Clinical diagnosis and typing of systemic amyloidosis in subcutaneous fat aspirates by mass spectrometry-based proteomics

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ABSTRACT

Examination of abdominal subcutaneous fat aspirates is a practical, sensitive and specific method for the diagnosis of systemic amyloidosis. Here we describe the development and implementation of a clinical assay using mass spectrometry-based proteomics to type amyloidosis in subcutaneous fat aspirates. First, we validated the assay comparing amyloid-positive (n=43) and -negative (n=26) subcutaneous fat aspirates. The assay classified amyloidosis with 88% sensitivity and 96% specificity. We then implemented the assay as a clinical test, and analyzed 366 amyloid-positive subcutaneous fat aspirates in a 4-year period as part of routine clinical care. The assay had a sensitivity of 90%, and diverse amyloid types, including immunoglobulin light chain (74%), transthyretin (13%), serum amyloid A (%1), gelsolin (1%), and lysozyme (1%), were identified. Using bioinformatics, we identified a universal amyloid proteome signature, which has high sensitivity and specificity for amyloidosis similar to that of Congo red staining. We curated proteome databases which included variant proteins associated with systemic amyloidosis, and identified clonotypic immunoglobulin variable gene usage in immunoglobulin light chain amyloidosis, and the variant peptides in hereditary transthyretin amyloidosis. In conclusion, mass spectrometry-based proteomic analysis of subcutaneous fat aspirates offers a powerful tool for the diagnosis and typing of systemic amyloidosis. The assay reveals the underlying pathogenesis by identifying variable gene usage in immunoglobulin light chains and the variant peptides in hereditary amyloidosis.

Introduction

Because systemic amyloidosis is a multiorgan disease with significant morbidity and mortality early diagnosis is critical for effective management of patients.[1-3] The disease presents insidiously with subtle signs and symptoms, and most frequent clinical findings can mimic cardiac, renal and neurological diseases. For this reason, quick, sensitive and specific methods to screen for the possibility of systemic amyloidosis are necessary. Microscopic examination of Congo red-stained abdominal subcutaneous fat aspirates is one of the widely used methods for the screening and diagnosis of systemic amyloidosis.[4-8] Subcutaneous fat aspiration (SFA) is simple, quick, can be performed in an outpatient setting, and has a high specificity and sensitivity for the diagnosis of systemic amyloidosis. However, although the diagnosis of amyloidosis can be established from the SFA specimen, identification of the specific amyloid type, which is essential for management of systemic amyloidosis, has not been possible until recently. We have recently developed a clinical assay using mass spectrometry (MS)-based proteomic analysis that can diagnose and classify amyloidosis in formalin-fixed paraffin-embedded (FFPE) tissues with high sensitivity and specificity.[9] The assay has been used extensively for typing amyloidosis at the Mayo Clinic, and nationally through the reference laboratory services of Mayo Medical Laboratories.[10-19] We and others have shown the possibility of applying proteomic approaches including MS-based proteomics for typing amyloidosis in SFA specimens in a research setting.[20-25] In this study, we show the clinical validation and implementation of an MS-based proteomic assay for the diagnosis and typing of amyloidosis in SFA specimens in a 1988 Clinical Laboratory Improvement Amendments (CLIA) accredited laboratory setting. The assay not only provides diagnostic information but in many cases also sheds light on the underlying pathogenesis by identifying the detailed proteome of the amyloid deposits.

Methods

Cases

The study included two distinct cohorts. The first cohort, referred to as the test validation cohort, comprised 95 patients prospectively investigated for systemic amyloidosis by SFA screening. Under a protocol approved by the Mayo Foundation Institutional Review Board (MFIRB) and after informed consent, an extra specimen was obtained for MS-based proteomic analysis from the patients in this cohort. The second cohort, referred to as the clinical cohort, comprised 366 consecutive patients diagnosed with systemic amyloidosis by Congo red reactivity in SFA specimens. In the clinical cohort, MS-based proteomic analysis was performed as part of the routine clinical work–up, and the data were retrospectively reviewed for this study as part of a protocol approved by the MFIRB.

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Subcutaneous fat aspiration for diagnosis of systemic amyloidosis

Specimens were obtained, stained with Congo red and interpreted according to previously established protocols described elsewhere.[5] For the validation cohort one half of each specimen was used to make a smear and stained with Congo red. The other half, irrespective of whether the smear was positive for amyloid by Congo red, was processed for MS-based proteomic analysis. For the clinical cohort, one half of each specimen was used to make a smear and stained with Congo red. The other half was processed for MS-based proteomic analysis only if the first half stained positive for amyloidosis, as required by the established clinical practice and test validation criteria.

Mass spectrometry-based proteomic analysis of subcutaneous fat aspirates

The SFA specimens were processed for MS analysis using a modification of a previously established protocol.[26] Briefly, the aspirate was solubilized and digested in trypsin and used for protein identification by nano-flow liquid chromatography electrospray tandem MS.[9] Peptide spectra present in the raw data files were matched against a composite protein sequence database using three different search engines (Sequest[27], X!Tandem[28] and Mascot[29]). The composite database contains protein sequences obtained from the SwissProt database selected for human subspecies, known human immunoglobulin variant domains obtained from ImMunoGeneTics database,[30] known amyloidogenic mutations collected from literature and common contaminants. Reversed protein sequences are appended to the database for estimating the false discovery rates of the identifications[31]. Resulting peptide identifications were combined, filtered, and assembled into protein sequences using Scaffold software (Proteome Software, Portland, OR, USA). Further details are provided in the *Online Supplementary Methods.*

Measurement of serum immunoglobulin free light chains

Serum immunoglobulin free light chain (FLC) was quantified using a serum FLC assay (Freelite; The Binding Site, San Diego, CA, USA) performed on a Dade-Behring Nephelometer (Deerfield, IL, USA) as described previously.[32]

Statistical analysis

The relative amounts of apolipoprotein E (APOE), apolipoprotein A1 (APOA1), apolipoprotein A4 (APOA4) and serum amyloid P-component (SAP) between the Congo red-negative/no amyloid (n=32) and Congo red-positive/amyloid (n=43) cases were compared in the validation cohort using the Mann-Whitney U test. Associations between the amount of patient's FLC and immunoglobulin kappa or lambda spectra identified by MS-based proteomic assay were examined using Spearman rank correlation. Further details are provided in the *Online Supplementary Methods*.

Results

Characteristics of the cases in the validation cohort

The validation cohort consisted of 43 females and 52 males with an average age of 62 years. From this cohort, 43 specimens were determined to be Congo red-positive and 52 were determined to be Congo red-negative (Table 1). All Congo red-positive cases had clinical features consistent with systemic amyloidosis and were, therefore, considered to be "true positive". In this set, amyloidosis was classified based on previously established gold standard criteria that included exhaustive clinical and pathological work-up. Forty (93%) cases were classified as due to immunoglobulin light chain (AL) and three (7%) as transthyretin-related (ATTR).

The Congo red-negative group fell into two distinct clinical categories. Thirty-two had no clinical evidence of systemic amyloidosis after extensive clinical work-up and were, therefore, "true negative" cases. Of these, 46% had underlying myeloma, 15% lymphoma and 38% were being investigated for peripheral neuropathy or other suspicion for amyloidosis. In the remaining 20 Congo red-negative cases, a clinical diagnosis of systemic amyloidosis was established at other anatomic sites. These cases were included in the analysis to investigate evidence for an amyloid proteome in the absence of Congo red-positive deposits.

Mass spectrometry-based proteomic analysis of subcutaneous fat aspirates types systemic amyloidosis with high sensitivity and specificity

MS-based proteomic analysis of SFA specimens from 75 patients (43 cases with systemic amyloidosis and Congo red-positive SFA, and 32 patients with no evidence of systemic amyloidosis) was performed and analyzed blindly without knowledge of the patients' clinical diagnosis or the results of Congo red staining of the SFA (Table 1). On average 670 proteins were identified per case. MS-based analysis was conclusive for an amyloid type in 38 cases. In 37 of these cases, the SFA specimens contained Congo red-positive amyloid, the patients had systemic amyloidosis and in each case MS-based proteomic analysis accurately predicted the amyloid type established by the clinical gold standard. One case was false positive with a proteome diagnostic of AL-kappa amyloidosis, but the SFA specimen was Congo red-negative and the patient did not have clinical evidence of systemic amyloidosis. This patient had very high levels of serum FLC (kappa, 285 mg/dL), suggesting that serum contamination may have been the cause of the false-positive result. In six cases of systemic amyloidosis with Congo red-positive aspirates, no proteome indicative of amyloidosis was detected and these cases were, therefore, false negatives by MS analysis.

In this validation cohort, the sensitivity of the MS-based proteomic assay was 88%, with a specificity of 96%, and an accuracy of 91%. Overall the positive predictive value in this cohort was determined to be 98% and the negative predictive value was determined to be 81%.

Elevated levels of serum free light chain do not interfere with mass spectrometry-based proteomic amyloid typing

Because we observed one false positive case that was attributed to high levels of serum FLC secondary to an underlying plasma cell myeloma, we next sought to deter-

Table 1. Validation cohort: amyloidosis typing by MS-based proteomic analysis.

Specimens (n=75)	AL-lambda	AL-Kappa	ATTR	NEG
CR+ n=43 "Systemic amyloidosis"	29(67%)	6(14%)	2(5%)	6 (14%)
CR- n=32 "No systemic amyloidosis"		1 (3%)		31(97%)

CR+: Congo red-positive; CR-: Congo red negative, AL: immunoglobulin light-chain amyloidosis; ATTR: transthyretin amyloidosis; NEG: negative for amyloidogenic peptides.

mine whether there was a relationship between the serum FLC values and the immunoglobulin light chain spectra counts identified by MS-based proteomic analysis (*Online Supplementary Figure S1*). No correlation was identified between the lambda FLC values and lambda spectra numbers or between the kappa FLC values and kappa spectra numbers (Spearman ρ=0.45; *P*=0.0061).

The presence of apolipoprotein E, serum amyloid P-component and apoliprotein A4 provides a universal proteomic signature for diagnosis of amyloidosis in subcutaneous fat aspirates

We next sought to identify a universal proteomic signature that could distinguish SFA specimens involved by systemic amyloidosis from uninvolved specimens irrespective of specific amyloid type by analyzing the differential presence of four proteins (APOE, SAP, APOA1, APOA4) in Congo red-positive and -negative fat aspirates. APOE, SAP and APOA4 were differently expressed in amyloid *versus* non-amyloid specimens (*P*<0.0001) (Figure 1A-C). In contrast, apolipoprotein AI (APOA1), which served as a control protein for this analysis, was not differentially expressed (*P*<0.98) (Figure 1D). The odds of finding at least one of the APOE, APOA4, or SAP proteins was significantly higher (odds ratio=36.3; *P*<0.0001) in Congo red-positive cases than in Congo red-negative cases. The detection of at least two peptide spectra representing at least two of the three proteins (SAP, APOE and APOA4) was, therefore, considered to be the universal proteomic signature for involvement of subcutaneous fat by amyloidosis, and could serve as an excellent surrogate for the diagnosis of systemic amyloidosis with a sensitivity similar to that of Congo red staining.

Table 2. Clinical cohort: amyloidosis typing by MS-based proteomic analysis.

Amyloidosis Type	N=366
AL- lambda	212 (58%)
AL-kappa	60 (16%)
ATTR	46 (12%)
AA	5 (1%)
AGel	3 (1%)
AIns	3 (1%)
ALys	1 (1%)
Inconclusive	36 (10%)

AL: immunoglobulin light-chain amyloidosis; ATTR:, transthyretin amyloidosis; AA: reactive secondary; AGel: gelsolin familial amyloidosis; AIns: insulin amyloidosis; ALys: lysozyme familial amyloidosis.

The proteomic amyloid signature may be a more sensitive tool for the diagnosis of amyloidosis in fat aspirate specimens than Congo red staining

Our cohort included 20 cases in which SFA specimens were Congo red-negative but in which the patients were diagnosed with systemic amyloidosis at another anatomic site (SFA Congor red-false negative). Having established the presence of SAP, APOE and/or APOA4 proteins as a universal proteomic signature for amyloidosis, we investigated whether we could identify the proteomic signature of amyloidosis in Congo red-negative SFA specimens. Thirteen out of 20 SFA Congo red-negative specimens (65%) from patients with systemic amyloidosis showed a proteome consistent with AL (8 AL-lambda, 5 AL-kappa). In all cases, the amyloid types identified by MS were identical to the amyloid type identified by the clinical gold

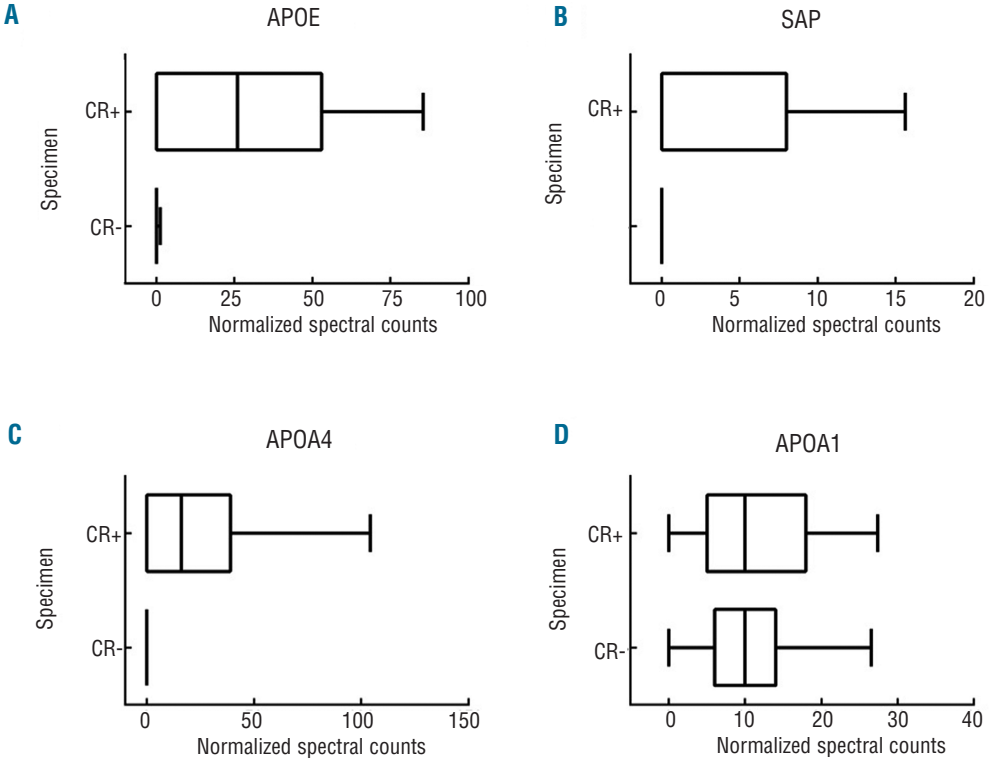

Figure 1. Identification of a proteome specific for amyloid deposition in SFA specimens. Distribution of the normalized protein spectral counts (the median and percentile distribution) for three amyloid markers (A) APOE, (B) SAP and (C) APOA4 and one control marker (D) APOA1 are shown for Congo red (CR) negative/no amyloid (n=31) and CR-positive/amyloid (n=43) cases of the validation cohort. APOE, SAP and APOA4 are only seen in SFA specimens involved by systemic amyloidosis and, therefore, provide a proteomic signature for amyloidosis. In contrast, APOA1 is present in SFA specimen not involved by amyloidosis suggesting that it is a constituent of normal adipose tissue.

standard at the time. These results suggest that MS-based proteomic analysis may be a more sensitive method for diagnosis than Congo red staining and light microscopy interpretation.

Characteristics of the cases in the clinical cohort

Given the high sensitivity and specificity of the MS-based proteomic analysis for diagnosing systemic amyloidosis, we implemented the method in 2008 as a diagnostic test in a CLIA-accredited laboratory environment specifically to identify the amyloid type once the diagnosis of amyloidosis had been established in SFA specimens by conventional Congo red staining.

In a 4-year period (2008-2012), 2160 SFA specimens were obtained at the Mayo Clinic to screen for the possibility of systemic amyloidosis. Of these, 366 specimens were interpreted as Congo red-positive and were submitted for amyloid typing by MS-based proteomic analysis (Table 2). The sensitivity for successful amyloid typing on Congo red-positive specimens was 90% (330 out of 366). In 10% (36 out of 366) of the cases no specific amyloid type could be detected. Only four of these cases showed the presence of the universal amyloid proteome (APOE/SAP/APOA4) suggesting that in most cases failure of MS-based proteomic analysis were due to sampling differences. As expected, the most frequent type of amyloidosis was AL (n=272, 74%), followed by ATTR (n=46, 13%). Additionally we identified AA, AGel (gelsolin), ALys (lysozyme) and AIns (insulin). Genetic analysis showed that cases of AGel and ALys had mutations involving the respective coding genes, *GSN* and *LYZ*. All insulin cases were patients suffering from insulin-dependent diabetes mellitus, who administered abdominal subcutaneous insulin injections. Representative MS–based proteomic read-outs for each amyloid type are shown in Table 3.

In 20 cases data obtained from the previously established MS-based proteomic analysis for FFPE biopsies from sites other than subcutaneous fat aspirates were available. There was 100% concordance between the results obtained from SFA and FFPE specimens. These results show that SFA MS-based proteomic analysis for typing amyloidosis can be applied with high sensitivity and specificity in a routine clinical setting.

Mass spectrometry-based proteomic analysis can detect clonotypic peptides representing immunoglobulin light chain variable regions in AL-amyloidosis

Using the MS-based proteomic assay, the amyloid type is determined by the presence of peptides representing immunoglobulin light chain constant regions. Although identification of constant regions provides an excellent

Table 3. Representative MS data from seven different types of systemic amyloidosis identified in the clinical cohort. Amyloidogenic proteins identified in this set of cases are listed first, followed by the most abundant stromal proteins seen in all cases. The numbers indicate the number of peptide spectra observed for each protein. Apolipoprotein E (row 1), Apolipoprotein A4 (row 2), and serum amyloid P component (row 7) are present in every sample. In contrast, each sample contains only one amyloidogenic protein. In two cases of AL amyloidosis, MS-based assay identified not only the immunoglobulin light chain constant region but also a clonotypic immunoglobulin variable region fragment.

#	Identified proteins (545)	ATTR	AA	AL-L	AL-K	AIns	ALys	AGel
1	Apolipoprotein E	21	76	61	53	136	31	3
2	Apolipoprotein A-4	21	7	36	55	69	33	22
3	Transthyretin	**174**						
4	Serum amyloid A-1 protein		**143**					
5	Ig lambda-2 chain C regions			**140**				
6	Ig kappa chain C region				**139**			
7	Serum amyloid P-component	11	47	27	11	23	2	4
8	Insulin					**107**		
9	Ig lambda chain V-IV region			**74**				
10	Lysozyme C						**46**	
11	Gelsolin							**41**
12	Ig kappa chain V-I region				**3**			
13	Collagen alpha-3(VI) chain	175	161	252	208	278	154	165
14	Basement membrane-specific heparan sulfate	87	117	101	91	53	135	122
15	Collagen alpha-1(VI) chain	81	49	95	104	124	55	61
16	Vimentin	54	76	47	31	64	120	60
17	Collagen alpha-2(VI) chain	45	35	75	64	90	45	54
18	Hemoglobin subunit beta	56	77	51	42	26	33	29
19	Membrane primary amine oxidase	45	58	32	28	27	63	56
20	Perilipin-1	48	46		9	24	90	62
21	Annexin A2	39	47	35	21	43	63	33
22	Actin, alpha skeletal muscle	20	44	25	60	36	55	17
23	Hemoglobin subunit alpha	55	44	43	42	17	26	23
24	Myosin-9	39	21	56	20	45	38	36

marker for typing amyloidosis, the variable region, which carries the clonotypic unique sequence, is the pathogenic part of the molecule. Because peptides from the variable regions are unique in each case, and not necessarily represented in reference databases, it may be difficult to identify them within the raw MS spectra data files. To overcome this difficulty we supplemented reference databases with sequences from our in- house databases as well as sequences from the ImMunoGeneTics database®. Using the composite database we identified clonotypic peptides from immunoglobulin light chain variable regions in approximately 75% of the cases (Table 4). These findings show that MS–based proteomic analysis can determine light chain peptide sequences from clonotypic variable-region genes confirming the presence of an underlying clonal plasma cell population.

Mass spectrometry-based proteomic analysis can detect the variant peptides representing gene mutations in hereditary ATTR amyloidosis

Similar to immunoglobulin light chain variable regions, variant peptide sequences caused by pathogenic mutations in hereditary amyloidosis are not represented in reference databases and are not, therefore, readily detectable by conventional database searches. To investigate whether we could identify the presence of variant peptide sequences, we curated a customized protein database that contains all known pathogenic peptide sequence variants associated with hereditary amyloidosis including 110 mutations for ATTR. The MS data were interrogated using this database in 46 cases of ATTR. No variant TTR sequences were identified in 16 cases. The remaining cases showed a variety of amino acid substitutions, *TTR* Thr60Ala being the most frequent. When germline DNA sequencing information was available, it confirmed the findings in every case in which MS detected a TTR peptide sequence abnormality (Tables 5 and 6). In three cases, proteomic analysis did not detect an abnormal TTR peptide sequence whereas the germline DNA sequencing found *TTR* gene mutations. In one case the DNA sequencing indicated an Asp18Asn mutation which is in an area that lacks tryptic peptides of appropriate size for detection by MS and it would not, therefore, be expected to be detected. The second case revealed an Ala36Pro mutation via DNA sequencing but the mass shift that is induced matches the variable modification for oxidation that is allowed in our search strategy and was not, therefore, detected. In the third case, Val30Met was present in an area that could be identified by MS analysis; however, the case had only a very weak signal for TTR suggesting that the tissue analyzed had very little amyloid and thus the probability of detecting the mutated peptide was reduced. Overall these findings show that pathogenic alterations in TTR peptide sequences can be identified by MS-based proteomic analysis in hereditary ATTR amyloidosis. Although the MS-based proteomic assay as it is performed in the clinical laboratory may not detect some of the rare genetic variants of ATTR, it has 100% accuracy if an abnormal peptide is detected.

Discussion

Congo red-stained SFA specimens provide an effective tool for screening and primary diagnosis of systemic amyloidosis. In this study, we demonstrate that SFA specimens can be used for the typing of systemic amyloidosis in a routine clinical setting in a CLIA-accredited laboratory environment with a high sensitivity and specificity. The ease of sample acquisition and preanalytic handling rivals the FFPE tissue-based methodology developed by and widely used by our laboratory.[9]

Our clinical test validation cohort comprised two groups; one with Congo red-positive SFA specimens (n=43) and the other with Congo red-negative specimens (n=32). In this cohort, the test performed with high sensitivity (88%), specificity (96%), and accuracy (91%). There was only one false positive case that had a proteome suggestive of AL-kappa in a patient with very high levels of kappa FLC due to an underlying multiple myeloma. This false result was attributed to serum contamination. The preanalytical parameters of the MS-based proteomic assay for SFA specimens differ significantly from the MS-based proteomic clinical assay for FFPE specimens previously developed and widely used by our laboratory. In SFA specimens, the diagnosis of amyloidosis was established by Congo red staining of one part of the specimen, and MS-based proteomic was performed on the other half. Unlike the FFPE-based assay no microdissection step was performed. For this reason the SFA test had a similar specificity to that of the FFPE-based test (98% *versus* 100%) but had a lower sensitivity (88% *versus* 100%) because of uneven distribution of amyloid material between the two halves of the sample.

Compared to other studies published on proteomic analysis of SFA specimens for the diagnosis of amyloidosis, our approach offers a number of advantages.[23-25] These

Table 4. Immunoglobulin light chain variable region usage in AL amyloidosis in the clinical cohort. There was marked bias for immunoglobulin light chain variable region gene usage. The majority of AL-lambda specimens contained either VλIII or VλVI gene regions while the AL-kappa specimens contained the VκI gene region.

Amyloid type		N. patients (%)	V-GENE and C-GENE (IMGT/GENE-DB symbols)
AL λ		**212/272 (78)**	
	VλI	31/212 (15)	IGLV1
	VλII	28/212 (13)	IGLV2
	VλIII	**51/212 (24)**	**IGLV3**
	VλIV	1/212 (0.5)	IGLV4
	VλVI	**47/212 (22)**	**IGLV6**
	no V-region	54/212 (25)	
	C-region	212/212 (100)	IGLC3 (151), IGLC2 (30) IGLC1 (27), IGLC7 (4)
AL κ		**60/272 (22)**	
	VκI	**30/60 (50)**	**IGKV1**
	VκII	1/60 (2)	IGKV2
	VκIII	5/60 (8)	IGKV3
	VκIV	9/60 (15)	IGKV4
	VκVI	1/60 (2)	IGKV6
	no V-region	14/60 (23)	
	C-region	60/60 (100)	IGKC

Nomenclature based on IGMT and Swiss-Prot databases.

include much simplified preanalytical processes which require very little tissue and provide a 24-hour turnaround time, a simple clinical read-out algorithm, a computational pipeline specifically customized for amyloidosis-associated variant proteins missing from reference databases, clinical validation and an operational infrastructure within a CLIA-compliant regulatory framework.

Most of the cases we studied in the test validation cohort were AL, which is associated with increased serum FLC levels. Because we encountered one false positive case, attributed to sample contamination by circulating FLC rather than amyloid-bound immunoglobulin, we investigated the relationship between serum FLC levels and immunoglobulin light chain spectra. We saw no correlation between serum FLC levels and proteomic results, indicating that the assay results were not affected by serum FLC levels systematically, and represented amyloid-bound immunoglobulin.

Although the causative protein is the main constituent of amyloid plaques, the amyloid proteome is complex and a number of other proteins are frequently co-deposited. Of these SAP is present in all amyloid fibrils and is thought to promote fibril formation and stabilization. APOE, APOA1 and APOA4 have also been consistently identified as constituents of various types of amyloidosis. We investigated the possibility of using these proteins as a universal proteomic signature for amyloidosis. This was particularly important in the case of SFA specimen analysis, as one half

Table 5. Representative MS data from ten cases of hereditary and one case of age-related ATTR amyloidosis. Amyloidogenic proteins and variant TTR peptides identified in this set of cases are listed first, followed by the most abundant stromal proteins seen in all cases. The numbers indicate the number of peptide spectra observed for each protein. TTR (row 1) and apolipoprotein E (row 2) are present in every sample. Apolipoprotein A-4 (row 3), and serum amyloid P component (row 4) are present in ten out of 11 samples. In contrast, in each of the hereditary ATTR cases only one variant TTR peptide is detected. In each case the variant peptide identified by MS matched the mutation identified by DNA sequencing. As expected no variant peptides with mutation was identified in the age-related ATTR sample with the wild-type gene.

#	Identified proteins (320)	T60A	S50R	L58H	E54G	V122Del	V122I	S52P	P24S	V30A	V30M	Wild type
1	Transthyretin	254	219	108	124	242	62	172	87	51	50	233
2	Apolipoprotein E	59	61	17	37	34	8	52	20	39	26	29
3	Apolipoprotein A-4	26	47	19	62	45		37	26	5	36	26
4	Serum amyloid P-component	31	21	15	8	25	8	38	7	17		20
5	TTR Thr60Ala ACT-->GCT	**86**										
6	TTR Ser50Arg AGT-->AGG		**46**									
7	TTR Leu58His CTC-->CAC			**22**								
8	TTR Glu54Gly GAG-->GGG				**19**							
9	TTR Val122Del GTC loss					**10**						
10	TTR Val122Ile GTC-->ATC						**16**					
11	TTR Ser52Pro TCT-->CCT							**16**				
12	TTR Pro24Ser CCT-->TCT								**12**			
13	TTR Val30Ala GTG-->GCG									**10**		
14	TTR Val30Met GTG-->ATG										**11**	
15	Collagen alpha-3(VI) chain	147	244	181	230	226	322	285	151	71	197	149
16	Basement membrane specific heparin sulfate	77	125	98	160	146	122	189	127	38	35	101
17	Collagen alpha-1(VI) chain	66	77	76	104	72	114	125	59	29	70	48
18	Vimentin	50	49	42	73	65	113	132	47	120	81	72
19	Annexin A2	34	33	40	43	30	92	80	29	47	129	50
20	Perilipin-1	23	42	35	61	51	74	105	35	58	86	48
21	Collagen alpha-2(VI) chain	44	72	47	81	58	66	85	42	23	51	39
22	Hemoglobin subunit beta	43	28	80	38	30	145	54	30	52	29	62
23	Actin, cytoplasmic 1	33	30	38	36	35	70	83	39	57	52	28
24	Membrane primary amine oxidase	25	31	54	56	41	64	68	32	22	69	34

of the specimen was used for Congo red staining and the other half for proteomic analysis. We observed that the presence of peptide spectra representing at least two of the three proteins, APOE, SAP and/or APOA4 was an excellent surrogate biochemical marker for the presence of amyloid. In contrast, APOA1, a normal constituent of fat tissue, cannot be used as a marker for amyloidosis.

Armed with this proteome signature, we investigated 20 patients with known systemic amyloidosis but Congo red-negative SFA specimens. We found that approximately half of these cases had the universal amyloid signature consistent with amyloidosis despite a Congo red-negative SFA specimen. From our validation study showing 88% sensitivity, we would expect approximately 10% discordance between the Congo red result and a universal amyloid proteome results, most likely due to biological heterogeneity and sampling. We observed a much higher frequency of the universal amyloid proteome in patients with systemic amyloidosis with negative SFA suggesting that proteomic analysis may be a more sensitive tool than Congo red staining for the diagnosis of systemic amyloidosis in SFA specimens, and may detect submicroscopic deposits. Although it is premature to suggest the use of proteomic analysis as a primary screening tool for the diagnosis of amyloidosis, the combination of proteomic analysis with Congo red staining may offer the greatest sensitivity. The detection of a universal amyloid proteome in cases of SFA specimens that are equivocal or negative for Congo red staining could be used as a prompt for further sampling of the fat tissues or target organ biopsies, particularly when there is a strong clinical suspicion of amyloidosis.

Once the clinical test was validated and implemented, we used it routinely in our amyloidosis practice. The clinical implementation was limited to only Congo red-positive SFA specimens. The test performed with high sensitivity (90%) similar to that in the validation cohort and previous studies using Congo red staining.[5,8,33] In a small subset of the patients, for whom proteomic data from both an SFA specimen and a separate FFPE tissue specimen were available, there was 100% concordance. As expected AL and ATTR were the most common types of systemic amyloidosis in our clinical cohort. We also identified a small number of AA cases but also rare cases of unexpected hereditary amyloidosis such as AGel and ALys, which were confirmed by genetic analysis. Interestingly, we also discovered AIns in SFA specimens from three patients with insulin-dependent diabetes mellitus. Subcutaneous AIns amyloidosis is a well-recognized but infrequent complication of subcutaneous insulin injections for the management of insulin-dependent diabetes mellitus.[34,35] Patients often present with masses in the areas of repeated insulin injection, and the insulin amyloid remains localized to these sites. The only significant clinical consequence of such a diagnosis appears to be increasing insulin resistance due to poor diffusion of the injected insulin through the amyloid mass if the area continues to be used for repeated insulin injections.[36,37] However, localized AIns amyloidosis poses a risk for a misdiagnosis of systemic amyloidosis if SFA specimens are used for screening without performing amyloid subtyping on the specimen.[38] Because the clinical features of insulin-dependent diabetes mellitus with renal or peripheral nerve disease may overlap with those of systemic amyloidosis, in particular AL amyloidosis, this is a major clinical concern. In this context, the availability of an MS-based proteomic assay has been invaluable for accurate diagnosis and clinical management.

In addition to identifying the specific amyloid types, the MS-based proteomic approach (so-called "shot-gun proteomics") used in this clinical assay also identifies all other proteins and peptides that are part of the amyloid deposits, and the fat tissue microenvironment. Although we have not used this information for primary clinical diagnosis, we explored whether additional information could be gained regarding the underlying molecular pathogenesis. Initially, we focused on two areas; identification of immunoglobulin variable region usage in AL amyloidosis, and identification of pathogenic TTR peptide sequence variants in hereditary ATTR amyloidosis. Using protein databases enhanced for the variants of interest, we were able to determine the immunoglobulin light chain variable-region peptides in 75% of AL amyloidosis cases. Consistent with the published data based on the sequencing of immunoglobulin genes of clonal plasma cells in AL amyloidosis,[39-41] the variable gene usage was markedly biased favoring VλIII or VλVI for AL-lambda and VκI for AL-kappa. In AL amyloidosis, immunoglobulin light chain variable-region usage has been associated with organ involvement patterns and, as a consequence, clinical outcome.[41] Availability of such information through proteomic analysis may be helpful in risk prediction and clinical decision-making.

We were also able to identify the amyloidogenic TTR amino acid sequence variants associated with hereditary ATTR amyloidosis. Although the most common familial ATTR peptide sequence variant is Val122Ile, found in the African-American population,[42] the two most common alterations observed in our specimens were Thr60Ala[43] and Val30Met[43]. This is likely due to the demographics of the patients from whom our specimens were derived. The other variants, each detected in one case, were Val122Ile, Pro24Ser[44], Ser50Arg[45], Glu54Gly[46], Val30Ala[47], Ser52Pro[48], Leu58His[49] and Val122Del[50]. While this method was very specific (100%) it had limited sensitivity since a number of rare mutations affecting residues not detectable by the current proteomic method were encountered. Alterations that are present in the TTR protein but are not within a tryptic peptide or mutations

Table 6. Pathogenic peptide sequence variants in hereditary ATTR amyloidosis identified by MS-based proteomic analysis.

TTR variant	Cases	Confirmed by sequencing
Pro24Ser	3	2/2
Val30Met	5	4/4
Val30Ala	1	NA
Ser50Arg	3	3/3
Ser52Pro	1	NA
Glu54Gly	3	2/2
Leu58His	1	NA
Thr60Ala	8	6/6
Val122Ile	4	2/2
Val122Del	1	1/1
Total	**30**	**20/20**

NA: not available.

that do not result in a significant mass shift compared to the normal protein will not be identified. Despite this shortfall the TTR sequence variant information proved very helpful in routine clinical practice to demonstrate the physical presence of the pathogenic protein in the amyloid deposits and to direct downstream genetic screening, especially for those patients for whom hereditary ATTR amyloidosis was not initially suspected.

In summary, MS-based-proteomic analysis of SFA specimens for the screening and diagnosis of systemic amyloidosis is a powerful clinical tool. The assay provides unprecedented sensitivity and specificity for the diagnosis and typing of amyloidosis, including rare hereditary and iatrogenic variants. In addition to specific diagnostic information, the assay provides insights into underlying pathogenesis, which may be important for downstream clinical decisions.

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