Identification of Transthyretin Variants by Sequential Proteomic and Genomic Analysis


Background: Transthyretin-associated hereditary amyloidosis (ATTR) is an inherited disease in which variants in the primary structure of transthyretin (TTR, prealbumin) lead to the extracellular polymerization of insoluble protein fibrils, causing organ failure and ultimately death when major organs are involved. We have developed an integrated approach to molecular diagnosis with initial analysis of intact plasma TTR by electrospray ionization mass spectrometry (MS) and referral of positive samples for DNA sequence analysis and real-time PCR to confirm the common Gly6Ser polymorphism.

Methods: Samples from 6 patients previously diagnosed with ATTR and from 25 controls with or without polyneuropathy were analyzed in a blinded fashion for the presence of variant TTR. TTR protein was extracted with an immunoaffinity resin from 20 μL of archived plasma samples. The purified TTR was reduced with tris(2-carboxyethyl)phosphine and analyzed by MS. The appearance of two peaks (or a single peak shifted in mass indicative of a homozygous variant), including the wild-type mass of 13761 Da, was indicative of the presence of a variant, and the individual was referred for DNA sequence analysis.

Results: MS analysis of intact reduced TTR correctly identified each of six samples known to contain variant TTR. These results were corroborated by subsequent DNA sequence analysis. Additionally, all Gly6Ser polymorphisms were correctly called based on the +30 mass shift and an equal relative abundance of the +30 polymorphism relative to wild-type TTR. No false-positive results were seen.

Conclusions: This referral method eliminates the necessity of sequencing most samples and allows screening for the familial forms of amyloidosis in a broad patient population in a timely fashion. This method correctly identified all previously known variants and also identified a novel variant, Val94Ala.

Transthyretin-associated hereditary amyloidosis (ATTR) is an inherited disease in which mutations in the gene coding for transthyretin (TTR, prealbumin) produce an altered primary sequence in the protein (1). These variant proteins cause the eventual formation of insoluble protein fibrils (amyloid) in various tissues and organs. The deposited amyloid eventually leads to organ dysfunction and ultimately death when major organs such as the heart are involved. Currently, the only therapeutic intervention for this disorder is liver transplantation because the liver is the major site of TTR synthesis and secretion. There is some evidence that livers transplanted early in the disease give better outcomes (2–4). TTR circulates in blood as a homotetramer, and some thought has been given to developing drugs to stabilize the tetramer to prevent amyloid formation (5).

ATTR is a late-onset disease, developing in the third to seventh decade (1). The symptoms in patients with ATTR are often difficult or impossible to distinguish from other

1 W.M. Keck FT-ICR Mass Spectrometry Laboratory, Mayo Proteomics Research Center, Rochester, MN.
2 Department of Hematology, 3 Department of Laboratory Medicine and Pathology, 4 Peripheral Neuropathy Research Center, Department of Neurology, and 5 Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN.

*Address correspondence to Dr. Thibodeau at: Department of Molecular Genetics, Mayo Clinic College of Medicine, 200 First Street, SW, Rochester, MN 55905. E-mail stthibodeau@mayo.edu; and to Dr. Muddiman at: Medical Science Building 3-115, Mayo Clinic College of Medicine, 200 First Street, SW, Rochester, MN 55905. Fax 507-284-9261; e-mail muddiman.david@mayo.edu.

Received February 21, 2004; accepted May 17, 2004.
Previously published online at DOI: 10.1373/clinchem.2004.033266

6 Nonstandard abbreviations: ATTR, transthyretin-associated hereditary amyloidosis; TTR, transthyretin; MS, mass spectrometry; PBS, phosphate-buffered saline; TCEP, tris(2-carboxyethyl)phosphine; Tm, melting temperature; and SCA, senile cardiac amyloidosis.

Copyright © 2004 by The American Association for Clinical Chemistry
varieties of polyneuropathy and from other types of amyloid diseases, such as those associated with immunoglobulin light chain and apolipoprotein A1. Misdiagnosis of amyloidosis is a growing concern (6).

There are several methods to detect the presence of variants in TTR at the gene or protein level. At the DNA level, both direct DNA sequence analysis and indirect screening methods such as single-strand conformation polymorphism analysis have been used. Unfortunately, base substitutions do not always lead to a DNA conformational change that will be detected by single-strand conformation polymorphism analysis. Several reports have indicated that obtaining the altered electrophoretic mobility requires multiple attempts, and in one instance, 10% of the analyses were false negatives (7, 8). As a result, DNA sequence analysis continues to be the gold standard for mutation analysis at the DNA level.

Among the methods for protein analysis, classic isoelectric focusing or the D1-D method of Altman, in which a first dimension is nondenaturing electrophoresis followed by hybrid isoelectric focusing in the presence of urea in the second dimension, has been used (9, 10). Although the latter method has been reported to distinguish between TTR monomers with the same pI, it has not found widespread use. Additionally, classic isoelectric focusing is not capable of distinguishing the most common variant, Val30Met, because there is no change in the pI of the protein.

Mass spectrometry (MS) has gained widespread use in the analysis of ATTR (7, 11–19). Both matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry, have been used to accurately measure TTR and its associated variants (14). The mean mass of the wild-type TTR is 13 761.4 Da. TTR variants are detected and assigned based on their mass shift. TTR has a single cysteine residue that readily forms adducts with other molecules, including cysteine, sulfite, cysteinylglycine, and glutathione. These adducts often comprise the majority of species found in the circulation, with free TTR constituting only a small fraction of the total. Because these adducts unnecessarily complicate the resulting mass spectra, it has been found beneficial to reduce these adducts before analysis, converting all circulating forms into free TTR (20, 21). It should be noted that high-performance MS provides the most accurate characterization of TTR and has the potential for multiple analyte detection, but it is currently not broadly available in a clinical chemistry environment (22).

Online analysis of TTR by MS does not allow for the reduction step and the subsequent desired simplification of the mass spectrum by elimination of multiple adducts (19). The assay described here is performed in a 96-well format, which allows for the rapid preparation of purified/reduced TTR for subsequent mass spectral analysis. We propose an interactive strategy for TTR mutation analysis that includes MS followed by DNA sequence analysis for confirmation of positive results (Fig. 1). The protocol outlined here allows for the MS detection of TTR variants and confirmation by DNA analysis of only those samples containing a variant. Additionally, the common (12%) Gly6Ser polymorphism is observed by its high relative abundance and +30 Da mass shift.

**Materials and Methods**

**HPLC-MS SYSTEMS**

A PE Sciex API 150LC/MS with TurboIonSpray ion source was used for mass detection. The Sciex was equipped with a Perkin-Elmer Series 200 autosampler and two PE Series 200 micropumps (Perkin-Elmer). Solvent A was 10 g/L acetic acid–methanol-acetonitrile (98:1:1 by volume), and solvent B was 5 g/L acetic acid/0.2 g/L trifluoroacetic acid–methanol–acetonitrile (5:48:48 by volume). A Shimadzu System controller (SCL-10Avp) was used to control a Shimadzu LC-10Advp pump (Shimadzu Scientific) that pumped an isocratic mixture water–methanol–trifluoroacetic acid (90:10:0.1 by volume) at 50 μL/min. This pump was connected to the autosampler and loaded the injected sample into a Valco six-port two-position valve (VICI Inc.) into which was plumbed a Phenomenex Security Guard column with a Widepore C₄ (Butyl), 4 mm × 2 mm (i.d.) cartridge (Phenomenex). The cartridge was washed for 3 min with the eluant going to waste before the valve switched to the second position.

The cartridge containing the desalted TTR was then in line with the PE pumps and the mass spectrometer.

An organic gradient (0–3 min, 0% B; 3–6 min, 0–75% B; 6–12 min, 75% B; 12–14 min, 75–95% B; 14–16 min, 95–0% B) eluted the bound TTR into the mass spectrometer for analysis. The mass spectrometer source potential was set to 5500 V with a desolvation temperature of 120 °C. The orifice potential was 51 V. All spectra were collected as a 1.7-s scan from 800-2500 Da. The scans containing the TTR spectra were summed, and the BioSpec Reconstruct algorithm was used to transform the summed multiply charged spectra to a relative mass scale. An output mass of 13 000–14 500 Da was used with a 1-Da bin width.

**TTR IMMUNOAFFINITY PURIFICATION**

Approximately 8.2 mg of rabbit anti-human TTR (DakoCytomation), dialyzed against phosphate-buffered saline (PBS), was coupled to 410 mg of Poros AL resin (Applied Biosystems) according to the manufacturer’s bulk coupling instructions. We diluted the resulting immunoaffinity resin to 10 mL with PBS containing 2 g/L Na₃N and added 100 μL of PBS and 40 μL of the prepared anti-TTR resin suspension to each well of a polystyrene 96-well plate. We then added 20 μL of the plasma to be analyzed to each prepared well. The plate was covered with laboratory film and agitated for 1 h with sufficient motion to maintain the resin in suspension.

After incubation, the contents of each well were transferred to a 10 μm fritted reservoir attached to a base plate [Isolute Array Base Plate (part no. 120-1000-P1) and Isolute Array 10 μm Fritted Reservoirs (part no. 120-1040-
The resin was separated from the plasma by centrifugation (2000 rpm for 2 min; acceleration factor 5) on a Beckman/Coulter Allegra 21 Centrifuge with S2096 Microplate rotor (Beckman Coulter, Inc.). The filtrate was discarded, and the resin was washed twice with 200 μL of PBS. The filtrate was discarded each time. We prepared a clean collection plate by adding 10 μL of 100 mM tris(2-carboxyethyl)phosphine (TCEP) to each sample collection well. The fritted reservoirs were then placed on top of this collection plate, and 55 μL of 100 mM glycine (pH 2.5) was added to the resin. The resin was agitated slightly, and after 5–10 min, the glycine was collected by centrifugation (2000 rpm for 2 min; acceleration factor 5; higher acceleration caused the Poros resin to pass through the frit). The collection plate was agitated slightly to assure mixing of the TCEP with the sample. After 20 min at 55 °C, the sample plate was inserted directly into the autosampler, and 45-μL injections were made.

The tissue samples analyzed in the blinded study came from a bank of tissues obtained in the Peripheral Neuropathy Research Center (N536797) and included patients with Finnish type amyloidosis and with amyloidosis of various types as well as neuropathy patients and healthy controls. All samples were deidentified for this study, which was approved by an Institutional Review Board (IRB Protocol 2263-01).

**DNA Extraction**

DNA was extracted from control and transformed patient lymphoblast cell lines by a standard organic phenol–chloroform method. All DNA samples were quantified with a DU® 650 spectrophotometer (Beckman Coulter), and concentrations were adjusted to 0.25 μg/μL with 1× Tris-EDTA, pH 8.0.

**PCR Amplification and Sequencing**

The following PCR conditions were used for amplification of all four exons of the human TTR gene. PCR reactions consisted of 250 ng of genomic DNA, 0.50 μM each forward and reverse primer, 2.0 mM MgCl₂, 2.0 μL of 10× PCR Buffer (Applied Biosystems), 0.20 mM each deoxynucleotide triphosphate (Applied Biosystems), and 1 U of AmpliTaq GOLD polymerase (Applied Biosystems) in a total volume of 20 μL. The following primer pairs were used:

Exon 1 (245 bp): TTR-1E (5’-TCAGATGGCAGG-GATAAG-3’) and TTR-1D (5’-CAAAGCTGGAAG-GAGTCAC-3’)

---

**Fig. 1.** Flow diagram for the clinical analysis of TTR variants.

The majority of samples will show no mass difference and can be presumed to be wild type (left branch). If a clinician still suspects amyloid, the archived buffy coat can be analyzed for the presence of mutations by sequencing of the TTR exons. The presence of a variant (middle branch) would indicate the need for DNA sequencing of the four TTR exons to determine the exact mutation. A +30 variant with a high ratio relative to the wild type is probably the Gly6Ser polymorphism and would be analyzed by real-time PCR (RT-PCR; right branch). A single peak at +30 would be indicative of homozygous Gly6Ser. The percentages of each branch are based on the mutation incidence in the general population and do not take into account any referral bias.
Exon 2 (296 bp): TTR-2E (5’-CTTGTTCGGCTCCA-GATTTTC-3’) and TTR-2D (5’-TGAGCTCTCTTC-TACCAAGTG-3’)

Exon 3 (281 bp): TTR-3C (5’-TGTTCCTCCTCAGTG-CG- TAACCT-3’) and TTR-3D (5’-TACAGCTTCTCTTG- GTAAGCT-3’)

Exon 4 (309 bp): TTR-4C (5’-GGACCTCCGGTCTG- CAGT-3’) and TTR-4D (5’-TGCGTGAACCTTCAA- CATAGC-3’).

The thermocycling conditions consisted of initial denaturation at 95 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 sec, with a final hold of 72 °C for 10 min. All reactions were run on the Thermo Hybaid MultiBlock System (Thermo Electron).

The forward and reverse PCR products for each exon were used in subsequent sequencing reactions. Before sequencing, all primers and deoxynucleotide triphosphates that remained from the PCR were removed by treatment with shrimp alkaline phosphatase (USB) and exonuclease I (USB). Sequencing reactions were done according to manufacturer’s instructions for the BigDye® Terminator (Ver. 1.1) Cycle Sequencing reagent set (Applied Biosystems) at one-half the recommended volume. The sequencing conditions consisted of initial denaturation at 95 °C for 2 min, followed by 23 cycles of 95 °C for 15 s and 60 °C for 4 min. Unincorporated fluorescent dye terminators were removed by gel filtration with Sephadex G-50 (Amersham Biosciences). We then diluted 1 μL of purified sequencing reaction with 9 μL of Hi-Di formamide (Applied Biosystems). The diluted sequencing products were denatured at 95 °C for 2 min and then cooled on ice/water slurry for 5 min before being loaded on an ABI 3100 for capillary electrophoresis system.

CAPILLARY ELECTROPHORESIS

Samples were analyzed on the ABI 3100 according to the standard ABI rapid sequencing protocol with an uncoated 36-cm capillary array of 16 and Performance Optimized Polymer 6 (POP-6™, Applied Biosystems) separation medium. Briefly, each sample was subjected to a 20-s injection at 1.5 kV and electrophoresed at 50 °C for 40 min at 15 kV. Data were collected with the ABI Prism 3100® Data Collection software, Ver. 1.1 (Applied Biosystems), with virtual filter E. Data were analyzed with SeqScape, Ver. 2.0, software (Applied Biosystems) and Mutation Surveyor™ software (SoftGenetics).

REAL-TIME PCR

The Gly6Ser real-time PCR and genotyping were performed on the LightCycler instrument (Roche Applied Science). Dual fluorescent probes were used to generate the fluorescent resonance energy transfer signal that can be detected by the LightCycler instrument. These consisted of a donor “anchor” probe and an acceptor “mutation detection” probe. The anchor probe, G6S-ANC (5’-ACGTGTTCTTCTACACCCAGGC-3’), was labeled with the donor fluorophore fluorescein at the 3’ end. The mutation detection probe, G6S-DET (5’-CCAGTGATC-CAGTGTTCTCTCTGTG-3’), which was complementary to the G6S mutant genotype, was labeled with the acceptor fluorophore LC-Red640 at the 5’ end and phosphorylated at the 3’ end. The following primer pairs were used to generate a 211-bp PCR product: TTR-2E (5’-CTTGTTC-TGCTCCTCATTTCTCTGTG-3’) and TTR-2F (5’-GCTGCCAG-GTTGTTCTCAG-3’).

PCR reactions were carried out in glass capillaries in a total volume of 20 μL. Each reaction included ~50 ng of genomic DNA, 6 μL of sterile PCR-grade water (Sigma), 2 μL of 10× PCR buffer (Applied Biosystems), 0.20 mM each deoxynucleotide triphosphate (Applied Biosystems), 1.5 mM MgCl₂, 1 mg/mL bovine serum albumin (Promega), 100 μL/mL glycerol (Sigma), 0.50 μM each of the forward and reverse primers, 0.20 μM anchor probe, 0.20 μM mutation detection probe, and 3 U of Taq polymerase (Applied Biosystems). The LightCycler fluorometer gain was set at 15 for channel F2. The thermocycling program was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 0 s, 55 °C for 15 s, and 72 °C for 15 s, with a ramp rate for all steps of 20 °C/s. Genotyping was performed by melting curve analysis. The melting consisted of 95 °C for 30 s, followed by 45 °C for 90 s (both 20 °C/s ramp rate) and then 80 °C for 0 s with a 0.1 °C/s ramp rate and continuous acquisition of emitted fluorescence in channel F2 (640 nm). Melting curves were analyzed with the LightCycler, Ver. 3.5, software (Roche).

Results

TTR variant analysis requires less than 20 min/sample for preparation, chromatography, and mass spectral analysis (96-well format). Both plasma and serum are appropriate samples for the analysis (data not shown). Although the resin is reusable, its disposal after a single use would not be cost-prohibitive. As shown by Nepomuceno et al. (22), the purity of the immunoaffinity-captured TTR from plasma is very high. Although some high-molecular-weight proteins are observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, they generally contribute only background noise to the spectrum. The mass-transformed spectra (i.e., zero charge state) of wild-type TTR before and after treatment with the reducing agent TCEP are shown in Fig. 2, A and B, respectively. Although other reductants are available (e.g., 2-mercaptoethanol and dithiothreitol), we found that TCEP works well at pH 2.5, is odorless, undergoes air oxidation at a much slower rate (23), and is much more effective in reducing the TTR sulfite adducts.

Shown in Fig. 3 are mass-transformed single-quadrupole mass spectra of samples from seven patients carrying different variants (Fig. 3, A–F), a sample with the Gly6Ser polymorphism (Fig. 3G), and the wild type (Fig. 3H). The mass shifts indicative of the presence of variant TTR are readily apparent in these mass spectra. In addition, the
use of TCEP greatly simplified the interpretation of these spectra (Fig. 2).

Overall, there was excellent correlation between the mass shift based on DNA sequence data and the measured mass shift (Fig. 4). In all cases, the mass difference between the measured and theoretical mean mass was ±2 Da.

Interestingly, when we plotted the ratio of the variant abundance to the wild-type abundance, the four Gly6Ser polymorphisms analyzed had a ratio ≥0.85. However, all of the amyloid-associated TTR variants had a ratio ≤0.85 (data not shown).

Because of the ~12% prevalence of the TTR Gly6Ser polymorphism, the most efficient method to verify the mass spectral identity is real-time PCR. A real-time PCR assay specific for the Gly6Ser polymorphism is more rapid and cost-effective than DNA sequencing. Representative results for real-time analysis of the Gly6Ser polymorphism on the LightCycler are shown in Fig. 5. For this analysis, at the conclusion of thermocycling the temperature was slowly increased with a resulting loss of fluorescence resonance energy transfer signal as a result of the dissociation of the hybridization probes from the target PCR DNA strand. The negative derivative of fluorescence over time as a function of melting temperature (Tm) was then compared as a function of melting temperature (Tm). For this assay, the Tm of wild-type samples was 58.6 °C, and the Tm of samples containing the Gly6Ser polymorphism was 62.6 °C. The curves were easily distinguished and could be used to confirm the MS analysis. Samples containing the wild type and the Gly6Ser polymorphism as well as samples that were homozygous for the polymorphism could be distinguished (Fig. 5).

The single-quadrupole mass spectra and DNA sequence analysis of the corresponding TTR gene for a patient who presented with amyloid symptoms are shown in Fig. 6. The mass shift of −28 Da was in agreement with the DNA sequence call of a Val-to-Ala substitution in codon 94. This substitution would give a theoretical mass shift of −28 Da that is congruent with data from MS. This TTR variant has not been reported previously.

Intraassay comparison of the Val94Ala mutation analyzed 10 times on the same day showed a mean (SD) measured mass difference of −28.1 (0.3) Da. An interassay comparison again on day 5 showed a mean mass difference of 28.7 (0.2) Da. We also looked at the variation in mass measurement of 49 wild-type TTR samples analyzed on the same day. A mean mass of 13 761.5 Da with a 95% confidence interval of 0.2 Da (theoretical mass = 13 761.4 Da) was obtained for these 49 samples, indicating excellent precision.

The highest single-day throughput to date by this MS analysis has been 140 plasma samples. The total number of samples analyzed to date exceeds 700. There was at least one documented autosampler failure on a highly used autosampler.
As described, our methodology does not identify variants with mass differences ≤10 Da because of limitations in the resolving power of the instrumentation. These variants could result from a single amino acid change, a double variant, or a variant that does not circulate (24). A double variant was not detected by single-quadrupole MS. This individual contained a cis Gly6Ser (+30 Da shift in protein mass) and a Val30Ala (−28 Da shift in protein mass) for an overall +2 Da shift, whose isotopic distribution overlapped with wild-type TTR. Importantly, this variant was detected by liquid chromatography–electrospray ionization Fourier-transform ion-cyclotron-resonance MS as reported by Nepomuceno et al. (22).

More than 200 samples have had all four TTR exons sequenced successfully, and the real-time PCR assay has recently been validated with success.

Discussion

A recent report indicated there are ~100 known variants of the TTR protein (25). The pathogenic variants are associated with protein plaques (amyloid) that cause organ dysfunction and ultimately can lead to death. These plaques result when the circulating TTR tetramer forms fibrils containing β-pleated sheet structures (1). Because the liver is the major source of synthesis and secretion into the circulation, there are no currently available treatments except for liver transplantation. Recently, Klabunde et al. (5) have shown that nonsteroidal antiinflammatory drugs and structurally similar compounds are strong inhibitors of fibril formation from TTR in vitro. Thus, there is reason to believe that therapies other than transplantation will be available in the future.

MS has gained widespread use in the analysis of ATTR (7, 11–19). Our data clearly demonstrate how MS can distinguish between wild-type and variant forms of TTR. This is especially true of TTR that has been completely reduced (Fig. 2). The sulfhydryl adducts greatly complicate the mass spectrum and subsequent interpretation.

The mass spectrum of TTR is simplified and the signal-to-noise ratio increases as a result of the signal collapsing into one mass channel (two in the case of a heterozygote) after reduction (Fig. 2). An online analysis has been reported for TTR analysis, but this methodology does not allow for the reduction step and thus gives an unnecessarily complex mass spectrum (19).

Several investigators, after finding a variant by examining the intact protein by electrospray ionization MS, subsequently performed peptide analysis to identify the tryptic peptide fragment containing the variant (11, 12, 26–28). Once the fragment was identified, the corresponding exon was typically sequenced to confirm the mass spectral interpretation. Although tryptic peptide analysis does indicate the specific exon to be sequenced, the savings in time and expense in sequencing one exon instead of all four exons are limited in our opinion. The major time and cost savings come by avoiding sequencing those samples that do not contain a mutation.

It has previously been observed that the circulating concentrations of variant TTR are lower than those of wild-type TTR (13, 28). Our results also support this observation. However, the Gly6Ser polymorphism, which is present in the Caucasian population with a 12% prevalence, is usually present in close to equal abundance (Fig. 3G). When the ratio of variant to wild-type is examined for pathogenic variants, the majority of the variants we have analyzed have a variant to wild-type ratio that is ≤0.85. The Gly6Ser polymorphism is present in almost all cases at a ratio ≥0.85. In the current set of samples, four contained the Gly6Ser polymorphism. All four samples were correctly identified as having the Gly6Ser polymorphism before DNA sequence analysis. The correct calls as polymorphisms were deduced from the +30 mass shift and the relative ratio of variant to wild-type TTR. On the basis of its ability to distinguish this polymorphism and because of the high prevalence of the Gly6Ser polymorphism in the Caucasian population, we suggest that a cost-effective alternative would be single-nucleotide polymorphism analysis with probes specific for the Gly6Ser
polymorphism (e.g., LightCycler). We have been able to develop a real-time PCR assay, as shown in Fig. 5, using the LightCycler. Individuals who are wild-type, heterozygous, or homozygous for the Gly6Ser polymorphism can be easily differentiated because there is a 4℃ difference in the $T_m$ between the wild type and the Gly6Ser polymorphism.

One interesting observation in the current blinded study was the high percentage of Gly6Ser polymorphism in the group having some type of neuropathy (3 of 15; 20%). In the control group, only 1 of 10 (10%) contained the Gly6Ser polymorphism. Our population size is too small to claim any direct association between this polymorphism and any type of neuropathy. However, wild-type TTR has been associated with disease. The fact that even wild-type TTR forms amyloid over time suggests that the Gly6Ser polymorphism might be associated with some type of pathology that has yet to be identified and understood.

Senile cardiac amyloidosis (SCA) is a nonhereditary disorder found in 95% of octogenarians (29, 30) and is clinically symptomatic in ~25% of these individuals (31). The amyloid fibrils in SCA are composed of wild-type TTR and TTR fragments. It is not possible to differentiate between SCA and ATTR on clinical features alone; therefore, it is critical to have the ability to exclude the presence of a variant form of TTR to differentiate between SCA and ATTR. Given the relative incidence of SCA compared with ATTR, confirmation of wild-type TTR in elderly individuals with amyloidosis is likely to be a more common use of this methodology than is detection of variants.

Our interactive strategy likely will allow us to examine more patients with unknown causes of polyneuropathy to determine whether a true association exists between the Gly6Ser polymorphism and neuropathy. Additionally, patients with light chain amyloidosis can also be screened to ensure that there is no underlying TTR amyloidosis, and patients who present with SCA can easily be confirmed.

Mass spectral analysis is not able to ascertain mass differences in samples that are isobaric (e.g., Leu to Ile) or that have mass shifts that are <10 Da on a quadrupole mass spectrometer. The resolving power required to distinguish a ±3 Da or larger mass shift at $m/z$ 1721 (the [M + 8 H]+ ion) is available on modern time-of-flight mass spectrometers. Fortunately, the known TTR variants with mass shifts ≤10 Da are not common, and they constitute only 10% of the 100 known variants. If all potential single-amino-acid variants are examined, 48 of
380, or 12.6%, would have a variant that is $\leq 10$ Da. This gives a theoretical minimum sensitivity of $\sim 87\%$.

The plasma sample containing the Val94Ala variant was easily identified by this methodology by use of serial dilution (data not shown). The TTR concentration in this sample was 150 mg/L (reference interval, 19–38 g/L). A 1:5 dilution corresponded to a TTR concentration of 30 mg/L, which is twice as low as the lower limit of detection for the nephelometric assay (70 mg/L). Thus, we were able to detect wild-type and variant TTR from a plasma sample with a total TTR concentration of 30 mg/L; lower concentrations of plasma TTR could be detected by increasing the volume of plasma in the assay.

The protocol outlined here was useful in characterizing a new protein variant of ATTR (Fig. 6). The MS-based assay indicated a mass shift of $-28$ Da. DNA sequence analysis corroborated the mass spectral analysis by showing a Val94Ala ($-28$ Da) variant. This patient initially presented with symptoms of peripheral neuropathy and later developed symptomatic heart failure. A subsequent cardiac biopsy revealed amyloid deposits. A thorough search for a monoclonal protein was negative, and screening for additional types of amyloid led to the discovery of the Val94Ala TTR variant.

To reduce the overall cost of working-up a patient with a possible diagnosis of ATTR, we propose an interactive strategy for TTR mutation analysis, as shown in Fig. 1. In this approach, all samples submitted for TTR variant analysis are first subjected to MS analysis of the intact protein. On the basis of the population being tested, the majority of these samples would contain only wild-type TTR (Fig. 1, left branch). A much smaller percentage of the samples would contain a variant (Fig. 1, middle branch). These positives would be referred for DNA sequence analysis. On the basis of population frequencies, $\sim 12\%$ of samples would contain the Gly6Ser single-nucleotide polymorphism (Fig. 1, right branch). A smaller percentage would be homozygous for the Gly6Ser polymorphism. Current techniques, such as real-time PCR (e.g., the LightCycler, which was used in this study), can be used to confirm the presence of this variant quickly and inexpensively (Fig. 5). The rapid, low-cost, and easily interpreted MS analysis followed by DNA sequence confirmation affords a cost-effective strategy providing definitive determination of TTR variants.

The ability to rapidly and relatively inexpensively screen for TTR variants could allow us to screen a larger patient population in the early stages of symptom development. We have currently analyzed almost 800 samples by MS analysis and 280 by DNA sequence analysis during the course of our validation studies. The only sample we are aware that the quadrupole MS analysis did not identify was a cis double mutant in which no mass shift was apparent because the overall mass shift was $\leq 10$ Da [Gly6Ser ($+30$ Da) + Val30Ala ($-28$ Da); $\Delta$ mass = $+2$ Da shift in protein mass]. However, Nepomuceno et al. (22) identified this novel mutation by use of internally calibrated liquid chromatography–dual-electrospray ionization Fourier-transform ion-cyclotron-resonance MS and a fitting procedure to determine the monoisotopic mass of the immunoaffinity-purified TTR. Although our antibody has been able to capture all of the variants we have tested to date, others have reported that some variants do not circulate. The Asp18Gly variant has been found to be undetectable in serum and cerebral spinal fluid (24). Presumably, the Asp18Gly variant is degraded before secretion from the liver. Additionally, the Tyr114Cys variant was not detectable by radial immunodiffusion, although the antibody was shown to have good reactivity toward the variant TTR (21). Rare instances such as these two examples, plus the fact that our analysis is limited to mass shifts $\geq 10$ Da, require careful interpretation and reporting to include appropriate caveats. The most common variants do have mass shifts $\geq 10$ Da and will be detected. Our protocols as outlined in Fig. 1 also allow referral of samples directly to DNA sequencing when amyloid is suggested and MS analysis does not detect the presence of a variant.

Actual referral patterns will undoubtedly change the referral percentages shown in Fig. 1. However, we would predict that the relative proportions would remain about the same. The majority of samples will have no variant, a large percentage will have the Gly6Ser polymorphism and be confirmed by real-time PCR, and a small fraction will require subsequent DNA sequencing of the TTR exons.

In conclusion, we propose that mass spectral analysis should be the first step in screening for the presence of TTR variants. All positive samples should be referred for DNA sequence analysis. All samples indicating a $+30$ Da mass shift and a mutant/wild-type ratio $>0.85$ could be analyzed by current single-nucleotide polymorphism analysis protocols (e.g., real-time PCR) before any sequence determinations. The methodology is robust, allows multiple samples to be purified simultaneously, and can be performed on single-quadrupole mass spectrometers, which are increasingly available in most clinical laboratories.

We are grateful to the Department of Laboratory Medicine and Pathology (Mayo Clinic) and the National Institute of Neurological Disorders and Stroke (Grant NS36797) for their generous financial support. We also thank Diane Klein for assisting in sample collection, Diana Ayerhart for assisting in manuscript preparation, and Dr. Frank Crow for sample analysis.

References
2. Ando Y, Tashima K, Tanaka Y, Nakazato M, Ericzon BG, Duraj FF,