

Amyloidogenicity and Clinical Phenotype Associated with Five Novel Mutations in Apolipoprotein A-I

Dorota Rowczenio,* Ahmet Dogan,[†]
Jason D. Theis,[†] Julie A. Vrana,[†]
Helen J. Lachmann,* Ashutosh D. Wechalekar,*
Janet A. Gilbertson,* Toby Hunt,*
Simon D.J. Gibbs,* Prayman T. Sattianayagam,*
Jenny H. Pinney,* Philip N. Hawkins,* and
Julian D. Gillmore*

From the National Amyloidosis Centre, Centre for Amyloidosis and Acute Phase Proteins, Division of Medicine, Royal Free Campus, University College London, London, England; and the Division of Anatomic Pathology,[†] Mayo Clinic, Rochester, Minnesota*

The phenotype of hereditary apolipoprotein A-I amyloidosis is heterogeneous with some patients developing extensive visceral amyloid deposits and end-stage renal failure as young adults and others having only laryngeal and/or skin amyloid, which may be of little clinical consequence. Clinical management and prognosis of patients with systemic amyloidosis depend entirely on correct identification of the fibril protein, such that light chain amyloidosis (AL, previously referred to as “primary”), the most frequently diagnosed type, is treated with chemotherapy, which has absolutely no role in hereditary apolipoprotein A-I amyloidosis. We report five novel apolipoprotein A-I variants, four of which were amyloidogenic and one of which was incidental in a patient with systemic AL amyloidosis. Interestingly, only one of four patients with apolipoprotein A-I amyloidosis had a family history of similar disease. Laser microdissection and tandem mass spectrometry-based proteomics were used to confirm the amyloid fibril protein and, for the first time in apolipoprotein A-I amyloidosis, demonstrated that only mutated protein as opposed to wild-type apolipoprotein A-I was deposited as amyloid. The clinical spectrum and outcome of hereditary apolipoprotein A-I amyloidosis are reviewed in detail and support the need for sequencing of the apolipoprotein A-I gene among patients with apparent localized amyloidosis in whom IHC is nondiagnostic of the fibril protein, even in the absence of a family history of disease. (*Am J Pathol* 2011, 179:1978–1987; DOI: 10.1016/j.ajpath.2011.06.024)

Amyloidosis is a rare disorder characterized by extracellular deposition of fibrillar protein that results in a progressive disruption of structure and function of affected tissues and organs. Amyloidosis is a remarkably heterogeneous disease; it can be acquired or hereditary and systemic or localized.

The most common form of systemic amyloidosis is AL (light chain, previously referred to as “primary”), in which the fibrils are derived from monoclonal immunoglobulin light chains and consist of the whole or part of the variable (V_L) domain. The prognosis of systemic AL amyloidosis is generally worse than in other amyloid types, although there is marked heterogeneity in the extent of organ involvement and rate of disease progression. Treatment of AL amyloidosis is with chemotherapy for which there is no role in other amyloid types.

Hereditary systemic amyloidosis is a rare autosomal dominant condition caused by deposition of variant proteins as amyloid fibrils. The term “hereditary nonneuropathic systemic amyloidosis” (Online Mendelian Inheritance of Man no. 105200) was coined by Ostertag¹ in 1932 following the discovery of two families with dominantly inherited renal amyloidosis. Mutations in the genes encoding apolipoprotein A-I (apoA1),^{2–12} apolipoprotein A-II (apoAII),¹³ fibrinogen A α -chain,^{14–17} and lysozyme¹⁸ have since been identified as the cause of hereditary renal amyloidosis in different kindreds (Online Mendelian Inheritance of Man no. 105200). The clinical amyloidosis syndromes that accompany the various mutations in these different genes are diverse with respect to age at onset, mode of presentation, pattern of organ distribution, rate of progression, and prognosis. Indeed, certain apoA1 variants are associated with neuropathy^{19,20} such that the nomenclature, which includes the term “nonneuropathic,” is confusing and probably no longer appropriate.

Supported by the UK Department of Health.

Accepted for publication June 15, 2011.

The licensed patent rights to perform protein extraction from paraffin-embedded tissue for the mass spectrometry-based amyloid typing test were granted by Expression Pathology, Inc.

Address reprint requests to Julian D. Gillmore, M.D., Ph.D., National Amyloidosis Centre, Centre for Amyloidosis and Acute Phase Proteins, Royal Free Campus, UCL Medical School, Rowland Hill St., London NW3 2PF, England. E-mail: j.gillmore@ucl.ac.uk.

Patients with hereditary apoAI amyloidosis typically present with hypertension, proteinuria, and renal impairment⁶ and frequently develop extensive visceral deposits that affect the liver, spleen, and kidneys, with occasional involvement of the heart, nerves, larynx, and gastrointestinal tract. Although the condition may lead to end-stage renal disease and other organ failure, the natural history of the disease is often slow, contrasting systemic AL amyloidosis in which the median patient survival without therapy is approximately 6 to 15 months from diagnosis.^{21,22}

Here we report five novel apoAI variants, four of which were associated with hereditary systemic amyloidosis and one of which was nonamyloidogenic, and an incidental finding discovered in a patient with systemic AL amyloidosis. The importance of correct identification of the amyloid fibril protein is highlighted, and the novel use of mass spectrometry to identify apoAI amyloidosis is reported. We review the UK National Amyloidosis Centre experience and published literature regarding the clinical spectrum and outcome of hereditary apoAI amyloidosis.

Materials and Methods

Five unrelated patients were referred to the UK National Amyloidosis Centre after discovery of amyloid deposits and underwent detailed investigations to elucidate the cause. Informed consent was obtained from all patients, and clinical care was in accordance with the Declaration of Helsinki.

Patients

A 35-year-old English man presented with hoarseness (patient 1). Vocal cord nodules were discovered at laryngoscopy and excised. A 51-year-old English woman presented with a soft tissue mass growing on her palate (patient 2). A 29-year-old Polish woman presented with edema (patient 3). A 77-year-old English woman presented with hypertension and was noted to have proteinuria and renal impairment (patient 4). A 56-year-old English man presented with proteinuria and progressive renal impairment (patient 5).

Histology and IHC

Biopsy specimens of vocal cord and testicles from patient 1, palatal mass from patient 2, rectum from patient 3, and kidney from patients 4 and 5 were stained at the UK National Amyloidosis Centre. Sections, 6- μ m thick, were stained for amyloid deposits with Congo red and viewed under crossed polarized light.²³ Immunohistochemistry (IHC) staining of the amyloid deposits was performed using monospecific antibodies reactive with serum amyloid A protein, lysozyme, apoAI, fibrinogen A α -chain, transthyretin, and κ and λ immunoglobulin light chains, as previously described.²⁴ Specificity of staining was confirmed by prior absorption of the antiserum with pure antigen in each case, and positive and negative controls were included in each run.

Direct DNA Sequencing

Genomic DNA from all five patients was extracted from whole blood treated with EDTA.²⁵ Exons 3 (c.5352 to c.5711) and 4 (c.6116 to 6859) of the *APOAI* gene (National Center for Biotechnology Information accession no. NG_012021) were amplified by PCR performed with Ready-To-Go tubes (Amersham Pharmacia Biotech, Piscataway, NJ). PCR amplification of exons 3 and 4 was performed with the following oligonucleotides: exon 3 forward 5'-GGCAGAG-GCAGCAGGTTTCTCAC-3' (c.5352–5374) and reverse 5'-CCAGACTGGCCGAGTCCTCACCTA-3' (c.5688–5711) and exon 4 forward 5'-CACTGCACCTCCGCG-GACA-3' (c.6116–6134) and reverse 5'-CTTCCCGGT-GCTCAGAATAAACGTT-3' (c.6835–6859). The total volume of PCR reaction was 25 μ L, and it consisted of the following: 2 μ L of DNA (at a concentration of 100 ng/ μ L), 1 μ L of each primer (at a concentration of 10 μ mol/L), and 21 μ L of water. PCR cycling conditions were as followed: initial denaturation at 96°C for 5 minutes; five cycles of 96°C for 30 seconds and 72°C for 30 seconds; 35 cycles at 96°C for 30 seconds, 72°C for 30 seconds, and 72°C for 1 minute; and final elongation step at 72°C for 7 minutes. Negative and positive controls were included.

The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Velno, the Netherlands) according to the manufacturer's protocol and sequenced with the ABI Big-Dye Terminator v 3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA). The primers 5'-GATCTCAGCCCACAGCTGGCC-3' (c.5417–5437) and 5'-AGGGCTCACCCCTGATAGGCTG-3' (c.6144–6166) were used for sequencing of exons 3 and 4, respectively. *APOAI* gene sequences were analyzed on the ABI 3130xl Genetic Analyzer, using Sequencing Analysis Software, version 5.4 (Applied Biosystems, Carlsbad, CA).

Laser Microdissection and Tandem Mass Spectrometry-Based Proteomics

The proteome of amyloid deposits was analyzed by combining microdissection and mass spectrometry-based proteomics. Sections (10- μ m thick) of formalin-fixed, paraffin-embedded tissues were placed on DIRECTOR slides (Expression Pathology, Rockville, MD). Sections were air dried and then melted, deparaffinized, and stained in hematoxylin followed by Congo red.

Congo red-stained sections were examined for the presence of amyloid deposits under fluorescence (B/G/R filter cube; Leica, Wetzler, Germany). Positive areas were microdissected into 0.5-mL microcentrifuge tube caps containing 10 mmol TRIS/1 mmol EDTA/0.002% Zwittergent 3–16 (Calbiochem, San Diego, CA) using a Leica DM6000B Microdissection System (Leica). For each case, two to four different microdissections were collected, and each microdissection contained 50,000 to 60,000 μ m² of the tissue section. Collected tissues were heated at 98°C for 90 minutes with occasional vortexing. After 60 minutes of sonication in a waterbath, samples were digested overnight at 37°C with 1 μ g of trypsin

(Promega, Madison, WI). The trypsin-generated digests were reduced with dithiothreitol and separated by nano-flow liquid chromatography–electrospray tandem mass spectrometry using a ThermoFinnigan LTQ Orbitrap Hybrid Mass Spectrometer (Thermo Electron, Bremen, Germany) coupled to an Eksigent nanoLC-2D HPLC system (Eksigent, Dublin, CA). A 0.25- μ L trap (Optimize Technologies, Oregon City, OR) packed with Michrom Magic C-8 was plumbed into a 10-port valve. A 75 μ m \times 15 cm C-18 column was used for the separation using an organic gradient from 6% to 86% in 55 minutes at 400 nL/min. The Thermo-Fisher Scientific (Waltham, MA) MS/MS raw data files were searched using three different algorithms (Mascot, Sequest, and X!Tandem) and the results assigned peptide and protein probability scores. The results were then combined and displayed using Scaffold (Proteome Software, Portland, OR). All searches were conducted with variable modifications and restricted to full trypsin-generated peptides, allowing for two missed cleavages. Peptide mass search tolerances were set to 10 ppm and fragment mass tolerance to 1.00 Da. The search algorithms used to identify peptides interrogate the human SwissProt database, which does not contain the mutated sequences identified in this study. To show that the peptides containing the mutation were part of amyloid deposits, these sequences were added to the human SwissProt database using an in-house script, and a second-round data analysis was performed. Peptide identifications below the 90% confidence level were not considered in our analysis.

Radiolabeled SAP Scintigraphy

All five patients underwent whole body anterior and posterior scintigraphic imaging 24 hours after administration of ^{123}I -labeled serum amyloid P component (SAP) using a GE Infinia Hawkeye (GE Healthcare, Chalfont St Giles, UK) gamma camera, as previously described.²⁶ The labeled SAP study results were interpreted by a panel of physicians with experience of more than 10,000 SAP scans.

Results

Clinical Findings

Patient 1 presented with a hoarse voice and no other significant history. He underwent direct laryngoscopy and excision of multiple nodules on the vocal cords and aryepiglottic folds, which were discovered to be amyloid on histologic analysis. On direct questioning there was no history of hemoptysis, dyspnea, rashes, petechiae, weight loss, anorexia, or edema. There was no history of paraesthesia or postural dizziness. The only medical history was of hypogonadism and azoospermia for which he had undergone a testicular biopsy 8 to 10 years previously. He did not take regular medication, and there was no family history of similar illness, cardiac disease, or renal disease. Clinical examination was unremarkable. Baseline investigations showed normal blood cell count, clotting profile, renal, and liver function. There was no

evidence of a plasma cell dyscrasia by sensitive nephelometric serum free light chain assay,²⁷ conventional electrophoresis and immunofixation of serum or urine, or bone marrow examination. There was no proteinuria, and ECG and echocardiography did not reveal evidence of cardiac amyloid infiltration.

Patient 2 presented with a 4-year history of a gradually enlarging mass on the palate within an area of scarring from a childhood injury. There had been no bleeding or pain associated with the lump, and the patient remained otherwise well. She experienced an occasional dry mouth and dry eyes and very rare tingling in her fingertips, with no other features to suggest a peripheral neuropathy or carpal tunnel syndrome. There was no history of weight loss or autonomic symptoms. There was no family history of amyloidosis, renal disease, or cardiac disease. Clinical examination was unremarkable. She had a medical history that included celiac disease, dermatitis herpetiformis, and multinodular goiter for which she had undergone a partial thyroidectomy at the age of 29 years. Shortly before her presentation with the lump in the palate, she had developed a recurrent clinically overt goiter. There was no evidence of a plasma cell dyscrasia by serum free light chain assay, conventional electrophoresis and immunofixation of serum or urine, or bone marrow examination. There was no proteinuria, and ECG, echocardiogram, and N-terminal pro-B-type natriuretic peptide were not suggestive of cardiac amyloid infiltration.

Patient 3 presented with edema and was discovered to have subnephrotic range proteinuria (1.68 g/24 h) and stage 3 chronic kidney disease (CKD). A kidney biopsy was performed in 2008 and revealed amyloidosis, thought on initial IHC in her local hospital to be AA type. The results of investigations to identify an underlying inflammatory condition were all negative. Treatment with colchicine was commenced empirically, and she was referred to the UK National Amyloidosis Centre. There was no history of chronic inflammatory disease in her or her family and no family history of kidney disease. There was no history to suggest peripheral or autonomic neuropathy and no dyspnea. Baseline investigations revealed normal blood cell count, clotting profile, and liver function. There was no evidence of a plasma cell dyscrasia. An echocardiogram demonstrated a 12-mm interventricular wall thickness but normal diastolic dysfunction and good left ventricular systolic function, but there was mild thickening of her heart valves; overall, there was no definite evidence of cardiac amyloidosis, but it could not be completely excluded. There was insufficient tissue remaining in her renal biopsy specimen to definitively confirm the amyloid type, and there was doubt about the diagnosis of AA amyloidosis; therefore, a rectal biopsy was requested.

Patient 4 presented with hypertension and was discovered to be nephrotic (7.9 g/24 h; serum albumin, 24 g/L) with CKD stage 3 on a background of type 2 diabetes mellitus and ischemic heart disease. A renal biopsy specimen revealed amyloid deposits in the glomeruli and interstitium. At presentation, she was experiencing paraesthesia in the feet and minimal dyspnea on exertion. There was no evidence of a plasma cell dyscrasia. Cardiac investigations did not suggest amyloid cardiomyop-

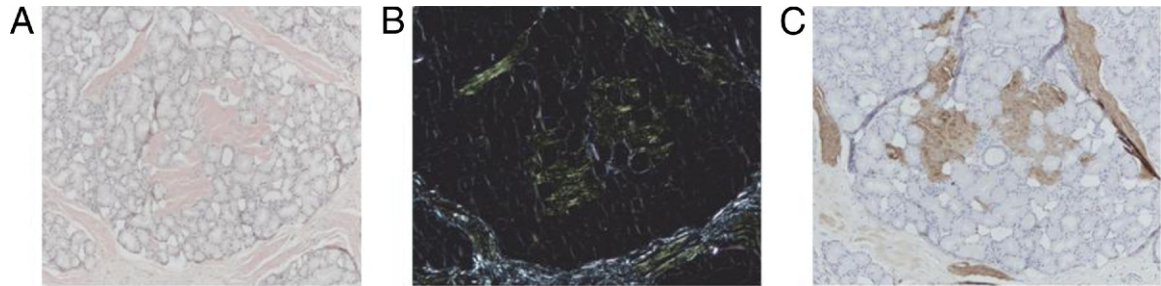


Figure 1. Palate biopsy specimen from a patient with apoAI amyloidosis. Staining with Congo red viewed under bright field light (**A**) showed amorphous eosinophilic deposits, which showed apple green birefringence when viewed under crossed polarized light (**B**) diagnostic of amyloid. The amyloid deposits stained with an antibody against apoAI (**C**), and staining was completely abolished by prior incubation of the antiserum with pure apoAI, thus confirming apoAI as the amyloid fibril protein.

athy. The patient's sister was subsequently discovered to have amyloid deposits on a renal biopsy specimen, undertaken for proteinuria.

Patient 5 presented with edema and was discovered to have proteinuria, advanced CKD, and mildly obstructive derangement of liver function tests. A renal biopsy specimen revealed amyloid deposits. There was mild breathlessness on exertion but no clinical evidence of peripheral or autonomic neuropathy. Nephelometric serum free light chain assay revealed a marked κ excess (κ , 393 mg/L; λ , 30 mg/L). There was no paraprotein by serum or urine immunoelectrophoresis, and bone marrow examination revealed less than 5% clonal plasma cells. Cardiac investigations did not

show any evidence of cardiac involvement by amyloid. The patient received 15 days of high-dose dexamethasone for presumed AL amyloidosis, which substantially suppressed the κ serum free light chain concentration. Despite this, however, there was a progressive decline in renal function and the patient commenced hemodialysis.

Histology and IHC

Extensive amyloid deposits were identified in the vocal cord and testes of patient 1, the palate of patient 2, the rectum of patient 3, and the kidneys of patients 4 and 5 by their pathognomonic green birefringence when

Figure 2. Partial DNA sequences of exons 3 and 4 of the *APOAI* gene. **Right column** shows five novel mutations indicated by **arrows**; four single nucleotide substitutions: A175P (**A**), F71Y (**B**), E34K (**C**), S36A (**D**), and a frameshift mutation H155MfsX46 (**E**). **Left column** shows the corresponding wild-type sequences.

A

#	Identified proteins (189)	UNIPROT Identifier	Molecular Weight	Control	Patient 1		Patient 2		Patient 3		Patient 4	
					S1	S2	S1	S2	S1	S2	S1	S2
1	Apolipoprotein A-1 O5=Homo sapi...	APOA1_HU...	31 kDa		103	96	67	80	32	37	18	7
2	Vitronectin O5=Homo sapiens GN...	VTNC_HUM...	54 kDa		67	56	59	83	31	28	27	15
3	Serum albumin O5=Homo sapiens...	ALBU_HUM...	69 kDa		23	19	56	85	28	65		
4	Apolipoprotein E O5=Homo sapie...	APOE_HUM...	36 kDa		17	15	50	61	19	10	40	23
5	Hemoglobin subunit beta O5=Ho...	HBB_HUMAN	16 kDa						73	53		
6	Serum amyloid P-component O5=...	SAMP_HUM...	25 kDa		27	33	21	32	35	24	7	
7	Hemoglobin subunit alpha O5=Ho...	HBA_HUMAN	15 kDa						46	35		
8	Clusterin O5=Homo sapiens GN=C...	CLUS_HUM...	52 kDa		12	11	16	29	25	20		
9	Actin, alpha cardiac muscle 1 O5=...	ACTC_HUM...	42 kDa				5		17	33	6	
10	Collagen alpha-2(I) chain O5=Ho...	COL1A2_HU...	129 kDa		4		20	18	10	12	6	7
11	Apolipoprotein A-IV O5=Homo sa...	APOA4_HU...	45 kDa		17	19	23	29				
12	Collagen alpha-1(I) chain O5=Ho...	COL1A1_HU...	139 kDa		3		20	18	7	7	3	4
13	Lactotransferrin O5=Homo sapie...	TRFL_HUMAN	78 kDa					15		46		
14	Collagen alpha-3(VI) chain O5=Ho...	COL6A3_HU...	344 kDa							29		
15	Myosin-11 O5=Homo sapiens GN...	MYH11_HU...	227 kDa							21		

B

1	Apolipoprotein A-1 O5=Homo sapi...	APOA1_HU...	31 kDa		103	96	67	80	32	37	18	7
2	Apolipoprotein A-1 F95Y	ApoA1_F95...	2 kDa				15	14				
3	Apolipoprotein A-1 E58K	ApoA1_E58...	1 kDa						5	3		

Figure 3. Results of mass spectrometry–based proteomic analysis of amyloid deposits (A). The 15 most abundant proteins identified among four patients are listed. At least two different samples (S1 and S2) were run for each patient. The columns show the protein name, the UniProt identifier (protein accession code in the UniProt database, <http://www.uniprot.org>), the molecular weight of the protein, the results of the blank control sample, and two samples from each of the four patients. The numbers indicate number of total peptide spectra identified for each protein. ApoAI is the most abundant protein in this sample set, confirming apoAI as the amyloid fibril protein. Identification of mutated protein in amyloid deposits by mass spectrometry–based proteomics (B). After addition of the mutated sequences to the human SwissProt database using an in-house script and a second-round data analysis, the presence of mutated protein (a tryptic peptide carrying the altered amino acid sequence) within the amyloid deposits of patients 2 and 3 was demonstrated. The nomenclature for the location of the mutated amino acid includes the 24 amino acid signal peptide; thus, F95Y and E58K in the figure correspond to the F71Y and E34K protein variants, respectively.

stained with Congo red and viewed under crossed polarized light (Figure 1A–B). The amyloid from patients 1 through 4 stained specifically with antibodies to apoAI (Figure 1C), and staining was completely abolished by prior absorption of the antiserum with an excess of pure human apoAI. There was no other staining with antibodies to κ light chains and no staining with antibodies against other known amyloid fibril proteins, including lysozyme, transthyretin, serum amyloid A protein, or λ light chains. The amyloid from patient 5 stained with antibodies against both κ light chains and apoAI such that IHC results were inconclusive.

APOAI Gene Mutations

Genetic analysis of exons 3 and 4 of the *APOAI* gene revealed five novel mutations (Figure 2). Four were single nucleotide substitutions, c.595G>C, c.284T>A, c.172G>A, and c.178T>G, resulting in amino acid change: alanine to

proline at position 175, A175P in patient 1; phenylalanine to tyrosine at position 71, F71Y in patient 2; glutamic acid to lysine at position 34, E34K in patient 3; and serine to alanine at position 36, S36A in patient 5, respectively. The other novel variant p. His155-MetfsX46, discovered in patient 4, resulted from a frameshift mutation caused by a deletion of cytosine at position c.535. The new reading frame created by this single nucleotide deletion results in premature termination, and consequently the mutated protein was 44 nucleotides shorter than wild-type *apoAI*. The remainder of the *APOAI* gene in all five patients was identical to the published sequence.²⁸

Amyloid Typing by Laser Microdissection and Tandem Mass Spectrometry–Based Proteomics

Laser microdissection and tandem mass spectrometry showed that, in patients 1 through 4, apoAI was one of

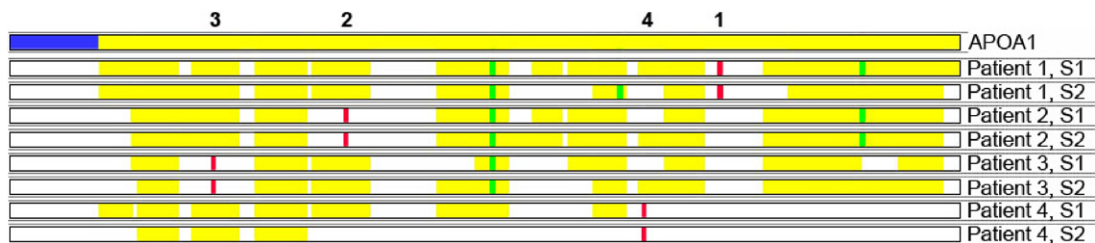


Figure 4. ApoAI protein coverage in apoAI amyloidosis. The top line represents the native apoAI protein (signal peptide in blue and secreted protein in yellow). The numbers above the top line indicate the individual patient's number throughout this article. Two samples (S1 and S2) from four patients are shown. The location of the amino acid residues affected by the mutations in each patient are represented by red bars and oxidized residues are represented by green bars. Most of the apoAI protein is deposited in patients 1, 2, and 3, but the tryptic peptides carrying the variant amino acid sequence are notably absent. In patient 4, where the mutation identified is predicted to cause a frameshift, only the N-terminal part of the protein before the mutation is present. The absence of the peptide carrying the mutated amino residue in each of these four patients indicates that the amyloid fibrils were composed solely of mutated protein and not wild-type apoAI. As discussed in the text, mutated peptides are not detected by the algorithms that only interrogate the data for wild-type sequences. If wild-type apoAI encoded by the normal allele had been incorporated into the amyloid deposits, these peptides would have been detected.

the most abundant proteins present in the amyloid deposits (Figure 3A). In contrast, there were no apoAI peptides present in the amyloid deposits of patient 5, which were composed of κ light chains. In addition to apoAI, other identified proteins included serum proteins, such as vitronectin and albumin, along with proteins that are known to be associated with all types of amyloidosis, such as apolipoprotein E and serum amyloid P component.

Identification of Mutated Protein in Amyloid Deposits by Tandem Mass Spectrometry–Based Proteomics

To show the presence of mutated proteins in the amyloid deposits, we searched the mass spectrometry raw data files using the human SwissProt database supplemented with the *APOAI* gene variants identified in this study. The mutated protein (a tryptic peptide carrying the altered amino acid sequence) was present in the amyloid deposits of patients 2 and 3 (Figure 3B). Detailed examination of the apoAI protein coverage showed that certain tryptic peptides, which would have been present had protein encoded by the normal allele been incorporated into amyloid deposits, were absent, indicating that only the protein encoded by the abnormal allele was amyloidogenic (Figure 4). Interestingly, the mutation in patient 1 affected an area of the protein that cannot be detected by the mass spectrometry–based proteomics technology used in the study due to the presence of numerous trypsin cutting sites in this area (Figure 4). In patient 4, the frameshift mutation in *APOAI* generates a novel C terminus amino acid sequence, which, theoretically, could be detected by mass spectrometry–based proteomics. Although this novel sequence was included in our supplemented database, we did not identify peptides representing the novel sequence, suggesting that this part of the protein was unstable and was most likely cleaved, generating a truncated apoAI protein that was deposited as amyloid.

SAP Scintigraphy

SAP scintigraphy did not show any visceral amyloid deposits in patient 1 and showed only renal amyloid deposits in patients 4 and 5. Subclinical hepatic amyloid deposits were detected in patient 2. A large amyloid load affecting the spleen and liver with an obscured kidney signal was identified in patient 3. It should be noted that amyloid deposits in the skin, vocal cord, heart, nerves, and testes do not show up by this technique.

Discussion

Determining the fibril protein remains a challenge in many patients with amyloidosis. Indeed, AL amyloidosis, the most commonly diagnosed systemic form of amyloid, is not infrequently a diagnosis of exclusion, which is complicated by the fact that there is considerable overlap between the clinical features of the different amyloid types. Furthermore, it is important to recognize that the

presence of a plasma cell dyscrasia, which occurs in approximately 3% of the population older than 50 years and approximately 5% of those older than 70 years,²⁹ in a patient with amyloidosis does not prove AL type and may be incidental to their amyloidosis.³⁰ The deposits in approximately 20% of patients with AL amyloidosis fail to stain with antibodies against κ and λ immunoglobulin light chains, presumably reflecting the fact that the fibrils consist of misfolded, variable light chain domains within which certain epitopes may be masked.^{31–33} Similarly, to confirm the amyloidogenicity of a novel mutation, even within a known amyloid fibril protein gene, one is required to demonstrate the protein composition of the amyloid fibrils. Until recently, this could only be achieved by IHC or direct protein sequencing. The latter is technically challenging and requires a substantial quantity of fresh tissue, which often cannot be obtained during life. Classification of amyloidosis by mass spectrometry–based proteomic analysis therefore represents a significant advance in amyloid diagnostics, enabling the fibril protein to be reliably identified from small quantities of formalin-fixed tissue.³³

The demonstration, by IHC and/or mass spectrometry, that the amyloid fibrils in four patients were composed of apoAI in conjunction with a typical phenotype of laryngeal involvement in patient 1, liver amyloidosis in patient 2, liver and renal manifestations in patient 3, and renal amyloidosis in patient 4 indicates that these novel *APOAI* mutations were the cause of their amyloidosis. The IHC was supported by mass spectrometry–based proteomics, which confirmed, for the first time in apoAI amyloidosis, that the amyloid fibrils were composed uniquely of variant protein. Interestingly, only one of these four patients had a family history of amyloidosis. The absence of a family history is apparently unusual in apoAI amyloidosis and might suggest a *de novo* mutation; however, variable penetrance is well recognized in other types of hereditary systemic amyloidosis.³⁴ We have not been able to obtain DNA from the parents of the three relevant patients to investigate this in more detail. Patient 5 presented a diagnostic challenge. In the presence of a κ light chain–secreting clonal dyscrasia and a novel *APOA1* mutation, the IHC was nondiagnostic of the amyloid fibril protein. Only laser microdissection of amyloid fibrils from fixed tissue and mass spectrometry enabled definitive confirmation of monoclonal κ light chain–derived (AL) rather than apoAI amyloidosis in this patient.³³ The mass spectrometry findings were later corroborated by SAP scintigraphic evidence of amyloid regression in response to successful suppression of κ light chain production with steroids, thereby excluding apoAI amyloidosis.

ApoAI (Mendelian Inheritance of Man no. 107680) is synthesized in the liver and small intestine and is encoded by a gene located on chromosome 11q23–q24. It is the major protein component of high-density lipoprotein in plasma, and it promotes efflux of cholesterol from cells. The *APOAI* gene sequence is composed of 8870 nucleotides (National Center for Biotechnology Information accession no. NG_012021) encoding a 267 amino acid primary transcript containing an 18-amino acid prepeptide and 6-amino acid propeptide connected to the

Table 1. Summary of *APOA1* Mutations Associated with Amyloidosis, Clinical Phenotype, and Outcome

<i>APOA1</i> variant	Ethnicity	Clinical presentation	Age at presentation, years/family history
Gly26Arg ^{2,19,36,37}	British Scandinavian	HTN, renal impairment, macroscopic hematuria	20–46/Y
Glu34Lys (current study)	Polish	Renal impairment, subnephrotic proteinuria	29/Unknown
Trp50Arg ⁴	Jewish (Ashkenazi)	Macroscopic hematuria, proteinuria, renal impairment	34/Y
Leu60Arg ³	British	HTN, renal impairment, macroscopic hematuria	24/Y
Leu64Pro ¹¹	Italian	Proteinuria, renal impairment	56–58/Unknown
Leu60_Phe71delins 60Val_61Thr ^{5,38}	Spanish	Hepatomegaly and abnormal LFT results	35–45/Y
Glu70_Trp72del ³⁹	British	HTN, renal impairment	18–21/Y
Asn74Lysfs ⁴⁰	German	Renal impairment, tumor of the right ovary	48/Unknown
Phe71Tyr (current study) Leu75Pro ^{12,40–42}	British Italian/German/Other	Palatal mass Hepatomegaly, renal impairment	51/Unknown 42–70/Y
Leu90Pro ⁸	French/American	Skin lesions, cardiomyopathy (later), dysphonia	37–51/Y
Lys107del ⁴³	Scandinavian	Angina	45/Unknown
Ala154fsX47 ⁴⁰	German	Renal impairment	58/Unknown
Leu170Pro ⁴⁰	German	Laryngeal amyloid deposits	52/Unknown
His155MetfsX46 (current study)	British	HTN, nephrotic syndrome, renal impairment	77/Unknown
Arg173Pro ⁷	American/British	Skin lesions, dysphonia, cardiomyopathy (later)	20–45/Y
Leu174Ser ^{9,20}	Italian/Dutch	Infertility, rash, cardiomyopathy, dysphonia	42–45/Y
Ala175Pro (current study)	British	Dysphonia	38/Unknown
Leu178His ^{10,20}	French	Dysphonia, cardiomyopathy	34/Y

(table continues)

A, adrenal glands; CABG, coronary artery bypass graft; CAD, coronary artery disease; ESRD, end-stage renal disease; GI, gastrointestinal; HTN, hypertension; L, liver; LFT, liver function test; OLT, orthotopic liver transplantation; PM, post-mortem; S, spleen; TIN, tubulointerstitial nephritis.

amino terminus of the 243 amino acid mature apoA1.³⁵ Although the precise mechanism for the amyloidogenicity of variant apoA1 remains unclear, it has been shown that mutated apoA1 undergoes proteolytic cleavage releasing the amyloidogenic N-terminal fragment from the full-length protein.^{5,7,9}

There are now 19 mutations in the *APOA1* gene known to be associated with hereditary systemic apoA1 amyloidosis, including the four novel amyloidogenic variants described here (Table 1). The phenotype of apoA1 amyloidosis may be

fairly aggressive and present in teenagers with CKD, hepatomegaly, cardiomyopathy, and sometimes neuropathy^{19,44} or may be extremely indolent and clinically insignificant. Interestingly, most C-terminal variants seem to be associated with hoarseness due to laryngeal amyloid deposits (Table 1),^{7,9,10,20,40} although there is even substantial heterogeneity in the age at onset and clinical manifestations between patients with identical mutations. G26R, which is the most frequently reported amyloidogenic apoA1 variant among Northern Europeans, is associated with amyloid de-

Table 1. *Continued*

Organ involvement by amyloid	SAP scintigraphic findings	Organs not involved to date	Clinical outcome
Kidneys, liver, peripheral nerves, GI tract (peptic ulcers)	Large load: liver, spleen, kidneys	Heart	Slowly progressive CKD, dialysis 11–20 years after presentation. Excellent outcome with transplantation of kidney with or without liver. Death at 47 years of age with renal failure reported. Death after 1–18 years reported
Kidneys, liver	Large load: spleen and liver, small kidney	Heart, nerves	Slowly progressive renal failure
Kidneys, liver, GI tract	Large load: liver, spleen, kidneys	Heart, nerves	Progressive renal failure, no reports of successful renal transplantation. Death at the age of 45 years (after 10 years), massive liver and renal amyloidosis
Kidneys, liver, testes, heart	Large or small load: liver, spleen, kidneys	Nerves	Good with single organ (kidney, heart) transplantation. Regression after liver transplantation. Death between 35 and 65 years of age from “amyloidosis”
Kidneys, liver	Large load: liver, spleen, kidneys	Heart, nerves	Progressive renal failure, death at 69 years of age with renal failure, good outcome with renal transplantation
Liver	Large load: liver, spleen	Heart, kidneys, nerves	Progressively abnormal LFT results (usually slow), liver failure, no reports of successful OLT. Death at 48–66 years of age, after 1–20 years
Kidneys, liver, choroid	Large load: liver, spleen, kidneys	Heart, nerves	ESRD 5 years after presentation. Death at 32 years of age and middle-age reported. Excellent outcome with renal transplantation. (No clinical sequelae of liver and spleen involvement.)
Kidneys, uterus, ovaries, pelvic lymph nodes, GI tract	Never performed	Not listed	Not mentioned
Liver, palate	Moderate load: liver	Heart, nerves, kidneys	Good outcome up to 52 years of age
Kidneys, liver, testes	Never performed	Heart, nerves, spleen	Gradually progressive liver amyloidosis, CKD, and TIN with death at 67–90 years of age, good outcome
Skin, heart, larynx	Never performed L, S, A at autopsy	Nerves, kidneys	Progressive cardiac failure and death at 41–70 years of age, no reports of successful heart transplantation
Aortic intimal amyloid; not known (PM study)	Never performed	Nerves, kidneys	Premature CAD, required CABG, and developed abdominal aortic aneurysm. Died at 68 years of age
Kidneys	Never performed	Not listed	Not mentioned
Larynx	Never performed	Not listed	Not mentioned
Kidneys	Small load: kidneys	Heart, liver	CKD, short follow-up
Kidneys, skin, heart, larynx	Small load with equivocal kidneys	Nerves, spleen	Progression of cardiomyopathy and renal failure, death at 52–63 years of age, good outcome with cardiac and renal transplantation
Skin, testes, heart, larynx, nerves	Small load: no visceral amyloid deposits	Nerves, liver, spleen	Progressive cardiomyopathy, death at 50–52 years of age, good outcome after cardiac transplantation
Larynx, testes	Small load: no visceral amyloid deposits	Heart, nerves, liver, spleen, kidneys	Good outcome up to 43 years of age
Larynx, skin, heart, nerves	Never performed	Liver, spleen	Progressive cardiomyopathy, death at 39 years of age

position in the peripheral nerves, as well as the kidneys, liver, and gastrointestinal tract.^{36,45} Patient 4 in this series presented with symptoms of possible neuropathy and is awaiting confirmatory tests. Two other apoA1 variants, L174S and L178H, are reportedly associated with varying degrees of neuropathy.²⁰ Here we report the sixth apoA1 variant to be associated with laryngeal amyloidosis, Ala175Pro. The nucleotide change in each of the previous kindreds occurred close to the carboxyl terminus (Leu170Pro,⁴⁰ Arg173Pro,⁷ Leu174Ser,^{9,20} Leu178His¹⁰)

apart from that in an Italian family with laryngeal, cardiac, and skin amyloidosis who carried the Leu90Pro apoA1 variant.⁸ His155MetfsX46 is the third reported amyloidogenic frameshift mutation in the *APOA1* gene. The other two, Asn74fs and Ala154fs, were recently described by Rocken and coworkers.⁴⁰ Interestingly, all three patients with frameshift mutations presented with renal manifestations.

In conclusion, we report here five novel apoA1 variants, four of which cause hereditary systemic amyloidosis. Mass spectrometry–based proteomics identified apoA1

as the main constituent of amyloid deposits in all four patients with pathogenic *APOAI* mutations and was successful in identifying the amyloid fibril protein as monoclonal light chain (AL) rather than apoAI in one patient who had both a clonal dyscrasia and a novel *APOAI* mutation whose IHC was nondiagnostic of the fibril protein. Minimal formalin-fixed amyloidotic tissue is required for the procedure, making this an invaluable new diagnostic tool for amyloid typing. The substantial phenotypic heterogeneity among patients with identical apoAI variants implies that other genetic and environmental factors influence clinical manifestations and support the need for *APOAI* gene sequencing in patients with apparent localized amyloidosis, particularly involving the larynx or skin.

Acknowledgments

We acknowledge all of the physicians and surgeons who were involved in the clinical care of the patients reported in this study.

References

- Ostertag B: Demonstration einer eigenartigen familiären paraamyloidose. *Zentralbl Aug Pathol* 1932, 56:253–254
- Jones LA, Harding JA, Cohen AS, Skinner M: New USA family has apolipoprotein AI (Arg26) variant. Edited by Natvig JB, Førre Ø, Husby G, Husebekk A, Skogen B, Sletten K, Westermark P. Dordrecht, Kluwer Academic Publishers, 1991, pp. 385–388
- Soutar AK, Hawkins PN, Vigushin DM, Tennent GA, Booth SE, Hutton T, Nguyen O, Totty NF, Feest TG, Hsuan JJ, Pepys MB: Apolipoprotein AI mutation Arg-60 causes autosomal dominant amyloidosis. *Proc Natl Acad Sci U S A* 1992, 89:7389–7393
- Booth DR, Tan SY, Booth SE, Hsuan JJ, Totty NF, Nguyen O, Hutton T, Vigushin DM, Tennent GA, Hutchinson WL, Thomson N, Soutar AK, Hawkins PN, Pepys MB: A new apolipoprotein AI variant. Trp50Arg, causes hereditary amyloidosis. *Q J Med* 1995, 88:695–702
- Booth DR, Tan SY, Booth SE, Tennent GA, Hutchinson WL, Hsuan JJ, Totty NF, Nguyen O, Soutar AK, Hawkins PN, Bruguera M, Caballería J, Solé M, Campistol JM, Pepys MB: Hereditary hepatic and systemic amyloidosis caused by a new deletion/insertion mutation in the apolipoprotein AI gene. *J Clin Invest* 1996, 97:2714–2721
- Persey MR, Booth DR, Booth SE, van Zyl-Smit R, Adams BK, Fattar AB, Tennent GA, Hawkins PN, Pepys MB: Hereditary nephropathic systemic amyloidosis caused by a novel variant apolipoprotein A-I. *Kidney Int* 1998, 53:276–281
- Hamidi Asl K, Liepnieks JJ, Nakamura M, Parker F, Benson MD: A novel apolipoprotein A-1 variant. Arg173Pro, associated with cardiac and cutaneous amyloidosis. *Biochem Biophys Res Commun* 1999, 257:584–588
- Hamidi Asl K, Liepnieks JJ, Hamidi Asl K, Uemichi T, Moulin G, Desjoyaux E, Loire R, Delpech M, Grateau G, Benson MD: Hereditary amyloid cardiomyopathy caused by a variant apolipoprotein A1. *Am J Pathol* 1999, 154:221–227
- Obici L, Bellotti V, Mangione P, Stoppini M, Arbustini E, Verga L, Zorzoli I, Anesi E, Zanotti G, Campana C, Viganò M, Merlini G: The new apolipoprotein A-I variant Leu¹⁷⁴ → Ser causes hereditary cardiac amyloidosis, and the amyloid fibrils are constituted by the 93-residue N-terminal polypeptide. *Am J Pathol* 1999, 155:695–702
- de Sousa MM, Vital C, Ostler D, Fernandes R, Pouget-Abadie J, Carles D, Saraiva MJ: Apolipoprotein AI and transthyretin as components of amyloid fibrils in a kindred with apoAI Leu178His amyloidosis. *Am J Pathol* 2000, 156:1911–1917
- Murphy CL, Wang S, Weaver K, Gertz MA, Weiss DT, Solomon A: Renal apolipoprotein A-I amyloidosis associated with a novel mutant Leu64Pro. *Am J Kidney Dis* 2004, 44:1103–1109
- Obici L, Palladini G, Giorgetti S, Bellotti V, Gregorini G, Arbustini E, Verga L, Marciano S, Donadei S, Perfetti V, Calabresi L, Bergonzi C, Scolari F, Merlini G: Liver biopsy discloses a new apolipoprotein A-I hereditary amyloidosis in several unrelated Italian families. *Gastroenterology* 2004, 126:1416–1422
- Benson MD, Liepnieks JJ, Yazaki M, Yamashita T, Hamidi Asl K, Guenther B, Kluve-Beckerman B: A new human hereditary amyloidosis: the result of a stop-codon mutation in the apolipoprotein AI gene. *Genomics* 2001, 72:272–277
- Benson MD, Liepnieks J, Uemichi T, Wheeler G, Correa R: Hereditary renal amyloidosis associated with a mutant fibrinogen α -chain. *Nat Genet* 1993, 3:252–255
- Uemichi T, Liepnieks JJ, Benson MD: Hereditary renal amyloidosis with a novel variant fibrinogen. *J Clin Invest* 1994, 93:731–736
- Uemichi T, Liepnieks JJ, Yamada T, Gertz MA, Bang N, Benson MD: A frame shift mutation in the fibrinogen A α -chain gene in a kindred with renal amyloidosis. *Blood* 1996, 87:4197–4203
- Hamidi Asl L, Liepnieks JJ, Uemichi T, Rebibou JM, Justrabo E, Droz D, Mousson C, Chalopin JM, Benson MD, Delpech M, Grateau G: Renal amyloidosis with a frame shift mutation in fibrinogen α -chain gene producing a novel amyloid protein. *Blood* 1997, 90:4799–4805
- Pepys MB, Hawkins PN, Booth DR, Vigushin DM, Tennent GA, Soutar AK, Totty N, Nguyen O, Blake CCF, Terry CJ, Feest TG, Zalin AM, Hsuan JJ: Human lysozyme gene mutations cause hereditary systemic amyloidosis. *Nature* 1993, 362:553–557
- Nichols WC, Gregg RE, Brewer HBJ, Benson MD: A mutation in apolipoprotein A-I in the lowa type of familial amyloidotic polyneuropathy. *Genomics* 1990, 8:318–323
- Hazenber AJ, Dikkers FG, Hawkins PN, Bijzet J, Rowczenio D, Gilbertson J, Posthumus MD, Leijmsma MK, Hazenber BP: Laryngeal presentation of systemic apolipoprotein A-I-derived amyloidosis. *Laryngoscope* 2009, 119:608–615
- Kyle RA, Gertz MA: Primary systemic amyloidosis: clinical and laboratory features in 474 cases. *Semin Hematol* 1995, 32:45–59
- Kyle RA, Gertz MA, Greipp PR, Witzig TE, Lust JA, Lacy MQ: A trial of three regimens for primary amyloidosis: colchicine alone, melphalan and prednisone, and melphalan, prednisone, and colchicine. *N Engl J Med* 1997, 336:1202–1207
- Puchtler H, Sweat F, Levine M: On the binding of Congo red by amyloid. *J Histochem Cytochem* 1962, 10:355–364
- Tennent GA, Cafferty KD, Pepys MB, Hawkins PN: Congo red overlay immunohistochemistry aids classification of amyloid deposits. Edited by Kyle RA, Gertz MA. Pearl River, New York, Parthenon Publishing, 1999, pp. 160–162
- Talmud P, Tybjaerg-Hansen A, Bhatnagar D, Mbewu A, Miller JP, Durrington P, Humphries S: Rapid screening for specific mutations in patients with a clinical diagnosis of familial hypercholesterolaemia. *Atherosclerosis* 1991, 89:137–141
- Hawkins PN, Lavender JP, Pepys MB: Evaluation of systemic amyloidosis by scintigraphy with ¹²³I-labeled serum amyloid P component. *N Engl J Med* 1990, 323:508–513
- Bradwell AR, Carr-Smith HD, Mead GP, Tang LX, Showell PJ, Drayson MT, Drew R: Highly sensitive, automated immunoassay for immunoglobulin free light chains in serum and urine. *Clin Chem* 2001, 47:673–680
- Shoulders CC, Kornbliht AR, Munro BS, Baralle FE: Gene structure of human apolipoprotein A1. *Nucleic Acids Res* 1983, 11:2827–2837
- Kyle RA, Therneau TM, Rajkumar SV, Larson DR, Plevak MF, Offord JR, Dispenzieri A, Katzmann JA, Melton LJ III: Prevalence of monoclonal gammopathy of undetermined significance. *N Engl J Med* 2006, 354:1362–1369
- Lachmann HJ, Booth DR, Bybee A, Gilbertson JA, Gillmore JD, Pepys MB, Hawkins PN: Misdiagnosis of hereditary amyloidosis as AL (primary) amyloidosis. *N Engl J Med* 2002, 346:1786–1791
- Novak L, Cook WJ, Herrera GA, Sanders PW: AL-amyloidosis is underdiagnosed in renal biopsies. *Nephrol Dial Transplant* 2004, 19:3050–3053
- Solomon A, Murphy CL, Westermark P: Unreliability of immunohistochemistry for typing amyloid deposits. *Arch Pathol Lab Med* 2008, 132:14–15
- Vrana JA, Gamez JD, Madden BJ, Theis JD, Bergen HR III, Dogan A: Classification of amyloidosis by laser microdissection and mass spectrometry-based proteomic analysis in clinical biopsy specimens. *Blood* 2009, 114:4957–4959
- Gillmore JD, Lachmann HJ, Rowczenio D, Gilbertson JA, Zeng CH, Liu ZH, Li LS, Wechalekar A, Hawkins PN: Diagnosis, pathogenesis, treatment, and prognosis of hereditary fibrinogen A alpha-chain amyloidosis. *J Am Soc Nephrol* 2009, 20:444–451

35. Brewer HB, Jr., Fairwell T, LaRue A, Ronan R, Houser A, Bronzert TJ: The amino acid sequence of human APOA-I, an apolipoprotein isolated from high density lipoproteins. *Biochem Biophys Res Commun* 1978, 80:623–630
36. Nichols WC, Dwulet FE, Liepnieks J, Benson MD: Variant apolipoprotein AI as a major constituent of a human hereditary amyloid. *Biochem Biophys Res Commun* 1988, 156:762–768
37. Vigushin DM, Gough J, Allan D, Alguacil A, Penner B, Pettigrew NM, Quinonez G, Bernstein K, Booth SE, Booth DR, Soutar AK, Hawkins PN, Pepys MB: Familial nephropathic systemic amyloidosis caused by apolipoprotein AI variant Arg26. *Q J Med* 1994, 87:149–154
38. Caballeria J, Bruguera M, Sole M, Campistol JM, Rodes J: Hepatic familial amyloidosis caused by a new mutation in the apolipoprotein AI gene: clinical and pathological features. *Am J Gastroenterol* 2001, 96:1872–1876
39. Persey MR, Booth DR, Booth SE, van Zyl-Smit R, Hawkins PN, Pepys MB: A new deletion mutation of the apolipoprotein AI gene causing hereditary amyloidosis. *Clin Sci* 1996, 90(Suppl 34):33P
40. Eriksson M, Schonland S, Yumlu S, Hegenbart U, von Hutten H, Gioeva Z, Lohse P, Buttner J, Schmidt H, Rocken C: Hereditary apolipoprotein AI-associated amyloidosis in surgical pathology specimens: identification of three novel mutations in the APOA1 gene. *J Mol Diagn* 2009, 11:257–262
41. Coriu D, Dispenzieri A, Stevens FJ, Murphy CL, Wang S, Weiss DT, Solomon A: Hepatic amyloidosis resulting from deposition of the apolipoprotein A-I variant Leu75Pro. *Amyloid* 2003, 10:215–223
42. Gregorini G, Izzi C, Obici L, Tardanico R, Rocken C, Viola BF, Capistrano M, Donadei S, Biasi L, Scalvini T, Merlini G, Scolari F: Renal apolipoprotein A-I amyloidosis: a rare and usually ignored cause of hereditary tubulointerstitial nephritis. *J Am Soc Nephrol* 2005, 16:3680–3686
43. Amarzguioui M, Mucchiano G, Häggqvist B, Westermark P, Kavlie A, Sletten K, Prydz H: Extensive intimal apolipoprotein A1-derived amyloid deposits in a patient with an apolipoprotein A1 mutation. *Biochem Biophys Res Commun* 1998, 242:534–539
44. Van Allen M, Frohlich J, Davis J: Inherited predisposition to generalized amyloidosis. *Neurology* 1969, 19:10–25
45. Joy T, Wang J, Hahn A, Hegele RA: APOA1 related amyloidosis: a case report and literature review. *Clin Biochem* 2003, 36:641–645