the use of laser-capture microdissection (LMD) to isolate regions of interest from FFPE tissues, followed by LC-MS/MS. By selectively excising CR-positive protein deposits, LMD enhances specificity of the amyloid proteome by reducing contribution from normal tissue. An additional advantage is the very small amount of CR-positive material that is required with the highly sensitive modern mass spectrometers. Successful application of LMD-LC-MS/MS in a clinical cohort was first reported in 2009, by Vrana et al from the Mayo Clinic Rochester, in a training cohort of 50 cases and a validation cohort of 41 cases.[9] An early independent study of LMD-LC-MS/MS for amyloid typing at Kumamoto University, Japan[10] demonstrated its superiority over immunohistochemistry in quantitating genetic variants. Since then, 13 cohort studies from Australia, Japan, United Kingdom, Czech Republic, Denmark, South Korea, and the United States independently confirmed the accuracy of LMD-LC-MS/MS in amyloid typing (Table).[9,11,12-22]

IMPACT OF PROTEOMICS ON PATIENT OUTCOMES

The advent of routine clinical use of LC-MS/MS for amyloid typing has had a profound effect on patient care. Evaluation of the overall impact of proteomics in amyloid typing was first highlighted in the Australian study by Mollee et al, in which 24% of the cohort's clinical treatment was altered as a result of the LC-MS/MS test.[14] The availability of new treatments for specific amyloidosis types, and recent findings of 2 amyloid types being present in a single patient,[23] further

TABLE. Diagnostic Studies Supporting Laser-Capture Microdissection-Assisted Liquid Chromatography Mass Spectrometry as the New Gold Standard for Amyloidosis Typing

Study, year	Clinic, country	Cohort size	Main findings
Vrana, 2009[9]	Mayo Clinic, USA	50 cases, 41 validation cohort	98% to 100% successful identification
Klein, 2011[11]	Mayo Clinic, USA	21 cases, nerve tissue	100% identification
Said, 2013[12]	Mayo Clinic, USA	147 cases, renal	97% identification
Gilbertson, 2015[13]	Mayo Clinic, USA National Amyloidosis Centre, London, UK	142 cases from 38 different organ sites	94% accuracy compared with 76% for IHC; 100% concordance
Mollee, 2016[14]	Princess Alexandra Hospital, Brisbane, Australia	138 cases, 35 different organ sites	94% identification rate compared with 39% for IHC; 97% concordance
Tasaki, 2017[15]	Kumamoto University, Japan	186 cases	96% consistent with clinical diagnosis
Park, 2018[16]	Samsung Medical Center, Seoul, South Korea	16 cases, compared shotgun with targeted proteomics method for 4 amyloid proteins	68% identification by shotgun proteomics, 100% identification by MRM-MS, 56% for IHC
Aoki, 2018[17]	Nippon Medical School, Tokyo, Japan	23 renal cases	91% accuracy Established 10 glomeruli as minimal requirement
Rezk, 2019[18]	National Amyloidosis Centre, London, UK	640 cases including 320 that could not be typed by IHC	85% identification rate 98% concordance with IHC
Holub, 2019[19]	University Hospital Olomouc, Czech Republic	11 cases with 2 organs per patient, across 5 organs	100% identification rate
Gonzalez Suarez, 2019[20]	Mayo Clinic, Rochester, USA	170 cases, renal	100% identification rate compared with 84.6% sensitivity and 92.4% specificity for immunofluorescence
Abildgaard, 2020[21]	Odense Amyloidosis Centre, Odense, Denmark.	106 cases from 6 organs	89.6% accuracy for typing, compared with immune-electronmicroscopy 91.6%
Dasari, 2020[22]	Mayo Clinic, Rochester, USA	16,175 cases, 31 organs	100% identification rate for 21 different amyloid types, amino acid substitutions identified with 100% specificity in hereditary cases

IHC, immunohistochemistry; MRM-MS, multiple reaction-monitoring/mass spectrometry.

highlight the need for highly sensitive and specific amyloid typing.

First, using a tiny amount of tissue, proteomics unambiguously identifies the amyloid type in a single assay with extremely high sensitivity and specificity, enabling rapid initiation of the correct treatment for the specific amyloid type. The critical role of proteomics as part of the multidisciplinary management of amyloidosis is exemplified by cases in which the patients would have received incorrect treatment without the LC-MS/MS test (see Box for example cases). In Case 1, a patient with cardiac amyloidosis and a concurrent monoclonal protein was presumed to have AL-type amyloid, but was subsequently found to have ATTRwt amyloid, based on proteomic analysis of upper gastrointestinal-tract biopsies. The incidence of both ATTRwt amyloidosis and monoclonal proteins increases with age; however, as ATTRwt and AL are distinct diseases, with distinct treatment modalities, establishing the correct diagnosis is of critical importance. In Case 2, a patient with diabetes and nephrotic syndrome, a monoclonal protein, and a CR-positive fat aspirate was presumed to have systemic amyloidosis of AL type but was subsequently found to have AIns (insulin)-type amyloidosis, based on proteomic analysis of the fat aspirate. In both cases, the patient avoided receiving inappropriate therapy for AL amyloidosis, thanks to the proteomics test.

Second, LC-MS/MS has been instrumental in the identification and

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characterization of new amyloid types. For example, several novel amyloid types, such as apolipoprotein (AApo) CII,[32,33] AApoCIII,[24] and AEnf,[25] were initially identified by LC-MS/MS. Much of our understanding of the clinicopathologic and demographic features of ALECT2 amyloidosis, which was established as a canonical amyloid type in 2010[26] and is now recognized as the third or fourth most common type of amyloid, is based on identification of cases by shotgun proteomics;[22,27,28] LC-MS/MS has also played a key role in our understanding of other new amyloid types, such as AApoAIV.[29,30]

Third, LC-MS/MS is capable of identifying amino acid sequence variants of amyloid proteins by using custom protein sequence database or a sequence tagging search strategy.[5,31] For example, mass spectrometry was instrumental in both identifying AApoCII as an amyloid type and in identifying 2 separate novel mutant Apoc2 peptides corresponding to Lys41Thr and Gln69Val pathogenic mutations.[24,25] Using LC-MS/MS, multiple amyloidogenic amino acid substitutions from a variety of amyloid types, including ATTR, AApoA1, AApoCII, AApoCIII, fibrinogen (AFib), hereditary gelsolin (AGel), and lysozyme (ALys), have been observed, and it is likely that additional novel mutant amyloid proteins will be uncovered in the future.[22] Although proteomics can detect amino acid substitutions in amyloid deposits with high sensitivity (known 92%; novel 82%) and specificity (known 100%; novel 99%),[31] the proteomics method for mutation detection remains to be clinically validated. Furthermore, given the heritability of genetic mutation, current patient-care protocols include verification of the mutation by gene (Sanger) sequencing coupled with genetic counseling.

TOWARD BROAD CLINICAL IMPLEMENTATION

Although targeted mass spectrometry is routinely used in clinical laboratories for small molecules, the LC-MS/MS amyloid-typing assay is the first semiquantitative shotgun proteomics platform that has been translated from research to clinical implementation. The complexity of the assay is a significant challenge, and its success can be hampered by myriad factors, including insufficient material for analysis caused by sample microdissection, recovery, or processing and interpretation of complex proteomic profiles. The diverse international clinical studies in the Table clearly demonstrate the ability of selected laboratories to establish a robust shotgun proteomics amyloid-typing assay, but several hurdles need to be overcome for broader clinical implementation. First, a robust sample-

BOX. Example Cases in Which Amyloid Typing by Proteomics Altered the Clinical Diagnosis and Treatment

CASE 1.

A 67-year-old man with cardiac amyloidosis was referred for autologous stem cell transplantation for amyloid light-chain (AL) amyloidosis. He had been diagnosed with cardiac amyloidosis on the basis of typical echocardiography and cardiac magnetic resonance imaging findings and positive myocardial uptake on bone scintigraphy. He was also noted to have a small IgG κ monoclonal protein in the serum and an abnormal free light-chain ratio. Recent endoscopic biopsies were retrieved and shown to have amyloid deposits in blood vessels, but immunohistochemical staining could not determine the amyloid type. Liquid chromatography/ mass spectrometry (LC-MS/MS) on these vessels demonstrated the amyloid to be composed of wild-type transthyretin. Without the proteomics test, the patient could have been subjected to unnecessary and hazardous autologous stem cell transplantation and be denied access to new, effective amyloidosis transthyretin (ATTR) therapies. Immunohistochemistry to type amyloid deposits is not always definitive, and both false positives and false negatives can be seen.

CASE 2.

A 64-year-old insulin-dependent patient with diabetes and nephrotic syndrome presented with G λ monoclonal protein in the serum and an abnormal free light chain ratio, suggestive of AL amyloidosis. A fat aspiration showed amyloid deposits, and the patient was referred for therapy; LC-MS/MS on the fat showed insulin-derived amyloidosis (A-Ins), which was attributed to repeated insulin injections at the fat aspiration site. The patient was ultimately determined to have diabetic nephrosclerosis. Without the proteomics test, the patient easily could have been given chemotherapy for AL amyloidosis, inappropriately. It is unlikely that, using immunohistochemistry, the true nature of the amyloid protein would have been identified.

processing platform should be established, with reference materials and quality standards, together with a quality management system, to ensure reproducibility over the long term, notwithstanding hardware and consumables changes. Second, suitable training and qualification for clinical laboratory personnel will be required. Finally, the robust performance of the technology needs to be disseminated to regulatory agencies to facilitate regulatory approval and to clinicians to increase referral and use.

As a step toward standardization and quality control for clinical translation, Theis et al[34] identified all key steps in the method that could alter the final amyloid protein identification report generated for clinical interpretation and developed quality metrics for each step. Reference ranges were derived, using reference quality-control materials included in each batch of patient samples. To ensure consistent performance of the LC-MS/MS method, standard operating procedures and blind proficiency tests were established for laboratory technicians. To ensure consistent case interpretation, reference amyloid proteome profiles from gold standard cases of various amyloid types were generated for training the pathologists. Furthermore, a continuous quality improvement procedure—with retrospective analysis of quality control metric data and amyloid case clinical interpretation data—was recommended.

These recommendations provide a clear roadmap for establishment of a highly reproducible and repeatable LC-MS/MS method for amyloid typing in a clinical setting. However, it is important to note that the shotgun proteomics amyloid-typing assay should not be used as an independent diagnostic test but instead serves as an antibody-independent ancillary tool that can provide unbiased information to the diagnosing physician. The final tissue diagnosis should be rendered by a surgical pathologist or hematopathologist, preferably with expertise in amyloidosis. In addition to the proteomic amyloid-typing result, the diagnosis should always take into account all clinical and histologic features.

CONCLUSION AND FUTURE PERSPECTIVES

For patients with systemic amyloidosis, unequivocal identification of the amyloid type is mandatory for optimal treatment. Through close collaborations among clinicians, pathologists, and proteomics researchers, mass spectrometry-based proteomics has become the new gold standard for amyloid typing, used in conjunction with current clinical and antibody-based tests at multiple centers internationally. In light of its clear benefit to patients, and with the availability of new treatments for specific amyloid types, mass spectrometry-based proteomics for amyloid typing should be implemented broadly. However, as establishment of this platform by a clinical laboratory is challenging and requires meticulous attention to standardization and quality control, as well as advanced bioinformatics, it should be undertaken only by institutions with sufficient resources and expertise to invest in this endeavor. For institutions that do not have this technology available in house, specimens can be referred to international specialist centers to perform this test. Furthermore, to standardize processes around the world, international efforts for methodology, workflow, and reporting standardization, as well as training in the consensus workflows, should be put in place for established mass spectrometry-based proteomics amyloid-typing clinical laboratories.

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Correspondence: Address to Ellen D. McPhail, MD, Department of Laboratory Medicine and Pathology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905 (mcphail.ellen@mayo.edu).

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