Author's response to reviews

Title: APOA5 Q97X mutation identified through homozygosity mapping causes severe hypertriglyceridemia in a consanguineous family.

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Version: 2 Date: 31 July 2012

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RESPONSE TO REVIEWERS

We have taken into account all the suggestions made by the reviewers. Here are the specific responses to them.

Referee 1

- In response to the comment of referee 1: “It is not properly explained why the authors used the genome wide linkage study approach as first line laboratory strategy. It is true that mutations in the known candidate genes explain a minority of cases with a severe hypetrglyceridemic phenotype but since the main aim of this study was to determine the genetic locus responsible for the severe HTG present in this consanguineous family the resequencing of the candidate genes in which pathogenic mutations have been previously described should have been considered as first line laboratory approach”

We agree with the referee that resequencing candidate genes is the most straightforward approach to find causal mutations. However, there are some important considerations that must be taken into account: first, sequencing the five most plausible candidate genes for severe HTG (LPL, APOC2, APOA5, LMF1 and GPIHBP1) would mean the analysis of 10 exons of the LPL gene, 4 exons of the APOA5, 13 exons of the LMF1, 4 exons of the GPIHBP1 and 5 exons of the APOC2 gene (a total of 36 exons). If no mutation is found in these genes, it would be logical to continue searching novel mutations in genes with common variants that have been associated with multifactorial HTG such as for example GCKR (19 exons) or other genes. If again the sequencing did not yield the causal mutation, then a next step might be to test for gain-of-function mutations in genes with previously-described loss-of-function mutations causing hypotriglyceridemia (for example, 6 exons of the ANGPTL3, 29 exons of the APOB gene, among others). Therefore, resequencing genes with traditional techniques (Sanger) may represent from a month of work if the disease mutation is found in the first candidate gene analyzed (usually, LPL) to more than one year if mutation is not found in the main candidate genes. It is important to say that if no mutation is found, there is no guidance from resequencing studies on the possible location of the unidentified disease gen.

In contrast with the above paragraph, the timeframe we have needed in this study in submitting DNA to genome-wide analysis, locating the causative gene and discovering the mutation was of approximately 6 months. This was possible because the homozygosity mapping directly led us to the chromosomal region in which the
causal mutation is located, regardless whether the causative gene has been previously described or not.

Given the powerful arguments presented above, we believe that our approach is a valid and represent a faster approach to locate causal recessive mutations in consanguineous families in comparison to traditional resequencing studies of candidate genes. We have included a whole paragraph (second paragraph in the “Discussion” section) explaining the advantages of homozygosity mapping.

- In response to the comment of referee 1: “The discussion section needs extensive revision focusing the findings of this work and shortening the section describing the mechanisms of action of ApoAV. Since a known mutation in one of the candidate genes was identified (Oliva et al, Charriere et al) it would be interesting to discuss the two different laboratory approach (gene resequencing vs genome wide) In terms of costs and time employed.

We agree with the comment of this referee. Therefore we have shortened the section of the Discussion in which we describe the action of Apo AV. Additionally; we have included part of the paragraph regarding to the response to the first suggestion of the referee 1. In this paragraph we discuss the two different laboratory approaches (resequencing versus homozygosity mapping). In the discussion section, we have also included a more extensive discussion about the phenotypes of family members with heterozygous and homozygous genotypes.

- In response to the comment of referee 1: “figure 3 and figure 4 may be moved to the supplemental data section since they do not add major information”

We agree with the referee and we have moved both figures to the supplemental data section.

**Referee 2**

- In response to the comment of referee 2: “Why the authors did not used first a direct sequencing of these major genes involved in severe HTG? Was direct sequencing not easily accessible? Is this method really less expensive and faster?
Please, see the answers to the first and second comment from the referee 1. As mentioned before, we have included a paragraph on this topic in the Discussion section of the manuscript.

- In response to the comment of referee 2: “The limits of the method are not clearly explained. Does it need to have large families? Would it have identified compound heterozygotes?"

The main limitation of the strategy based on homozygosity mapping is that it requires consanguineous families highly suggestive for a recessive genetic defect. It is also important to notice that sometimes (especially in very small families), this method pinpoints a very large chromosomal region, in which hundreds of genes could be the carriers of the causative mutation. Therefore, finding the exact gene and causal mutation is not always guaranteed. In our study, clear evidence indicates that causal mutation was located in 11q23 and more specifically in the APOA5 gene.

Compound heterozygous are not detectable using this strategy, because they usually come from non-consanguineous families. Regarding the size of the study, it is noteworthy to mention that the discovery of mutations is possible using this strategy using a single family (Rojas et al. 2002). As is evident, statistical power for finding significant LOD scores are always increased with larger number of affected and unaffected relatives.

We have included all these comments in the Discussion section of the manuscript.

- In response to the comment of referee 2: “The method used for LPL activity is not indicated. Moreover, the results of LPL activity are not given in Figure 1 as said in the text. Was it a post-heparin LPL activity? LPL activity is a critical and difficult assay. The authors say in results that the LPL activity was normal in 2006? and in discussion that ?in 2006, even the proband had normal TG?. Normal LPL activity is probably explained by the fact that LPL activity was done when TG were normalized. The method, the results of LPL activity compared to normotriglyceridemic controls should be added and TG values at the time of LPL activity should be mentioned.

We agree with the comment of the referee. The LPL assay performed in 2006 was a post heparin LPL activity assay. The laboratory technician in charge of this determination is not accessible today and regrettably we do not have many details about the exact methodology employed at that time we will not mention this data in the
article. Consequently, we have decided to remove all references to the LPL enzyme activity assay.

- In response to the comment of referee 2: “In discussion, the phenotypic variability of patients compared to previous reported cases should be more detailed. The probands exhibited very severe phenotypes (TG > 100 g/l) with several episodes of acute pancreatitis compared with Q97X reported by Priore Oliva and Charriere et al. Could you discuss this point and indicate if additional genetic variants have been studied to explain the severity of their phenotypes? (for example, APOE2 or E4 variants, LPL or apoC3 polymorphisms.)"

As both affected sisters come from a consanguineous marriage, it is highly possible that they share additional genetic variants that contribute to the severity of their phenotype. However, APOA5 mutation is likely the most influential mutation present in this family. We did not study additional genetic variants to confirm this except for common variants S19W and -1131T>C of APOA5 gene.

- In response to the comment of referee 2: “In discussion, the authors say affected cases (HTG> 5,000 mg/l) only showed wild type S19W and -1131T>C genotypes of APOA5?. The authors referred to homozygotes. The influences of these polymorphisms are probably minor in homozygotes with non sense mutations. Moreover in the French and Italian study, no truncated peptide was identified. On the contrary, as discussed by Charriere et al. in Atherosclerosis in 2009, the influence of the polymorphisms may be crucial in heterozygotes. The authors should analyze the influence of these polymorphisms in their heterozygous cases”

We agree with the referee. In the paragraph 9 of the Discussion section, it is stated that the presence of susceptibility alleles for HTG in APOA5 -1131T>C and S19W might be important in heterozygous carriers of Q97X when the mutation is located in a different haplotype than the susceptibility common variants APOA5 -1131C and 19W. In this family, only one heterozygous carrier of the mutation had a susceptibility variant of the APOA5 gene (19W), but he does not present a more severe disease than the other heterozygous subject who does not carry this variant. Since we found only one subject with this variant, it was not possible to analyze the influence of these polymorphisms on disease phenotype.
• In response to the comment of referee 2: “Are apoAV plasma values available in this family?”

During the last year, we were working very hard in trying to identify Apo AV in plasma via western-blot. However, we did not succeed probably because the low plasma APOA5 concentration. In any case, we think that the information provided by this test would not add relevant information regarding the main objectives of the research.

• In response to the comment of referee 2: “In table S4, for charriere el al (2009), clinical manifestations not described, is wrong. It is written in the original article. None of the patients with Q97X or L242P mutation suffered from acute pancreatitis or cardiovascular diseases

Thank you very much for this correction. We have modified the manuscript.

• In response to the comment of referee 2: “International units should be used for biological values (TG in mmol/l for example)”

According to the suggestion of the referee, we have now used international units for all biological determinations.

• In response to the comment of referee 2: “The authors could explain why proband 1 was treated by ezetimibe and nicotinic acid in addition to fibrates. It is an unusual treatment for hypertriglyceridemia. These treatments are usually used to treat hypercholesterolemia”

Proband 1 was treated with fibrates and nicotinic acid for her HTG. Ezetimibe was added because at one point she developed hypercholesterolemia as well. Nevertheless and according the suggestion of the referee, we have decided to mention exclusively the medications used to treat HTG, without mention to ezetimibe or other drugs aiming to control plasma cholesterol levels.

• In response to the comment of referee 2: “The authors could comment the fact that subject 8 (Q97X heterozygote) died of coronary heart disease, and if he had additional cardiovascular risk factor”
All the available information regarding cardiovascular risks factors of this person are indicated in the Subjects and Methods section.

- In response to the comment of referee 2: “The exact name of Oliva in reference n°47 is Priore OLiva”

The name of this author is properly referred in the reference 2.

- In response to the comment of referee 2: “In figure 1, the author should precise if all rs number referred to APOA5”

All “rs” numbers of common SNPs that are shown in the figure 1 corresponds to SNPs that are flanking the APOA5 gene. Therefore, none of them are within APOA5 genes, except of course APOA5 -1131T>C, APOA5-S19W and the mutation itself APOA5 Q97X. This information is now more clearly mentioned in the legend of figure 1.