Author’s response to reviews

Title: A new genetic variant of hereditary Apolipoprotein A-I amyloidosis: a case-report followed by discussion of diagnostic challenges and therapeutic options

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Author’s response to reviews:

Dear Editor,

Initially, we would like to thank the reviewers for their time to review our manuscript. Their comments will help to improve the paper. Please find below the point-by-point reply. All changes are tracked in the main manuscript and in the responses the comment-specific paragraph/sentence is presented.
Kazuchika Nishitsuji, Ph.D. (Reviewer 1):

1. Histological and/or immunohistochemical data and images preferably need to be included.

Reply: Unfortunately, no images are available to be included.

2. Which apoA1 fragment was accumulated or deposited in the patient liver? In most of AApoA1 amyloidosis, apoA1 fragments, not the full-length apoA1, reportedly deposit as amyloid fibrils in tissues/organs, and the cleavage of apoA1 sometimes may be critical for amyloid formation by destabilizing apoA1.

Reply: This is a very important comment and one that of course needs further analysis. Unfortunately we have not performed such studies and we have no data on which specific part of the protein is forming the amyloid fibrils. Based on the presence of another known mutation on the same position, we hypothesize that the fragment will not be different.

Olga Gursky (Reviewer 2):

1. Bottom of page 5, Line 58 and beyond: "...the main acceptor of high-density lipoprotein..." and the rest of this sentence is confusing and needs re-writing. ApoA-I is not an acceptor of HDL but its main structural and functional protein. Further, ref. 5 cites pre-beta apoA-I; this lipid-poor form contains little if any cholesterol. ApoA-I in the form of mature HDL mediates plasma transport of cholesterol (not "cholesterol trafficking" in general). HDL removes cholesterol from peripheral cells; how exactly does this action relate to hypogonadism and infertility? Please clarify.

Reply: The whole paragraph is now changed as following: “The APOA1 gene, located on chromosome 11q23-q24, encodes the primary form of apolipoprotein AI (ApoAI). ApoAI is synthesized as a pre-protein by the liver and the intestine, is cleaved by plasma protease, providing the long mature form and degraded mainly in kidney [5]. ApoAI is the major protein component of high-density lipoprotein particles in the plasma, implicating in cholesterol transport [5]. More than 50 variants of APOAI gene are known, of which more than 20 have been associated with hereditary ApoAI amyloidosis (AApoAI) [6]. Each specific mutation in APOAI gene results in the deposition of ApoAI in various tissues causing distinct clinical syndromes with different age of onset, pattern of organ involvement, progression rate, and prognosis [6]. Even among individuals with identical variants, the clinical spectrum of disease may be quite heterogeneous. According to the involved target-organs, individuals with AApoAI present manifestations from the liver, kidney, skin and the heart, up to the gonads and adrenal glands [7-10].”

2. Page 9 bottom line 58: "the clinical presentation provides a complex model of interactions between the produced amyloid fibrils and the involved tissues." Not sure if fibrils are biologically active species that cause pathology.

Reply: This sentence is now removed.
3. Bottom of page 5, line 48 "its clinical spectrum may be heterogeneous" Unclear what "it" refers to; please specify.

Reply: The whole paragraph is now changed; please check our reply in your first comment.

4. p. 8 line 46 "case report" has no hyphen; "for the first time" insert "the".

Reply: Both typing errors have been corrected as suggested by the reviewer.

Dorota M. Rowczenio (Reviewer 3):

1. Throughout the manuscript (Abstract, Background) the authors' state that mutation occur in the protein, which is a false statement. Mutations or genetic variants occur in the genes (DNA sequence) and result, in this case, in the amino acid substitution of Leucine to proline at position 60 of the mature ApoAI protein. The manuscript needs to be amended.

Reply: The manuscript has been revised and all these statements have been corrected.

2. When listing the variant proteins authors should include the apolipoprotein CII, and apolipoprotein CIII and beta-2-microglobulin.

Reply: Whenever the variant proteins are being listing, we now include the apolipoprotein CII, and apolipoprotein CIII and beta-2-microglobulin. The Reference 2 replaced by the following: “Jean D. Sipe, Merrill D. Benson, Joel N. Buxbaum, Shu-ichi Ikeda, Giampaolo Merlini, Maria J. M. Saraiva & Per Westermark (2016) Amyloid fibril proteins and amyloidosis: chemical identification and clinical classification International Society of Amyloidosis 2016 Nomenclature Guidelines, Amyloid, 23:4, 209-213.”

3. In the abstract conclusion needs re-writing. I don't think the current report extend the AApoAI phenotype, as the phenotype described in this patient (amyloid affecting the liver and testes) has already been reported in this type of hereditary amyloidosis.

Reply: The abstract conclusion was re-written as following: “This case-report enlarges the clinical picture of ApoAI-driven disease and its complex genetic background and in parallel suggests for a more systematic approach in any case with strong suspicion of hereditary amyloidosis.“

4. In the Background the authors state: More than 50 variants in the APOA1 gene encoding apolipoprotein AI (ApoAI) are known, of which 20 have been associated with ApoAI amyloidosis (AApoAI).

I don't think this numbers are correct; i.e. there is more than 20 mutations known to cause ApoAI amyloidosis.

Reply: According to http://www.amyloidosismutations.com/mut-aapoai.php, up to now there are 23 mutations known to cause ApoAI amyloidosis. We modified the sentence writing “more than 20” instead of 20.
5. Individuals with AApoAI present mainly manifestations due to liver, kidney, laryngeal, skin and myocardial involvement. Authors should change this sentence, as the English is incorrect.

Reply: The whole paragraph is now changed as following: "The APOA1 gene, located on chromosome 11q23-q24, encodes the primary form of apolipoprotein AI (ApoAI). ApoAI is synthesized as a pre-protein by the liver and the intestine, is cleaved by plasma protease, providing the long mature form and degraded mainly in kidney [5]. ApoAI is the major protein component of high-density lipoprotein particles in the plasma, implicating in cholesterol transport [5]. More than 50 variants of APOA1 gene are known, of which more than 20 have been associated with hereditary ApoAI amyloidosis (AApoAI) [6]. Each specific mutation in APOA1 gene results in the deposition of ApoAI in various tissues causing distinct clinical syndromes with different age of onset, pattern of organ involvement, progression rate, and prognosis [6]. Even among individuals with identical variants, the clinical spectrum of disease may be quite heterogeneous. According to the involved target-organs, individuals with AApoAI present manifestations from the liver, kidney, skin and the heart, up to the gonads and adrenal glands [7-10]."  

6. By the next generation sequencing, the proband was found to carry an heterozygous single-base substitution at the codon for residue 60 of the mature ApoAI protein from 5'-GCAAGCTGCGGA for Leu-60 or 5'-GCAAGGCCGCGGA for Pro-60. Authors should re-write this paragraph
- The proband was found to be a heterozygous for a single base substitution
- Edit the paragraph in accordance with suggestions in point 1.
- Furthermore, it should be mentioned that the DNA change is at position c.251T>C, changing the codon CTG to CCG, resulting in a change of amino acid from leucine to proline.

Reply: We re-wrote this paragraph as following: "To establish the diagnosis of hereditary AApoAI, mutation analysis of the APOA1 gene was performed. By the next generation sequencing, the proband was found to be heterozygous for a single-base substitution at position c.251T>C, changing the codon CTG to CCG. This change resulted in a amino acid substitution, from leucine to proline in the peptidic residue 60 of the mature ApoAI protein. Sanger sequencing was used for subsequent screening of the proband family members; his siblings (two brothers) and his mother lacked the mutation, but paternal DNA was unavailable."  

7. Sanger sequencing validation of all family members revealed that his siblings (two brothers) and his mother lacked the mutation, but paternal DNA was unavailable.  
In this case Sanger sequencing was used for subsequent screening of the proband family members not for validation.

Reply: This sentence was re-written according to reviewer suggestions: "Sanger sequencing was used for subsequent screening of the proband family members; his siblings (two brothers) and his mother lacked the mutation, but paternal DNA was unavailable."
8. **Discussion and Conclusions**
In this case-report we recognized a new subtype of hereditary AApoAI for first time in a Greek patient caused by an heterozygous mutation at codon 60 from CTG to CCG in the APOA1 gene leading to an amino-acid substitution from Leu to Pro in the mature ApoAI protein.
This is not a new subtype of hereditary AApoAI, but hereditary AApoAI caused by a novel mutation in the APOA1 gene, described, for the first time, in a patient of Greek ancestry.

The remaining of this paragraph needs to be edited in accordance with suggestions in point 1.

Reply: The initial paragraph of Discussion and Conclusion section was rewritten as following: "In this report, we recognized a case of hereditary AApoAI caused by a novel single nucleotide mutation c.251T>C in the APOA1 gene, described for the first time in a patient of Greek ancestry. This single-base mutation resulted in an amino acid substitution from leucine to proline in the mature ApoAI protein, leading to lower cleavage and increased deposition of ApoAI into the involved organs. In our case, the ApoAI-driven disease presented initially with liver involvement followed by the..."

9. .....and only one with isolated liver involvement (Leu60_Phe71delinsValThr) [7]
The reference (7) used here refers to (Leu60_Phe71delinsValThr)? In that case it is not correct.

Reply: The position of reference was changed appropriately.

10. Leu60_Phe71delinsValThr and Leu60_Phe71delins60Val_61Thr are the same variants so authors should be consistent with the nomenclature

Reply: We corrected the variants to be consistent.

11. In conclusion, the identification of this new APOA1 mutation broadens the spectrum of known genetic mutations associated with hereditary ApoAI amyloidosis with liver and gonadal involvement.

Reply: We changed the conclusion paragraph according to reviewer suggestion: “In conclusion, this is a case report presenting a previously unknown amyloidogenic single point mutation in ApoAI gene associated with hereditary ApoAI amyloidosis with liver and gonadal involvement.”

Vassiliki A. Iconomidou, PhD (Reviewer 4):

1. This Case study follows the CARE report. CARE guidelines should be suitably cited (for example: Gagnier J, Kienle G, Altman DG, Moher D, Sox H, Riley DS, and the CARE group. The CARE guidelines: consensus-based clinical case report guideline development. Journal of Clinical Epidemiology;67(1),46-51.)

Reply: The CARE guidelines are now cited according to the reviewer suggested reference.

2. Page 10. "The amyloidogenicity of these mutations and the variability in the clinical presentation provides a complex model of interactions between the produced amyloid fibrils and the
involved tissues" Authors are encouraged to elaborate on this statement by introducing a scheme of this hypothetical model.

Reply: This sentence was removed.

Giorgio Cavigiolio, Ph.D. (Reviewer 5):

1. The authors should provide more technical details of the Immuno EM measurements, e.g. Name and description of the antibody used for the detection of apoA-I and of the other antibodies tested with negative results.

Reply: The ImmunoEM was performed in the Pathology Unit, Fondazione Istituto Di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo and University of Pavia, Pavia, Italy; according to the standard protocols and techniques that have already been published (Blood 2015, 125(14):2239-2244 and Gastroenterology 2004, 126(5):1416-1422). Due to the limited space for this case report we did not provide additional details, which have already been published. The whole paragraph was modified as following: “Liver tissue sample was sent for typing of amyloid to Amyloidosis Research and Treatment Center, Foundation Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo and University of Pavia, Italy. By immunoelectron microscopy performed according to the standard already published methodology [11,12], the liver appeared heavily infiltrated by amyloid fibrils that reacted with anti-apoAI, while no immunoreaction was seen with other antibodies, supporting the diagnosis of AApoAI”

2. From the Immuno EM data, is there any extra information regarding the nature of the apoA-I deposits available? It would be very important to better identify the apoA-I peptide that produces the amyloid deposits. In particular, is it full-length apoA-I or a fragment of it? N-terminal fragment?

Reply: This is an important comment; unfortunately we do not have this information and additional work needed to be done to identify the specific fragment (if not the full length protein). Unfortunately, at this time this is not possible.

3. To better illustrate the single amino acid mutation identified in the apoA-I sequence of this patient, it is suggested to add a figure in which the N-terminal amino acid sequence of apoA-I (e.g. 1-80) is shown and the mutated residue clearly indicated. Even this simple graphic illustration would improve the understanding of the case at the molecular level. To put it in the context of the current knowledge, ideally, the other known amyloidogenic mutations involving the first 80 amino acids of apoA-I could be also marked in the same figure.

Reply: According to the recommendation of the reviewer we provide a figure in which the secondary structure of the protein and the known mutational spots are indicated. We added this in the Discussion section as following: “In general, each specific mutation results in a syndrome with different clinical manifestations and organ involvements [4]. Figure 1 presents the secondary structure of ApoAI, based on the UNIPROT database [16] and pinpoints on this pattern the exact sites of identified mutations [17].”
Best regards,

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