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## Laboratory Methods for the Diagnosis of Hereditary Amyloidoses

S. Michelle Shiller, Ahmet Dogan and W. Edward Highsmith, Jr.

*Department of Laboratory Medicine and Pathology, Mayo Clinic College of Medicine,  
Rochester, Minnesota  
United States of America*

### 1. Introduction

The majority of systemic amyloid disorders are acquired in nature and are most often secondary to plasma cell dyscrasias (AL amyloid), age-related accumulation of transthyretin (senile ATTR amyloid) or chronic inflammation (AA amyloid). A smaller, though significant, fraction of cases are due to inherited mutations in one of several amyloidogenic proteins. As described elsewhere in this volume, amyloid consists of fibrils composed of stacked proteins which have adopted a beta pleated sheet conformation. The mechanism by which a protein which has substantial alpha helical character refolds into a configuration with its primarily beta pleated sheet is unclear and is the subject of much ongoing research. It is clear, however, that specific amino acid substitutions in a small number of circulating proteins can accelerate or facilitate this process.

All of the hereditary amyloidoses (also known as familial or systemic amyloidoses) are inherited in an autosomal dominant manner, as is the case for other "gain of function" mutations in disorders such as Huntington disease, myotonic dystrophy, or the spinocerebellar ataxias. The dominant inheritance of the familial amyloidoses has implications for family members of an affected individual. First, careful attention to the family history will often reveal symptoms in one parent, which may or may not have come to medical attention. However, even in the setting of a thorough family history, hereditary amyloidosis can be missed and attributed to more common diseases. It is important to understand that siblings and children of an affected individual have a 1 in 2 chance of being affected themselves. Thus, after the identification of an amyloidogenic mutation in an individual, it is important to offer testing for at-risk family members so that appropriate monitoring can be carried out for mutation positive family members.

The hereditary amyloidoses involve deposition of amyloid in a variety of tissues. Typically, there is a proclivity for the vascular tree, particularly the microvasculature, and it can be a cause of cerebral hemorrhage. The kidneys and the heart are the most commonly involved organs. However, virtually any organ can become involved, with liver, spleen, thyroid, larynx, gastric wall, and salivary glands all described. Further, amyloid deposition can also occur in the soft tissue adjacent to the salivary gland, tracheobronchial tree, the tongue, and in the skin. On physical examination this diverse distribution manifests with a similar broad spectrum of features including macroglossia, peripheral edema, peripheral neuropathy, hypertension,

popular rash, and waxy papules. Patients may demonstrate congestive heart failure as part of the restrictive cardiomyopathy conferred due to the amyloid deposition within cardiac muscle. Additionally, gastric dysmotility occurs, and cerebral hemorrhage is documented, as previously mentioned.

Clinically, it may be difficult to distinguish amyloidosis that is secondary to overproduction of immunoglobulin light chains (AL amyloid), age-related ATTR amyloidosis or serum amyloid A (AA amyloid) from an amyloidosis that is hereditary in nature. As the treatment for the underlying cause of amyloidosis is drastically different for the different etiologies, it is critical that the amyloid be properly classified. Therapy for plasma cell disease can include chemotherapy and/or bone marrow transplant, and the therapy for AA amyloid involves addressing the underlying cause of inflammation, while the curative treatment for two varieties of familial amyloidosis, including the most common form due to mutant transthyretin (ATTR amyloid) is liver transplant.

To make a diagnosis, including identification of the protein being deposited as amyloid fibrils, it is typically necessary to obtain biopsy material from an affected organ or site. Most often, biopsies are obtained from either the bone marrow, subcutaneous fat (often of the abdomen), or the rectum. Following tissue acquisition, a variety of methods are used in identifying and characterizing amyloid protein. These include Congo Red staining, immunoperoxidase staining of histological tissue, mass spectrometry and genetic evaluation in cases of familial disease.

While the majority of systemic amyloidosis is due to transthyretin (TTR) mutations, identification of other genes involved in conferring aberrant protein folding with subsequent amyloid deposition have been identified. These additional genes have been documented in a substantially smaller number of individuals than TTR mutations and include ApoA1, ApoA2, gelsolin, lysozyme, and fibrinogen alpha (FGA). By and large, the most common symptom of these genetic variants is nephropathy. However, the gelsolin variant of disease does not involve the kidneys, rather it displays a predisposition for cranial nerve tissue, lattice corneal dystrophy, and cutis laxa of the facial skin. As gelsolin amyloidosis was originally identified in a large Finnish family, and is more common, but not limited to individuals of Finnish descent, it is often referred to as Finnish amyloidosis.

The differential diagnosis of systemic amyloidosis includes light chain disease, Sjögren's syndrome, rheumatoid arthritis, other inflammatory conditions,  $\beta$ 2-microglobulinemia, and Familial Mediterranean Fever, as well as other similar conditions. A discussion of a thorough diagnostic evaluation for these conditions is beyond the scope of this chapter. However, a few key laboratory tests can expedite the process: serum protein electrophoresis (assists in the diagnosis of light chain disease or  $\beta$ 2-microglobulinemia), the presence of antinuclear antibody and SSBLa>SSBRo by immunofluorescence for Sjögren's syndrome. The presence of rheumatoid factor points towards Rheumatoid Arthritis. Detection of serum amyloid A in amyloid deposits by immunohistochemistry elicits a definitive diagnosis of AA amyloid, or amyloid deposition of an inflammatory origin.

With respect to AL amyloidosis, while serum protein electrophoresis is the classical method of working up this diagnosis, ruling out hereditary amyloidosis through DNA interrogation is pivotal. Studies have demonstrated that individuals with hereditary amyloidosis may also demonstrate monoclonal immunoglobulins on serum protein electrophoresis in as many as 24% of patients. In the study by Lachmann et al., all of the patients had less than 0.2 g/dL of immunoglobulins in the serum, and none of the

patients had kappa or lambda free light chains by urine protein electrophoresis. Comenzo et al. had similar findings, with six percent of patients with a hereditary amyloidosis presenting definitive monoclonal gammopathies in a subject population of similar size. The differences between these two studies is that the patients with monoclonal gammopathy in the Lachmann et al. study demonstrated mutations in a variety of genes for hereditary amyloidosis, whereas in the Comenzo et al. study, all patients had TTR mutations. In the absence of DNA analysis, these patients with hereditary amyloidosis masquerading with a monoclonal gammopathy would be misdiagnosed, and the improper clinical management could be implemented.

## 2. Methods used for the evaluation of tissue amyloid

### 2.1 Congo red staining

The gold standard for the detection of the presence of amyloid is Congo Red staining (Figure 1). A paper by Cooper compared Congo Red staining against other techniques to detect amyloid at the time, and his findings demonstrated that the green birefringence demonstrated under polarized light was completely specific for the presence of amyloid. A positive Congo Red stain on a biopsy or fat aspirate does not give the specific precursor protein causing the amyloid deposition. It does, however, define the presence of amyloid, setting the stage for further diagnostic evaluation.

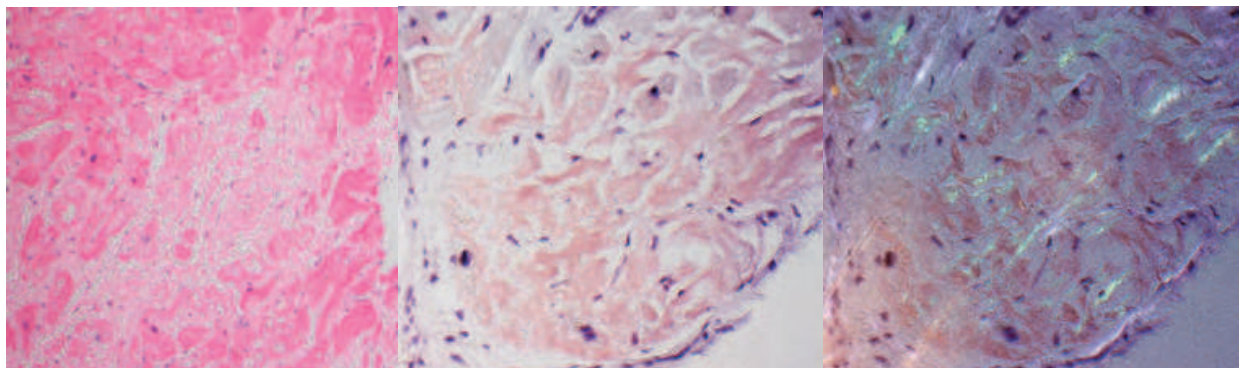


Fig. 1. Cardiac biopsy from a patient with TTR amyloidosis by hematoxylin and eosin (left), Congo Red (middle), Congo Red under polarized light with apple green birefringence (right). (Courtesy of Ahmet Dogan, MD, PhD; Mayo Clinic, Rochester, MN).

### 2.2 Immunohistochemistry

Immunohistochemistry (IHC) has been in use for many years to identify the specific protein involved in amyloid deposits identified by Congo red staining. In principle, IHC can differentiate the most common types of amyloid, light chain, serum amyloid A, and transthyretin (Figure 2). However, it is the authors' experience that, with the exception of serum amyloid A, the results can be equivocal and difficult to interpret. One of the



problems is that the epitopes that specific antisera recognize can be partially or even completely buried in the insoluble amyloid fibril. There is a lack of high affinity antibodies specific for amyloidogenic proteins in the beta pleated sheet conformation. Further, a trained eye for interpretation of the results is required, with the ability to discern a poor staining pattern (such as that seen with serum leakage into amyloid plaques), and to recognize nonspecific or non-contributory background staining (low specificity and sensitivity). In addition, the adverse effects of changing protein structure by crosslinking due to formalin fixation limits the utility of immunohistochemistry in detecting amyloid deposition (demonstration of this phenomena thus far has been limited to TTR). If TTR is identified, IHC is unable to distinguish between a mutant TTR protein in a case of familial amyloid and a wild-type protein in case of senile amyloid (see the discussion of TTR and ApoA2 amyloid below). Finally, typical IHC panels consisting of antibodies to kappa and lambda light chains, serum amyloid A, and TTR, cannot identify the presence of less common amyloids consisting of lysozyme, gelsolin, fibrinogen alpha, or apolipoproteins 1, or 2.

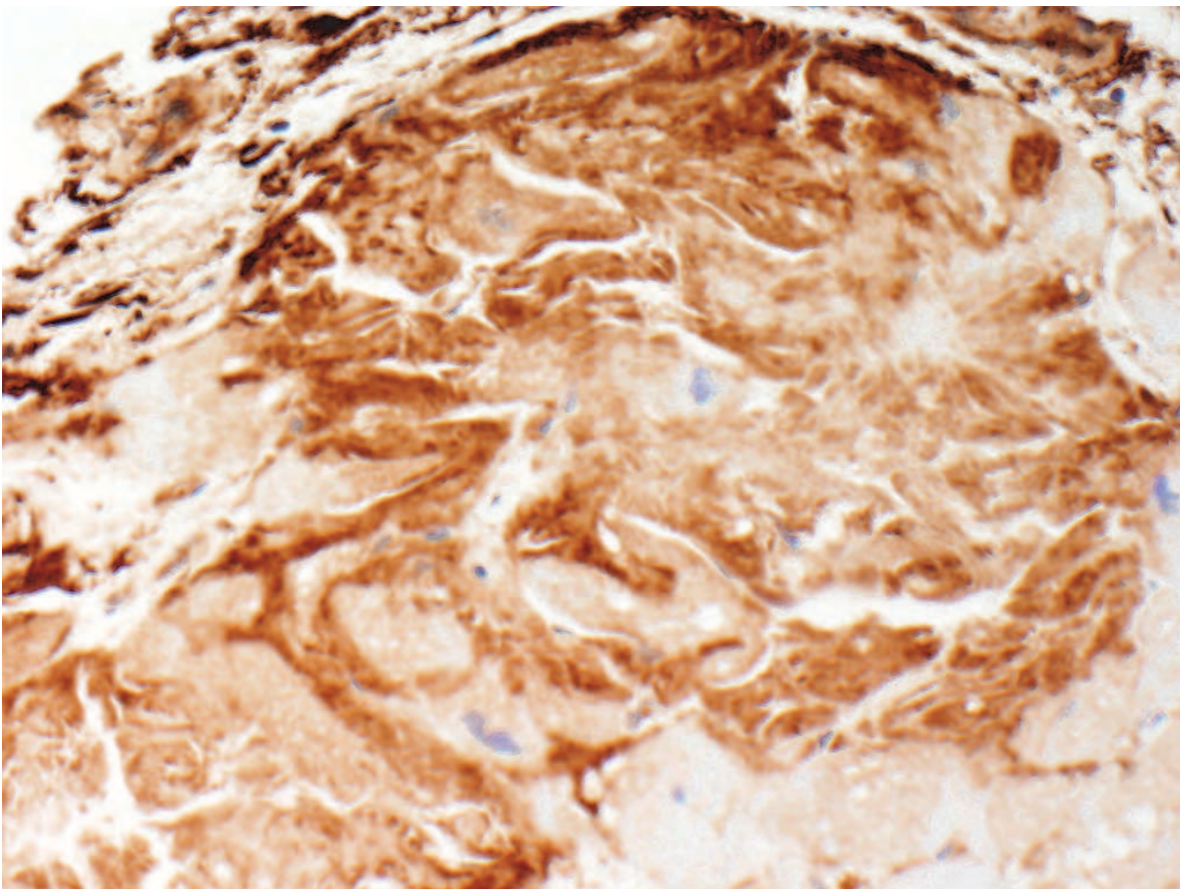


Fig. 2. Cardiac biopsy with TTR amyloid, positive for TTR by immunohistochemistry (photograph courtesy of Ahmet Dogan, MD, PhD; Mayo Clinic, Rochester, MN).

### 2.3 Electron microscopy

Ultrastructural examination (electron microscopy) of amyloid is not a common method of identifying this pathology. However, on ultrastructural examination, amyloid has a fibrillar pattern (Figure 3). Of note, there are several other fibrillary diseases of the kidney such as fibrillary glomerulonephritis and immunotactoid glomerulopathy, both of which are completely different entities than hereditary amyloid. Thus, fibrillary glomerular deposits by electron microscopy are non-specific, and additional studies are warranted to determine their etiology.

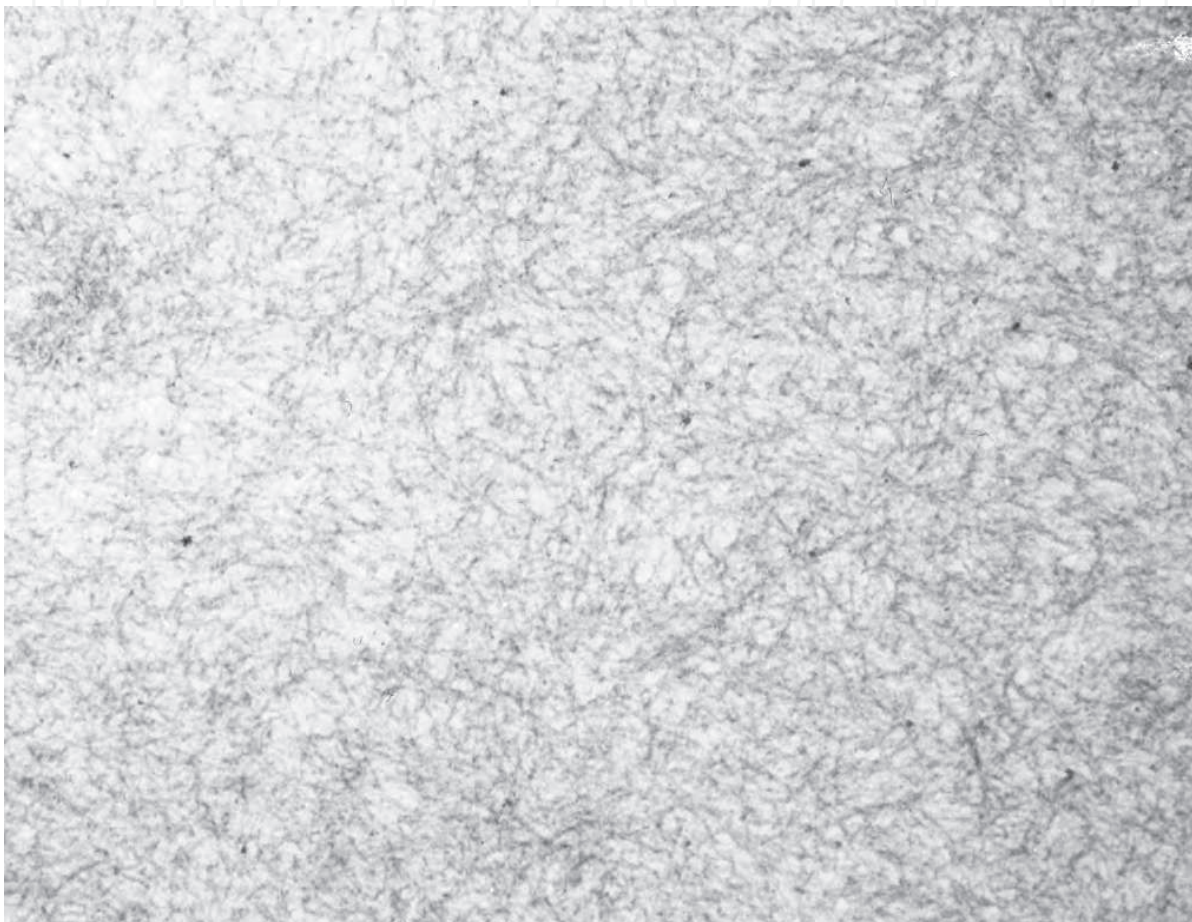


Fig. 3. Electron microscopy of amyloid fibrils measuring 6-10 nanometers in a patient with cardiac amyloidosis (photograph courtesy of Ahmet Dogan, MD, PhD; Mayo Clinic, Rochester, MN).

### 2.4 Mass spectrometry

More recently with new technological developments, mass spectrometry (MS) based proteomic methodologies have been applied to subtype amyloidosis. Initial studies using MS, similar to amino acid sequencing approaches, required large quantities of fresh or frozen tissue with a heavy amyloid load. However, in recent years, the sensitivity of MS based technologies has significantly improved and methods to extract proteins and peptides from small amounts of clinical biopsy specimens have been developed. This has led to development of a highly sensitive and specific clinical test for typing of amyloid deposits in paraffin embedded tissues. The approach incorporates laser capture microdissection (LCM)



of amyloid plaques up front which dramatically increases the amount of proportion signal coming from the amyloidogenic protein compared to the signal coming from the background tissue (Figure 4).

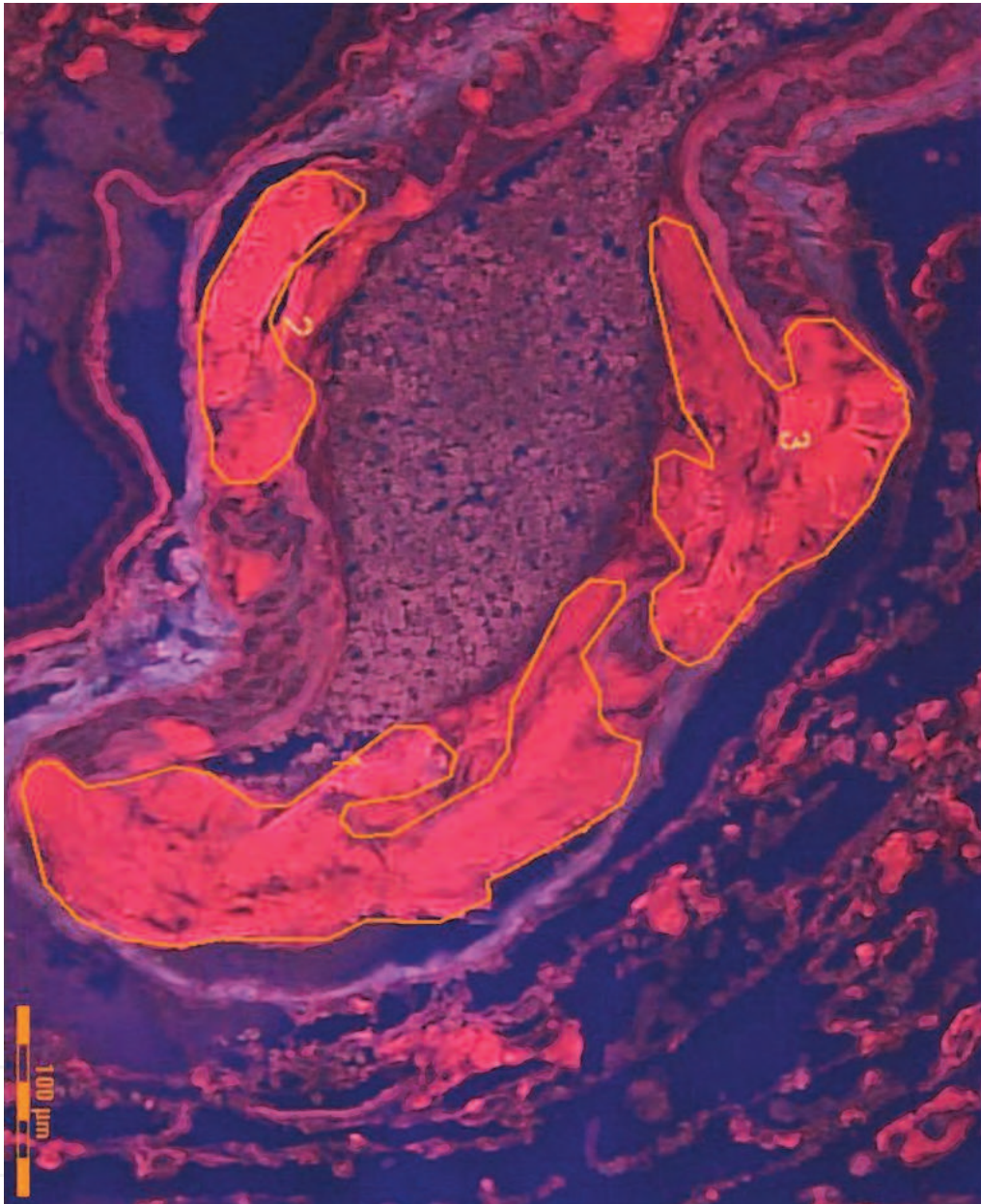


Fig. 4. AA amyloidosis showing predominantly vascular deposition with Congo red staining. For mass spectrometry based proteomic analysis, the areas circled by yellow lines are microdissected with laser and the microdissected fragments are collected in the tube cap ready for protein extraction and digestion into peptides suitable for tandem mass spectrometry (courtesy of Ahmet Dogan, MD, PhD; Mayo Clinic, Rochester, MN).

Once the amyloid plaques are captured, they are fragmented by heating and sonication to reverse crosslinking caused by formalin fixation, followed by digestion into peptide fragments by trypsin. Trypsin specifically cleaves the proteins following lysine and arginine residues and generates a peptide “soup.” For each protein a specific and reproducible set of peptides is

generated. The peptide solution is fractionated by high performance liquid chromatography and peptides are introduced into the mass spectrometer by a process called electrospray ionization (ESI). ESI provides the interface between the liquid environment of the HPLC and the high vacuum environment of the MS. It also adds charges to the peptides so that they can be detected and manipulated by MS. After ESI, the peptides are analyzed by tandem MS/MS. The first MS detects mass/charge ratio ( $m/z$ ) of each peptide (precursor ion) as they are focused in the mass spectrometer. Based on relative abundance and other preset criteria, a subset of the charged peptides are selected for collision-induced dissociation. This leads to fragmentation of the peptides into smaller charged particles (product ions). Mass/charge ratio of these fragments is captured by the second MS (tandem mass spectrometry, MS/MS). This raw MS data is then interrogated by bio-informatic algorithms which match the fragmentation pattern of each peptide to one of the theoretical tryptic peptides present in human proteome. In this way, amino acid sequence of each peptide analyzed by MS/MS is predicted with high specificity. Proteins are identified and displayed in order of relative abundance based on the total number of peptide spectra identified for each protein (Figure 5). Using this approach amyloid deposits can be subtyped by very high sensitivity and specificity (100% for both in the published validation set) (Vrana et al., 2009).

		Probability Legend:					
		over 95%	80% to 94%	50% to 79%	20% to 49%		
		0% to 19%					
Bio View:		Accession Number	Molecular Weight	1	2	3	4
#	Identified Proteins (463)						
1	Serum albumin OS=Homo sapi...	ALBU_HUMAN	69 kDa	58	79	59	52
2	Transthyretin OS=Homo sapie...	TTHY_HUMAN	16 kDa	26	26	26	25
3	Apolipoprotein E OS=Homo sa...	APOE_HUMAN	36 kDa	30	31	25	19
4	Serum amyloid P-component ...	SAMP_HUMAN	25 kDa	33	27	25	19
5	Apolipoprotein A-IV OS=Homo...	APOA4_HUMAN	45 kDa	24	28	17	18
6	Collagen alpha-1(I) chain OS...	CO1A1_HUMAN	139 kDa	14	16	24	22
7	Vitronectin OS=Homo sapiens...	VTNC_HUMAN	54 kDa	22	17	13	11
8	TTR Val30Met GTG --> ATG *A...	TTHY_Val30...	1 kDa	19	13	13	12
9	Complement C3 OS=Homo sap...	CO3_HUMAN	187 kDa	15	15	8	6
10	Collagen alpha-2(I) chain OS...	CO1A2_HUMAN	129 kDa	9	13	18	14
11	Complement factor H-related ...	FHR1_HUMAN	38 kDa	12	13	8	7
12	Ig gamma-1 chain C region O...	IGHG1_HUMAN	36 kDa	9	7	9	8
13	Fibulin-1 OS=Homo sapiens G...	FBLN1_HUMAN	77 kDa	8	10	9	6
14	Vimentin OS=Homo sapiens G...	VIME_HUMAN	54 kDa	6	12	6	7
15	Collagen alpha-3(VI) chain OS...	CO6A3_HUMAN	344 kDa	5	11	5	3
16	Ig kappa chain C region OS=...	IGKC_HUMAN	12 kDa	5	6	5	7
17	Katanin p60 ATPase-containin...	KATL1_HUMAN	55 kDa	2	3	4	
18	Trypsin-3 OS=Homo sapiens ...	TRY3_HUMAN	33 kDa	5	5	4	5
19	Midkine OS=Homo sapiens GN...	MK_HUMAN	16 kDa	6	5	2	1
20	Collagen alpha-1(III) chain O...	CO3A1_HUMAN	139 kDa	2	4	2	4

Fig. 5. Mass spectrometry based proteomic analysis of hereditary amyloidosis. Transthyretin (red arrow) is the most dominant amyloidogenic protein in all four microdissections studied. Gene sequencing confirmed Val50Met in this case. (courtesy of Ahmet Dogan, MD, PhD; Mayo Clinic, Rochester, MN).



### 3. Genetic evaluation of familial amyloidosis

#### 3.1 Genetic evaluation

Gene sequencing is the gold standard to detect aberrations such as substitutions, and small deletions and insertions at the nucleotide level. Sequencing is particularly useful when heterogeneity occurs in a disease, as with amyloidosis. The familial amyloidoses display both genetic heterogeneity (multiple genes being involved in a disease) and allelic heterogeneity (multiple mutations in the sample gene being able to cause the disease).

The first step in any DNA sequencing is the extraction, or purification, of DNA, typically from a peripheral blood sample. Many platforms are available for DNA extraction, the authors use the MagNaPure® LC (Roche Diagnostics). Following DNA extraction, the samples are prepared for PCR with primers specific to the gene of interest, and the standard PCR constituents (Taq polymerase, buffer, magnesium chloride and PCR-grade water). The authors perform a gel electrophoresis next to confirm the PCR reaction prior to proceeding with the sequencing assay. Next, the PCR product is treated, or “cleaned” to remove unincorporated primers and nucleotides. Again, there are multiple ways that this can be accomplished. The authors utilize shrimp alkaline phosphatase (to convert unincorporated deoxynucleotide triphosphates (dNTP's) into dephosphorylated products that will not interfere with the downstream sequencing reaction) and exonuclease (to digest unextended PCR primers into nucleotides to prevent unwanted extension during the sequencing reaction). The cleaned PCR product is next combined with a mixture of fluorescently labeled di-deoxynucleotide triphosphates and dNTP's (ex: BigDye® terminators [Applied Biosystems]), sequencing buffer, PCR grade water, and a thermostable DNA polymerase. After carrying out the sequencing reaction by thermal cycling and another purification step, this time removing unincorporated fluorescent material, the sample is analyzed by capillary electrophoresis. There are multiple software programs commercially available for base calling, alignments, and mutation detection. The authors use Mutation Surveyor® (Soft Genetics) (Figure 6).

### 4. Genes involved in hereditary amyloidosis

#### 4.1 Notes on nomenclature

The Human Genome Variation Society (HGVS) has proposed standard nomenclature for variation both at the nucleotide and the protein level ([www.hgvs.org/mutnomen/](http://www.hgvs.org/mutnomen/)). In this chapter, all mutations and variants will be discussed referring to protein sequence, or amino acid changes. The HGVS recommends that proteins be numbered starting with the initiator methionine as amino acid number one. Older literature often uses a different convention. Previously, the standard nomenclature was to number the first amino acid of the mature, processed protein as amino acid number one. For secreted proteins (such as those involved in familial amyloidoses), this numbering system neglected the signal peptides and propeptides that are cleaved from the amino terminus after translation as the protein is being processed by the cell for secretion. All of the amino acid changes discussed here will use the HGVS standard nomenclature.

For example, the signal peptide of the TTR protein is 20 amino acids in length. One mutation seen in TTR amyloid is Cys30Arg (new nomenclature). Using historical nomenclature, the mutation is termed Cys10Arg (subtract 20 amino acids that account for the signal peptide in

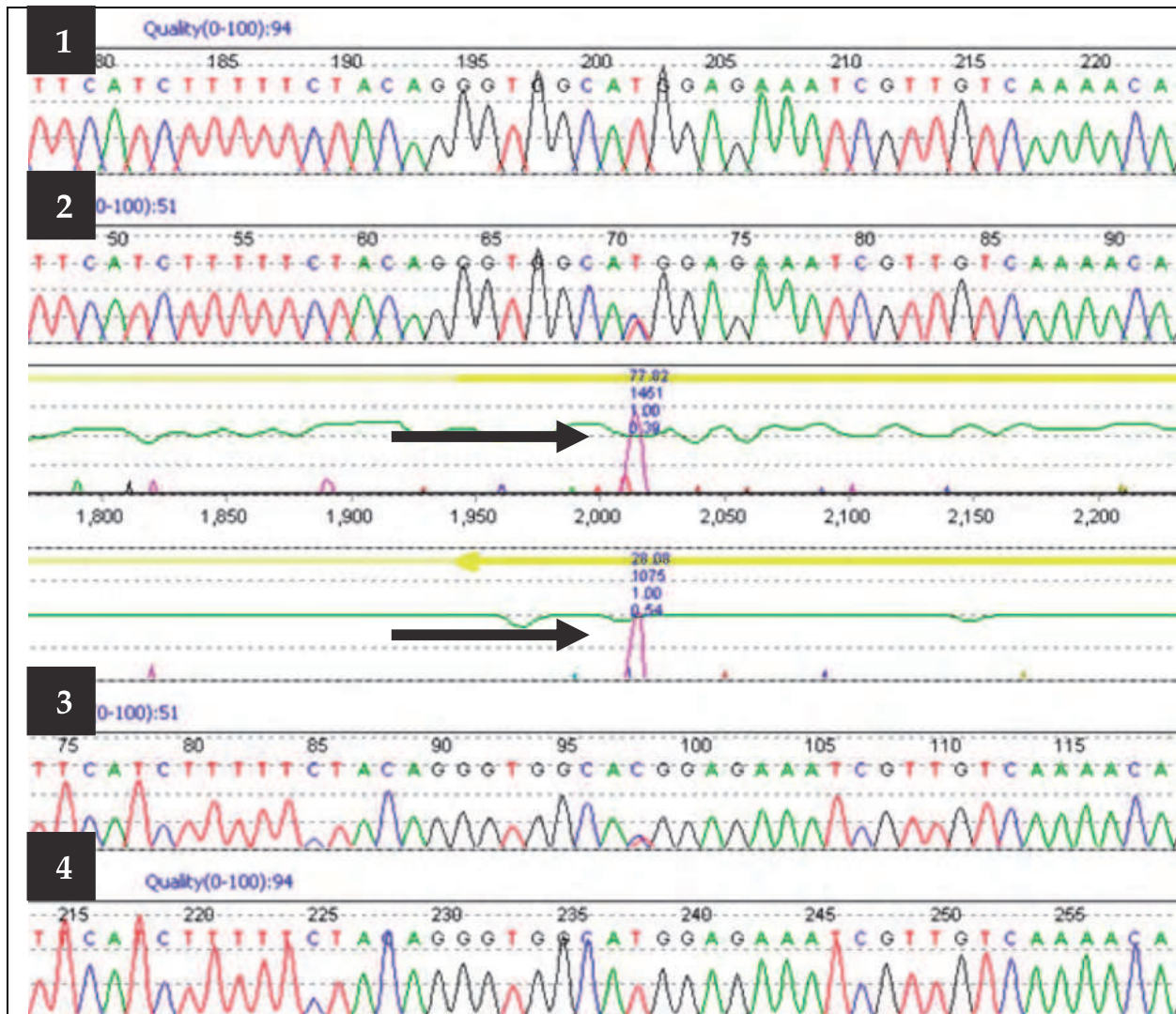


Fig. 6. Gene sequencing for lysozyme showing c. 388T>A, p. W130R, a pathogenic mutation. The top and bottom sequences (1 and 4) are the reference sequences against which the sample is compared. Sequences 2 and 3 are the patient sample. The top trace (1, 2) are sequenced in the forward direction, and the bottom trace (3, 4) in the reverse. The two middle traces are the subtraction plots between the reference sequence and the patient sample. The intronic region is indicated by the tan line, the yellow line indicates the exon. The pink peak is the location of the mutation (arrows), indicating substitution of a cytosine for a thymine. This nucleotide substitution alters the tryptophan (W) codon TGG to CGG, which codes for arginine (R).

the new nomenclature to derive this). Apolipoprotein A1 (ApoA1) is an example of a protein with a signal peptide and a propeptide. The signal peptide is 18 amino acids in length, and the propeptide is 6. Hence, to extrapolate the historical nomenclature from the new nomenclature for a mutation in ApoA1, Gly50Arg would be Gly26Arg. Please refer to Table 1 for a listing of all genes and the conversions.

Protein	Signal Peptide/Propeptide	Example (historical→new)
TTR	20	Cys10Arg→Cys30Arg
ApoA1	18/6	Gly26Arg→Gly50Arg
ApoA2	18/5	Stop78S→Stop101S
Gelsolin A	27	Asp187Tyr→Asp214Tyr
Fibrinogen Alpha	19	Arg554Leu→Arg573Leu
Lysozyme	18	W112R→W130R

Table 1. Table depicting nomenclature conversion from historical to new. The primary difference is that the new nomenclature includes all codons beginning with the initiating methionine, and the historical nomenclature utilizes only the mature protein. Hence, to convert to the new nomenclature requires adding the appropriate number of codons acting as signal peptides and propeptides, as indicated.

#### 4.2 Transthyretin (TTR)

Transthyretin (TTR) was the first protein identified in hereditary amyloidosis with amyloid deposition due to coding sequence missense mutations was transthyretin. Notably, it is by far the most common protein and gene involved in familial amyloidosis, accounting for between 95-98% of reported familial amyloid cases, and often presenting after the age of 50. TTR is a transport protein that has four exons, 127 amino acids and weighs 55kDa, and is synthesized predominantly in the liver. The function of TTR is to carry thyroxine (T4) and to participate in the thyroxine-retinol binding protein complex. Consequently, when TTR is mutated, aberrant protein folding results with deposition as described above with the clinical sequelae including most predominantly peripheral polyneuropathy, and/or cardiomyopathy (with or without eye and brain involvement).

Due to its predominantly hepatic synthesis, liver transplantation is the treatment of choice for patients with TTR amyloidosis. Since this treatment is vastly different than the cytotoxic chemotherapeutic regimens and/or bone marrow transplant indicated for AL amyloidosis, the correct diagnosis of these two disorders with supporting laboratory data is paramount. Also, senile amyloid deposition is often composed of wild-type TTR protein. In this case, the conversion of TTR into amyloid fibrils is not driven by pathogenic mutations. Gene sequencing is necessary to distinguish TTR type senile amyloid from a hereditary disorder.

Though more than 100 mutations have been reported in TTR, almost all of them are due to single base substitutions in the gene, located on chromosome 18. Common single base substitutions include V50M, L75P, L78H, T80A, and Y134H. A common three-nucleotide/single codon deletion is ΔVal142. Moreover, ethnic propensities exist such as the association of V142I with African Americans. Further, M33I is seen in the German population, A45T, Y89I, and Q112K segregate amongst the Japanese. Variants common in the United States are D38N, A45S, F53C, W61L, T69P, L75Q, A101T, and R123S. Phenotypic



clustering is seen in some codon changes (Table 2), and Tyr89Ile is the only double nucleotide substitution documented to date. Specifically, Tyr89Ile is seen in the Japanese population, with cardiac and connective tissue involvement, and autonomic neuropathy.

Mutation	Clinical Features	Geographic kindreds
Phe53Ile	Peripheral Neuropathy, Eye	Israel
Phe53Leu	Peripheral Neuropathy, Heart	USA
Phe53Val	Peripheral Neuropathy	UK, Japan, China
Ala65Thr	Heart	USA
Ala65Asp	Heart, Peripheral Neuropathy	USA
Ala65Ser	Heart	Sweden
Ile104Asn	Heart, Eye	USA
Ile104Thr	Heart, Peripheral Neuropathy	Germany, UK
Glu109Gln	Peripheral Neuropathy, Heart	Italy
Glu109Lys	Peripheral Neuropathy, Heart	USA
Val142Ile	Heart	USA
ΔVal142	Heart, Peripheral Neuropathy	USA
Val142Ala	Heart, Eye, Peripheral Neuropathy	USA

Table 2. Phenotypic correlations of TTR mutations along with segregation among particular geographic kindreds (adapted from Benson 2003 ).

### 4.3 Apolipoprotein A1 (ApoA1)

Apolipoprotein A1 (ApoA1), another protein involved with hereditary amyloidosis, contains four exons, 243 amino acids, weighs 28kDa, and is located on chromosome 11q23-q24. ApoA1 is synthesized in the liver and small intestine, conferring a plasma protein that is the main protein of high-density lipoprotein particles and has a key role in lipoprotein metabolism. As such, ApoA1 is important for the formation of high-density lipoprotein cholesterol esters, promoting efflux of cholesterol from cells. Mutations in ApoA1 can lead to one of two rare disease of lipoprotein metabolism, primary hypoalphalipoproteinemia or Tangier's disease; or, ApoA1 amyloidosis, depending on the mutation. Thirteen mutations are associated with ApoA1 amyloidosis, predominantly nucleotide substitutions. However, two are deletions, and one is a deletion/insertion mutation. Most of the deletions are in-frame, with the exception of Asn122fs and eAla202fs. Hence, the mechanism of amyloid production for all of the ApoA1 mutations involve aberrant folding, the unstable species produced with the Asn122fs and Ala202fs mutations is a truncated protein rather than a full length one.

The clinical presentation of amyloidosis consistent with ApoA1 involves the liver, kidney, larynx, skin and myocardium most commonly; rarely the testes and adrenal glands. The most common mutations to date include G50R, L99P, A197P, A199P, and L198S. Most of these mutations are present in Northern Europeans. Specifically, G50R is common among British, Scandinavians and North Americans, L99P in Italians, Germans, and North Americans, A197P in Americans and British, and L198S in Italian and Dutch individuals (Table 3).

Mutation	Clinical Features
Gly50Arg	Peripheral neuropathy, Nephropathy
Glu58Lys	Nephropathy
Leu84Arg	Nephropathy
Glu94_Trp96del	HTN, Nephropathy
Trp74Arg	Nephropathy
Del84-85insVal/Thr	Hepatic
Leu88Pro	Nephropathy
Del94-96	Nephropathy
Phe95Tyr	Palate
Asn98fs	Nephropathy, gastrointestinal
Leu99Pro	Hepatic
Leu114Pro	Cardiomyopathy, cutaneous
Lys131del	Aortic intima
Ala178fs	Nephropathy
Leu194Pro	Laryngeal
Arg197Pro	Cardiomyopathy, cutaneous, laryngeal
Leu198Ser	Cardiomyopathy
Ala199Pro	Laryngeal
Leu202His	Cardiomyopathy, laryngeal

Table 3. Common ApoA1 mutations (adapted from Benson, 2003; Eriksson, et al., 2009; Rowczenio, et al., In Progress).

#### 4.4 Apolipoprotein A2 (ApoA2)

Apolipoprotein A2 (ApoA2), similar to ApoA1, is an amyloidogenic protein involved with lipid metabolism. ApoA2, unlike ApoA1, can be found in senile amyloidosis. As is the case with TTR, gene sequencing is required to determine if ApoA2 deposition in a given case is due to deposition of a wild-type protein (senile amyloid) or a mutant one (familial amyloidosis). Structurally, it is a 77 amino acid, 17.4kDa protein located on chromosome 1p21-1qter. While comprised of four exons, three exons in ApoA2 are coding: exons 2, 3 and the 5' end of exon 4 (Alamut). The Apo A2 gene is one of the more recently described forms of hereditary amyloid, with a clinical picture of early adult-onset, rapidly progressive renal failure. The abrupt renal failure occurs in the absence of proteinuria and has no associated neuropathy. Mutations in the stop codon are the common genetic change resulting in a 21-amino acid extension at the carboxy terminus of the mature protein. All of these changes occur at codon 101 in exon 4 as follows: Stop101G, Stop101S, and Stop101R (Table 4). Geographically, these mutations are seen in North Americans, with the exception of Stop101R, which is also seen in Russians.

Protein	Mutation	Clinical Features
ApoA2	Stop78Gly	Nephropathy
	Stop78Ser	Nephropathy
	Stop78Arg	Nephropathy
Gelsolin A	Asp214Asn	PN, LCD
	Asp214Tyr	PN
Fibrinogen Alpha	Arg573Leu	Nephropathy
	Glu545Val	Nephropathy
	1629delG	Nephropathy
	1622delT	Nephropathy
Lysozyme	Ile74Thr	Nephropathy, petechiae
	Asp85His	Nephropathy
	Trp82Arg	Nephropathy
	Phe75Ile	Nephropathy

Table 4. Listing of common mutation for other amyloidogenic proteins. PN = Peripheral Neuropathy, LCD = lattice corneal dystrophy.

#### 4.5 Gelsolin A (GSN)

Gelsolin protein is associated with actin metabolism. Also known as brevin, or, actin-depolymerizing factor, it acts to prevent toxicity due to the release of actin into the extracellular space in the presence of cell necrosis. The gene is comprised of 17 exons and is located on chromosome 9q34 (centromeric to ABL); the protein weighs 82kDa. In the setting of familial/hereditary amyloidosis presents with unique features of neuropathy, particularly of the cranial nerves. Additionally, Gelsolin A has distinguishing clinical features that merit clinical, not genetic, subclassification of the disease. For example, some patients may have lattice corneal dystrophy, the “Meretoja” subtype, and cutis laxa of facial skin. Known pathogenic mutations include D214N in individuals from Finland, North America, Denmark and Japan, and D214Y (c. 654G>C), in individuals from Finland, Denmark, and the Czech Republic. The D214N and D214Y mutations permit exposure of an otherwise masked cleavage site, and is the initial step of amyloid formation. Both of these mutations result in the production of an aberrant, 68-kD fragment, likely a carboxy-terminal part of the protein which is suggested to be amyloidogenic.

The Meretoja subtype is associated with the D214N mutation, a single base mutation c. 654G>A (GAC>GAA), p. D214N (Asp214Asn). The pathogenic protein is comprised of 71 amino acids. Individuals that are heterozygous for this mutation may be asymptomatic early in life, with possibly only lattice corneal dystrophy (in their thirties); those who are homozygous may have significant manifestations of visceral involvement, specifically renal, including proteinuria and amyloid nephropathy with nephrotic syndrome by their twenties. Regardless of the genotype, the gelsolin variant of amyloid is classically associated with cranial neuropathy, possibly even bilateral, with additional phenotypic features rendering subclassification as described herein (Table 4).



#### 4.6 Fibrinogen alpha (FGA)

Synthesized in the liver, fibrinogen is a plasma glycoprotein with three structural subunits: alpha (FGA), beta (FGB), and gamma (FGG). Most research regarding fibrinogen has been in the context of hemostasis, where it has a primary functional role. Two rare diseases due to mutations in fibrinogen alpha confer bleeding disorders: afibrinogenemia and dysfibrinogenemia. Afibrinogenemia has an absence of fibrinogen due to a truncating mutation, and dysfibrinogenemia has decreased fibrin production due to a mutation at the cleavage site for thrombin to convert inactive fibrinogen to fibrin. The mutations seen with bleeding are different than those seen in FGA amyloid. Fibrinogen alpha is located on chromosome 4q28, with 6 exons, and varying amino acid lengths as determined by alternative splicing.

Phenotypically, FGA amyloidosis is associated with visceral involvement, specifically renal, with the manifestations including hypertension, proteinuria, and subsequent azotemia. Importantly, renal involvement in amyloid of this genetic origin is associated with rapidly progressive renal failure. Hence, detection of amyloid with a FGA mutation, like TTR, permits consideration of liver transplant for curative treatment, and, perhaps, could avoid the negative consequences of renal disease. Historically, renal transplantation, which has also been performed, has not had long-term success, thus, this paradigm-shift to hepatic transplantation, especially in light of the ability to detect the mutation, is a promising alternative for patients. Neuropathy has not been seen in this type of amyloidosis, and cardiomyopathy is reported in one case, thus far. Hence, neurologic and cardiac involvement would be the exception rather than the rule at this early phase of diagnosing FGA amyloid.

To date, there are four common mutations associated with FGA amyloidosis: two point mutations with pathogenic single amino acid substitutions, and two single nucleotide deletions yielding a frameshift in DNA transcription, with subsequent premature termination of protein synthesis. One point mutation, c. 4993 G>T, p. R573L has been identified in a Peruvian family, and another, c. 1674 A>T, p. E545V has been detected in individuals of American and Irish descent. Specifically, the E545V mutation is the one example of cardiac manifestations of FGA amyloid. One mutation, 1629delG, a deletion of the third base in codon 543, was detected in an American family with hereditary renal amyloidosis. Due to this mutation, a premature stop codon is created at codon 567. Individuals with the 1629delG mutation had a later onset of disease (later thirties and early forties) when compared to those with the R573L mutation. Finally, early renal disease with terminal renal failure has been documented in French kindred with a single nucleotide deletion c.1622T, with subsequent frameshift mutation at codon 541 and, similar to 1629delG, premature termination of protein synthesis at codon 567 (Table 4). This particular subtype, with its inherently aggressive sequelae, is particularly relevant to consideration of liver transplantation early in the course of disease.

#### 4.7 Lysozyme (LYZ)

Lysozyme is an enzyme that catalyzes the hydrolysis of certain mucopolysaccharides of bacterial cell walls. Specifically, it catalyzes the hydrolysis of the beta (1-4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine. Lysozyme is found in the spleen, lung, kidney, white blood cells, plasma, saliva, milk and tears. The gene is located on chromosome 12q15 and contains 4 exons. The 14.6kDa protein contains 130 amino acids.

In the same vein with regard to management, early detection of this amyloid variant alters the course of treatment in that individuals with this mutation experience a very early onset of renal disease, with rapid decline (this is similar, in some regards, to some variants of FGA). However, unlike FGA, benefit from renal transplantation has proven effective. Other manifestations include gastrointestinal involvement (peptic ulcer), cardiac disease, Sicca syndrome, and propensity towards petechiae, hemorrhage and hematoma, including hepatic hemorrhage. Uniquely, neuropathy is not a component of this type of amyloid, and in fact, might suggest a different variant, such as gelsolin, depending on the presentation.

The mutations documented thus far with lysozyme amyloid have their ancestral roots associated with the United Kingdom, France, America, and Italy (specifically, Piedmont, Italy). The D85H (Asp85His) mutation regionalizes to the United Kingdom, renal disease is the predominant symptom associated with this mutation. A tryptophan-to-arginine substitution at codon 82 (W82R) has been documented with a French family, with Sicca syndrome contributing to the phenotype in addition to renal manifestations. Two other mutations, Phe75Ile (F75I), and Trp82Arg (W82R), are described in an Italian-Canadian family, and an Italian family (Piedmont, Italy), respectively. The W82R variant had predominant gastrointestinal involvement; however, the same mutation in an English man presented with dramatic bleeding and rupture of abdominal lymph nodes (Table 4).

#### **4.8 LECT2: A new hereditary amyloidosis gene?**

The most recently described gene in systemic amyloidosis is LECT2. LECT2 is a leukocyte chemotactic factor whose synthetic origin is uncertain at this time (Benson 2010). Some studies indicate a hepatic origin as LECT2 is expressed in the adult and fetal liver, but follow-up immunohistochemical studies have detected LECT2 in many tissues of the body. LECT2 weighs 16.4kDa, is comprised of 133 residues (after cleavage of the 18 amino acid signal peptide), and is located on chromosome 5q31.1-q32.

Functionally, LECT2 can serve as a cartilage growth factor (chondromodulin II), as well as in neutrophil chemotaxis, as the name implies. With its role in neutrophilic chemotaxis, LECT2 has a presumable role in cell growth and repair after damage. Further, LECT2 has also been detected in hepatocellular carcinoma cell lines, suggesting a role in neoplasia, and also supporting its potential origin within hepatic tissue.

To date, LECT2 amyloidosis has been seen primarily in individuals of Mexican American ancestry. A study by Murphy et al. reported a series of 21,985 consecutive renal biopsies, of which 285 had positive Congo Red staining. Seven of ten cases with LECT2 renal amyloidosis were of Mexican descent. In some cases (typically reported in smaller studies), the amyloid was detected after longstanding, slowly progressive renal disease.

The case reported by Benson is a patient with a long history of slowly progressive renal failure, without a diagnosis of amyloidosis including its specific subtype until nephrectomy due to renal-cell carcinoma. Moreover, since its recent discovery, there is suggestion by Larsen et al. that the incidence of LECT2 amyloid might actually exceed TTR. While a polymorphism has been detected in all of the cases affected with LECT2 amyloid (Ile58Val), no pathogenic mutations are present to date. Thus, whether or not LECT2 will emerge under the category of systemic or hereditary amyloidosis is yet to be determined.

## 5. Conclusion

In summary, many laboratory techniques to detect and characterize the presence of amyloid are available. With these tools, the ability to detect the presence of amyloid has improved, as well as our ability to better understand the varying presentations and pathologic processes associated with the presence of amyloid.

While the understanding of amyloid continues to evolve, so does our ability to detect, diagnose, and treat the varying etiologies. Two techniques pivotal this progress are tissue mass spectrometry and gene sequencing. The refined finesse available utilizing mass spectrometry and gene sequencing continues to unravel the amyloid puzzle, and reveal more patients, with more unique phenotypic expression of disease. As our ability to identify and characterize systemic amyloidosis improves, and genotype-phenotype correlations become more clear, it will likely be possible in the future to explain seemingly unique manifestations of the disease, such as the cardiac specific presentation seen with the V142I TTR mutation.

The ultimate beneficiary of the utility of the refined laboratory diagnosis of amyloidosis is, of course, the patient. However, the information gathered due to test results is best handled in a multidisciplinary practice with well-established genetic counseling to educate the patient and family regarding the disease process, screening, and treatment considerations. At present, no direct pharmacologic therapy “cures” for the amyloid disease. However, understanding the origin of the proteins involved in the subtypes has achieved better control of this process in some types (TTR, FGA).

## 6. Acknowledgments

The authors would like to thank Steven R. Zeldenrust, MD, PhD Consultant, Division of Hematology, Mayo Clinic, Rochester, Minnesota for his critical review of the manuscript and clinical support; Jason Theis and Karen Schowalter for their assistance in the mass spectrometry and molecular genetics laboratories respectively, at Mayo Clinic, Rochester, Minnesota; Tad Holtegaard and Cindy McFarlin for their technical support, and Debbie Johnson for her administrative support; Mayo Clinic, Rochester, Minnesota.

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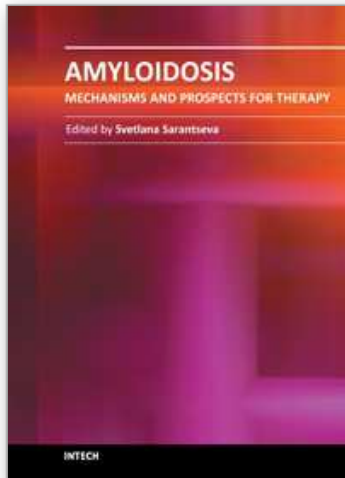
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Edited by Dr. Svetlana Sarantseva

ISBN 978-953-307-253-1

Hard cover, 216 pages

**Publisher** InTech

**Published online** 22, September, 2011

**Published in print edition** September, 2011

Amyloidoses are a heterogeneous group of diverse etiology diseases. They are characterized by an endogenous production of abnormal proteins called amyloid proteins, which are not hydrosoluble, form depots in various organs and tissue of animals and humans and cause dysfunctions. Despite many decades of research, the origin of the pathogenesis and the molecular determinants involved in amyloid diseases has remained elusive. At present, there is not an effective treatment to prevent protein misfolding in these amyloid diseases. The aim of this book is to present an overview of different aspects of amyloidoses from basic mechanisms and diagnosis to latest advancements in treatment.

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