Mass Spectrometric–Based Proteomic Analysis of Amyloid Neuropathy Type in Nerve Tissue

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Objective: To determine the specific type of amyloid from nerve biopsies using laser microdissection (LMD) and mass spectrometric (MS)–based proteomic analysis.

Design, Setting, and Patients: Twenty-one nerve biopsies specimens (17 sural, 3 sciatic, and 1 root amyloidoma) infiltrated by amyloid were studied. Immunohistochemical subtyping was unable to determine the specific amyloid type for these 21 cases, but the clinical diagnosis was made based on additional testing. Clinical diagnosis was made through evaluation of serum monoclonal proteins, biopsy of bone marrow for acquired diagnosis was made based on additional testing. Clinical diagnosis identified were diagnosed based on genetic confirmation in their first-degree relative. Congophilic proteins in the tissues of these 21 cases underwent LMD, were discussed into tryptic peptides, and were analyzed using liquid chromatography electrospray tandem MS. Identified proteins were reviewed using bioinformatics tools with interpreters blinded to clinical information.

Main Outcome Measure: Specific amyloid type was ascertained by LMD tandem MS and compared with clinical diagnosis.

Results: Specific types of amyloid were accurately detected by LMD/MS in all cases (8 cases of acquired monoclonal immunoglobulin light chain amyloidosis, 2 cases of gelsolin amyloidosis, and 11 cases of transthyretin amyloidosis). Incidental serum monoclonal proteins did not interfere with detection of transthyretin amyloidosis in 2 patients. Additionally, specific TTR mutations were identified in 10 cases by LMD/MS. Serum amyloid P-component and apolipoprotein E proteins were commonly found among all cases.

Conclusions: Proteomic analysis of nerve tissue using LMD/MS distinguishes specific types of amyloid independent of clinical information. This new proteomic approach will enhance both diagnostic and research efforts in amyloidosis and other neurologic diseases.


THE CLINICAL INVOLVEMENT, prognosis, and treatment of amyloid neuropathies are directly related to the specific causative amyloid protein.1,2 Congo red staining of amyloid demonstrates characteristic birefringence under polarized light,3 but subtyping amyloid can be challenging. Infiltration of amyloid typically occurs in distal peripheral nerves including autonomic small fibers,4 occasionally in isolated proximal plexus and spinal roots, which is referred to as an amyloidoma.5 Peripheral nerve tissue involvement is frequently the initial presenting feature of systemic amyloidosis.6,7 The known amyloid proteins associated with neuropathy include immunoglobulin κ or λ light chains, transthyretin (TTR), gelsolin, and, most rarely, apolipoprotein A1. The mechanisms that cause these proteins to transform from their precursors to amyloid fibril aggregation are not understood. Several proteins including serum amyloid P-component and apolipoprotein E8,9-10 have been associated with amyloid pathogenesis, and their identification is helpful in the diagnosis of amyloidosis. Acquired monoclonal immunoglobulin light chain (AL)–type amyloidosis caused by immunoglobulin κ or λ light chain deposition is the most common type of amyloidosis-associated neuropathy. The other types of amyloidosis affecting nerves are caused by mutations in the TTR gene (OMIM 105120) (ATTR) and the GSN gene (OMIM 105120) (AGEL) with autosomal
dominant inheritance, with ATTR being more common. Clinically, ATTR is not easily distinguished from AL amyloidosis, while AGEL has facial diplegia and corneal lattice dystrophy, which may be early distinguishing features. Inherited amyloidosis is frequently overlooked for complex reasons, including the following: (1) coexisting incidental monoclonal proteins; (2) lack of family history owing to varied age at onset and penetrance; and (3) small families or disrupted family dynamics. A large retrospective study demonstrated that up to 9% of patients with familial amyloid polyneuropathy were misclassified and treated for AL-primary amyloidosis.

The specific approach for treating amyloidosis depends on the type of amyloid. High-risk aggressive chemotherapy and stem cell transplantation may be used in AL-type amyloidosis, whereas liver transplantation and emerging protein stabilizing therapies have shown promising results in ATTR. For patients with AGEL, life is not threatened but treatment is limited, with no currently available therapeutics to prevent ongoing amyloid deposition. Various supportive therapies for certain symptoms have also been used, such as lubrication of the eyes and reconstructive surgery for facial and corneal abnormalities. Therapeutic small molecules are now being investigated based on the specific underlying cause. Incorrect diagnosis of the amyloid type can lead to substantial incurred risk from inappropriate treatment.

Subtyping the specific amyloid proteins by immunohistochemical staining has been challenging as the antigenic epitope may be lost by formalin cross-linking and circulating serum proteins (TTR and monoclonal proteins) may contaminate tissue preparations. Additionally, comparing the staining intensities of different amyloid antibodies is often required and can be problematic. Recently, liquid chromatography tandem mass spectrometry (LC-MS/MS) with laser microdissection (LMD) of amyloid plaques from formalin-fixed paraffin-embedded (FFPE) tissues has shown great promise in subtyping systemic and localized amyloid. In this study, we explore the utility of LMD and LC-MS/MS for the evaluation of peripheral nerve tissues infiltrated by amyloid.

### METHODS

The Mayo Clinic peripheral nerve laboratory records were searched for cases of amyloidosis with subtypes that could not be determined through immunohistochemical staining. All cases must be diagnosed with a specific amyloid type using various tests. The criteria for clinical diagnosis included the presence of serum monoclonal proteins consistent with AL-type amyloidosis and mutation detection in patients or in their first-degree relatives through DNA sequencing of TTR or GSN. We carried out DNA sequencing for 5 of 8 patients with AL-type amyloid neuropathy to exclude the possibility of TTR amyloidosis (Table). The FFPE nerve sections were used in amyloid diagnosis. All cases were reviewed and interpreted by nerve pathologists (C.J.K., P.J.D., and P.J.B.D.) with positive detection referring to 100% predicted probability based on 4 or more spectra confirmed on analysis.

### PATIENTS

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### Table. Clinical Diagnosis Compared With Mass Spectrometry Results

<table>
<thead>
<tr>
<th>Case No./Sex/Age at Biopsy, y</th>
<th>Serum Protein</th>
<th>Tissue</th>
<th>Clinical Diagnosis</th>
<th>DNA Sequencing</th>
<th>MS Result (Identified Mutation)</th>
<th>Amyloid-Associated Proteins Identified&lt;sup&gt;b&lt;/sup&gt;</th>
<th>TTR</th>
<th>SAP</th>
<th>APOE</th>
<th>Gelsolin</th>
<th>Ig λ</th>
<th>Ig κ</th>
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<tr>
<td>1/16/65</td>
<td>IgG λ</td>
<td>Sural</td>
<td>AL-Ig λ amyloidosis</td>
<td>Positive for TTR</td>
<td>AL-Ig λ</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>2/16/65</td>
<td>IgM λ</td>
<td>Sural</td>
<td>NT</td>
<td>AL-Ig λ</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>3/16/65</td>
<td>IgG λ</td>
<td>Sural</td>
<td>AL-Ig κ amyloidosis</td>
<td>Negative for TTR</td>
<td>AL-Ig κ</td>
<td>+</td>
<td>+</td>
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<tr>
<td>4/16/65</td>
<td>IgG κ</td>
<td>Sural</td>
<td>AL-Ig κ amyloidosis</td>
<td>Negative for TTR</td>
<td>AL-Ig κ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
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<td>IgG κ</td>
<td>Sciatic</td>
<td>Negative for TTR</td>
<td>AL-Ig κ</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>6/16/65</td>
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<td>Sural</td>
<td>Negative for TTR</td>
<td>AL-Ig κ</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>IgG κ</td>
<td>Root</td>
<td>Negative for TTR</td>
<td>AL-Ig κ</td>
<td>+</td>
<td>+</td>
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<tr>
<td>8/16/65</td>
<td>IgG κ</td>
<td>Sciatic</td>
<td>NT</td>
<td>AL-Ig κ</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>9/16/65</td>
<td>Negative</td>
<td>Sural</td>
<td>GEL amyloidosis</td>
<td>Asp187Asn</td>
<td>AGEL</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>10/16/65</td>
<td>Negative</td>
<td>Sural</td>
<td>TTR amyloidosis</td>
<td>Positive FH</td>
<td>AGEL</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>11/16/65</td>
<td>Negative</td>
<td>Sural</td>
<td>TTR amyloidosis</td>
<td>Positive FH</td>
<td>ATTR</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>12/16/65</td>
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<td>Sural</td>
<td>Val32Ala</td>
<td>ATTR (Val32Ala)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>13/16/65</td>
<td>Negative</td>
<td>Sural</td>
<td>Thr60Ala</td>
<td>ATTR (Thr60Ala)</td>
<td>+</td>
<td>+</td>
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<td>14/16/65</td>
<td>Negative</td>
<td>Sural</td>
<td>Ala109Ser</td>
<td>ATTR (Ala109Ser)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>15/16/65</td>
<td>Negative</td>
<td>Sural</td>
<td>Val32Ala</td>
<td>ATTR (Val32Ala)</td>
<td>+</td>
<td>+</td>
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<tr>
<td>16/16/65</td>
<td>Negative</td>
<td>Sural</td>
<td>Val30Met</td>
<td>ATTR (Val30Met)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>17/16/65</td>
<td>Negative</td>
<td>Sural</td>
<td>Phe64Leu</td>
<td>ATTR (Phe64Leu)</td>
<td>+</td>
<td>+</td>
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<tr>
<td>18/16/65</td>
<td>Negative</td>
<td>Sural</td>
<td>Ala97Ser</td>
<td>ATTR (Ala97Ser)</td>
<td>+</td>
<td>+</td>
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<tr>
<td>19/16/65</td>
<td>Negative</td>
<td>Sciatic</td>
<td>Thr60Ala</td>
<td>ATTR (Thr60Ala)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>20/16/65</td>
<td>IgG κ</td>
<td>Sural</td>
<td>Val30Met</td>
<td>ATTR (Val30Met)</td>
<td>+</td>
<td>+</td>
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<tr>
<td>21/16/65</td>
<td>IgG κ</td>
<td>Sural</td>
<td>Val30Met</td>
<td>ATTR (Val30Met)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

Abbreviations: AGEL, GSN-associated amyloidosis; AL, acquired monoclonal immunoglobulin light chain; APOE, apolipoprotein E; ATTR, TTR-associated amyloidosis; GEL, gelsolin; FH, family history; MS, mass spectrometry; NT, not tested; SAP, serum amyloid P-component; TTR, transthyretin; +, positive detection.

<sup>a</sup> Positive FH indicates that the patient had a first-degree relative with an unspecified TTR or GSN mutation.

<sup>b</sup> Positive detection refers to 100% predicted probability based on 4 or more spectra confirmed on analysis.
LMD amyloid areas reviewed by a hematopathologist (A.D.). Immunohistochemical preparations of all nerve tissues had been read in conjunction with clinical information including knowledge of monoclonal serum proteins and with all accessible record information. In contrast, the analyses of the LMD and LC-MS/MS results for all cases were interpreted by 3 of us (J.A.V., J.D.T., and A.D.), who were blinded to the clinical information. Clinical data, demographic characteristics, and follow-up information were obtained from retrospective record review and from prior consultation correspondence. This study was approved by the institutional review board at the Mayo Clinic.

SPECIMEN PREPARATION AND LMD

The LMD and LC-MS/MS methods have been previously summarized in full text and supplemental forms.4,20 All cases had Congo red birefringence identified prior to LMD from FFPE nerve tissue. The duration of storage for FFPE nerve tissues ranged from 2 weeks to 12 years. Ten-micrometer-thick sections were placed on DIRECTOR slides (Expression Pathology Inc, Gaithersburg, Maryland) for LMD. Two to four separate areas of amyloid deposition were microdissected and analyzed. Equal tissue volumes were collected (60 000 µm³) by LMD into 0.3-mL microcentrifuge tube caps containing 10mM Tris/1mM EDTA/0.002% Zwittergent 3-16 (Calbiochem, San Diego, California) using a DM6000B Microdissection System (Leica Microsystems, Wetzlar, Germany). Collected tissues were heated at 98°C for 90 minutes with occasional vortexing. Following 60 minutes of sonication in a water bath, samples were digested overnight at 37°C with trypsin (Promega Corp, Madison, Wisconsin).

PROTEIN IDENTIFICATION VIA MS

Trypsin digests of LMD amyloid were reduced with dithiothreitol and separated by nanoflow LC electrospray MS/MS using a Thermo Finnigan LTQ Orbitrap Hybrid Mass Spectrometer (Thermo Electron Corp, Bremen, Germany) coupled to an Eksigent nanoLC 2-dimensional high-performance LC system (Eksigent, Dublin, California). The MS/MS raw data were converted to DTA files using BioWorks version 3.2 software (Thermo Electron Corp) and correlated with theoretical fragmentation patterns of tryptic peptide sequences of the Swiss-Prot database (https://portal .biobase-international.com/cgi-bin/portal/login.cgi). The peptides were accepted using the ProteinProphet algorithm when greater than 90.0% probability occurred and at least 2 identified queried proteins were found.21,22 Identification of previously identified TTR mutations was additionally performed and not previously reported. Molecular weights of known TTR mutants were appended to the database of searched molecules for specific mutation identification in addition to available proteins in the Swiss-Prot database (https://portal .biobase-international.com/cgi-bin/portal/login.cgi).

IMMUNOHISTOCHEMISTRY

Immunohistochemical stains had been performed by standard approach20 with Autostainer (Dako North America, Inc, Carpinteria, California) using an EnVision + Dual Link or ADVANCE (Dako North America, Inc) detection system. Antibodies were directed against the following antigens, with the corresponding clones for the monoclonal antibodies specified: κ light chain (polyclonal; dilution, 1:6000; Dako North America, Inc), κ light chain (Auto ProEnzyme pretreatment; polyclonal; dilution, 1:2500; Dako North America, Inc), λ light chain (polyclonal; dilution, 1:2000; Dako North America, Inc), λ light chain (Auto Pro-Enzyme pretreatment; polyclonal; dilution, 1:3000; Dako North America, Inc), prealbumin (TTR) (polyclonal; dilution, 1:3000; Dako North America, Inc), serum amyloid A (clone MC-1; dilution, 1:1000; Dako North America, Inc), and serum amyloid P-component (polyclonal; dilution, 1:20; Biocare Medical LLC).

Twenty-one cases with a definitive clinical diagnosis were identified for LMD and LC-MS/MS analysis. Their clinical diagnosis and results from MS/MS are summarized in the Table. At the time of biopsy, the median age was 60 years (range, 32-76 years); there were 6 women and 15 men. In all cases, the congophilic amyloid-infiltrated nerve tissue stained positively on immunostaining for serum amyloid P-component, but subtyping for distinction of TTR, κ light chain, and λ light chain was not possible as illustrated by nonspecific staining in case 1 (Figure, A-D). In contrast, proteomic analysis by LMD/MS was able to accurately identify specific amyloid protein in the representative case 1 (Figure, F) and the other 20 cases tested. Identified amyloid proteins in 21 cases were consistent with the results from clinical diagnosis, and the results were concordant between different LMD areas (Figure, E). All AL-type amyloidosis cases showed identified specific serum monoclonal proteins through analysis by LC-MS/MS. One patient with AL-type amyloidosis (case 6) was diagnosed with a serum bclonal gammopathy of IgG κ and λ light chains, but only κ amyloid type was identified by LMD/MS. In 2 elderly patients with ATTR, incidental serum monoclonal proteins (case 20 with IgM κ light chain and case 21 with IgG λ light chain) did not interfere with TTR mutation (Val30Met) identification by LMD/MS. Two cases with AGEL (1 with the Asp187Asn mutation and 1 not sequenced) were identified. The patient with AGEL who had no sequencing finding had clinical features and a family history of gelsolin amyloidosis. She had facial diplegia and corneal lattice dystrophy for 10 years; these were characteristic of her father and 1 sister who both had positive GSN genetic testing results, but the specific mutation was not disclosed in records from another institution. The mutations identified by LMD/MS in 10 patients with ATTR are 100% concordant with the previous DNA sequencing results (3 with the Val30Met mutation, 2 with the Val32Ala mutation, 2 with the Thr60Ala mutation, 1 with the Ala109Ser mutation, 1 with the Ala109Ser mutation, and 1 with the Ala97Ser mutation). The patient with ATTR who had no sequencing finding had a report of a positive family history and unspecified TTR mutation confirmation in 1 first-degree relative based on the records from another institution. One patient (case 7), whom we previously described, had lumbar sacral root biopsy for an amyloidoma affecting the cauda equina; κ amyloid was identified in this case. Two cases (case 8 with κ AL-type amyloidosis and case 19 with ATTR) had neuropathy with focal sciatic enlargements or conduction blocks that led to proximal targeted fascicular nerve biopsy. On average, each case had
Identifying a specific causative amyloid protein from nerves and other tissues is often difficult but important in diagnosis and treatment. The results from this study extend LMD/MS application to nerves beyond application in other tissues described previously. In this article, we identified the specific amyloid subtype in 21 different nerve biopsies by LMD/MS without assistance from clinical information. The nerve immunohistochemical staining in these cases failed to determine the specific amyloid type despite assistance from available clinical information. With further validation and ongoing improvements, this approach has potential to reach the earlier-stated goal for MS/MS, namely biomarker discovery without clinical bias. Additionally, this study demonstrates that LMD/MS can determine not only amyloid type but also specific TTR mutations from infiltrated nerve tissue. Because TTR normally circulates in the serum, identifying the specific mutation from amyloid-infiltrated tissue provides greater specificity of the pathologic cause. However, the current limitations for mutation identification with LMD/MS are highlighted by our gelsolin cases and 1 of 11 TTR cases where a mutant amino acid sequence could not be determined.

The practical issues for LMD/MS mutation identification include the following: (1) the mutations must be known or available in protein databases; (2) amino acid changes must lead to alterations enough for detection; (3) if the mutations occur at the sites of tryptic digestion, the mutant protein may be too large to be detected; and (4) there must be adequate quantities of mutant protein for MS analysis. In the 3 cases where mutation identification had failed, the aforementioned issues are all potential limiting factors. As the number of reported mutations in protein databases increases and the protein concentration requirement decreases and the protein concentration requirement decreases, the number of reported mutations in protein databases increases and the practical issues for LMD/MS mutation identification decrease.
creases, some limitations will diminish but the others will remain until the technology advances further.

In 2 of our genetically confirmed ATTR cases (cases 20 and 21), coexisting incidental serum monoclonal proteins did not interfere with LMD/MS tissue diagnosis of ATTR. The occurrence of incidental monoclonal proteins in cases with familial amyloidosis was the main factor that led to misdiagnosis in one large study. Additionally, the specific IgG α amyloid subtype was identified in 1 patient with AL-type amyloidosis (case 6) diagnosed with bicalonal gammopathy. Our results suggest that LMD/MS analysis is unlikely to make a misdiagnosis from serum monoclonal protein contamination.

Using a probability-based algorithm for mapping amino acid sequences of detected peptides, it is possible to identify the amyloid subtype with high specificity and sensitivity through LMD/MS. Increasingly, application of LMD/MS facilitates or aberrant proteins to the deposition of fibril formation. Proteomic analysis with LMD/MS can help define not only the specific amyloid protein but also the proteins associated with pathogenic amyloid protein. Our data confirm that serum amyloid P-component and apolipoprotein E are associated with various amyloid proteins. In each of our cases, approximately 200 other proteins were identified; most of them are not known to be related to amyloidosis. The finding may provide further clues for disease pathogenesis. Broader application of LMD/MS analysis in other neurologic disorders is predicted for the diagnosis and understanding of disease pathogenesis.

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REFERENCES