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Description of a large family with autosomal dominant hypercholesterolemia associated with the *APOE p.Leu167del* mutation

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Abstract

Apo E mutants are associated with type III hyperlipoproteinemia characterized by high cholesterol and triglycerides levels. Autosomal Dominant Hypercholesterolemia (ADH), due to mutations in

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the *LDLR*, *APOB* or *PCSK9* genes, is characterized by an isolated elevation of cholesterol due to high levels of low-density lipoproteins (LDL).

We now report an exceptionally large family including 14 members with ADH. Through genome wide mapping, analysis of regional/functional candidate genes and whole exome sequencing, we identified a mutation in the *APOE* gene, p.Leu167del previously reported associated with sea-blue histiocytosis and familial combined hyperlipidemia. We confirmed the involvement of the *APOE* p.Leu167del in ADH, with (1) a predicted destabilization of an alpha-helix in the binding domain; (2) a decreased apo E level in LDL; and (3) a decreased catabolism of LDL.

Our results show that mutations in the *APOE* gene can be associated with *bona fide* ADH.

Keywords

Apolipoproteine E; Autosomal Dominant Hypercholesterolemia (ADH); mutation; low-density lipoproteins (LDL); linkage analysis

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Apolipoprotein E (apo E) is a 34 kDa glycosylated and excreted protein which is a component of chylomicrons, VLDL (very low density lipoproteins), IDL (intermediate density lipoproteins), LDL (low density lipoproteins), and HDL (high density lipoproteins), (OMIM +107741). Apo E is a ligand for the scavenger receptor B type 1 (SR-B1), and for all members of the LDL receptor family. The *APOE* gene accounts for a significant fraction of the variation in plasma cholesterol levels (Eichner JE et al. 2002). Apo E presents three major isoforms: E3 (the most frequent one), E2 and E4. Whereas apo E3 and apo E4 bind with similarly high affinity to the LDL receptor, the binding of apo E2 is 50- to 100- times weaker (Weisgraber KH et al. 1982). Apo E2 isoform is associated with lower LDL cholesterol, except for 5% of E2/E2 carriers who develop type III hyperlipoproteinemia (OMIM +107741), a relatively rare disease characterized by severely elevated cholesterol and triglyceride levels. Apo E4 isoform is associated with elevated total and LDL-cholesterol levels, due to the higher affinity of apo E4 for VLDL and LDL, whereas apo E2 and apo E3 lipoprotein-binding preference is for HDL (Hatters DM et al. 2006). This relative enrichment of apo E4 on VLDL particles is thought to lead to their accelerated uptake and the consequent down-regulation of LDL receptor expression. This, in turn, would account for the increased levels of LDL in the plasma of apo E4 subjects. In addition to isoforms E2, E3 and E4, rare apo E mutants have been described associated with type III hyperlipoproteinemia or lipoprotein glomerulopathy (OMIM +611771). Finally, a few rare apo E variants (p.Glu21Lys, p.Pro102Arg and, p.Leu270Glu) have been observed in probands with hyperLDLemia (type IIa hyperlipoproteinemia), but none co-segregated with hyperLDLemia in the families (Wardell MR et al. 1991, van den Maagdenberg AM et al. 1993).

The *APOE* p.Leu167del mutation was reported to be associated with sea-blue histiocytosis (OMIM #26960), characterized by splenomegaly, mild thrombocytopenia, and, in the bone marrow, numerous histiocytes containing cytoplasmic granules which stain bright blue with the usual hematologic stains. The *APOE* p.Leu167del mutation was also reported in subjects with familial combined hyperlipidemia (FCHL, type III dyslipoproteinemia) (Solanas-Barca M et al. 2012). FCHL (OMIM #144250) is characterized by elevated levels of either plasma cholesterol or triglyceride or both in members of the same family. And from time to time the profile can change in a given person. FCHL was shown to be distinct from autosomal dominant hypercholesterolemia (ADH, OMIM #143890) that is characterized by an isolated elevation of cholesterol bound to LDL giving rise to tendon xanthomas and premature morbidity and mortality from cardiovascular complications. ADH is genetically heterogeneous and associated with mutations in the well-known *LDLR* (low density

lipoprotein receptor) (FH, OMIM #606945), *APOB* (apolipoprotein B-100) (FDB, OMIM # 144010) and *PCSK9* (Proprotein Convertase Subtilisin/Kexin type 9, Abifadel M et al. 2003) genes. Subsequent work from our team has shown further heterogeneity with at least 19% of ADH families in which the disease is not associated with a mutation within one of these three genes (Marduel M et al. 2010). Indeed, new ADH loci were recently mapped at 16q22.1, *HCHOLA4* (Marques-Pinheiro A et al. 2010), and 8q24.22 (Cenarro A et al. 2011). While trying to identify the *HCHOLA4* gene, we investigated an exceptionally large ADH French kindred that proved to be unlinked to any of the known genes or loci. To identify this new ADH gene, we combined two strategies: the classic genome wide mapping/candidate gene analysis and the novel whole exome sequencing design (methods in supplemental data).

ADH families were recruited by the French National Research Network on Hypercholesterolemia. For probands, the following inclusion criteria were used: total and LDL-cholesterol values above the 95th percentile when compared to a sex- and age-matched French population (STANISLAS cohort (Siest G et al. 1998), B. Herbeth, G. Siest & S Visvikis-Siest, personal communication 2009), triglycerides below 1.5 mmol/L, personal and/or documented familial xanthomas and/or early coronary artery disease. Non *LDLR*/-*APOB*/-*PCSK9* probands were selected after direct sequencing of the *LDLR* and *PCSK9* gene coding sequences, MLPA analysis of the *LDLR* gene, and direct sequencing of parts of the *APOB* gene encoding the binding domain (exons 26 and 29). Norwegian dyslipidemic probands were recruited with the following inclusion criteria: non-*LDLR*/-*APOB*/-*PCSK9* carriers, total cholesterol above 8.5 mmol/L, LDL-cholesterol above 5.5 mmol/L and triglycerides below 3.0 mmol/L. The investigation performed here conforms with the principles outlined in the Declaration of Helsinki.

Among 9 non-*LDLR*/-*APOB*/-*PCSK9*/-*HCHOLA4* families we have identified, one was large enough to enable genome wide mapping of a new ADH gene (Marques-Pinheiro A et al. 2010). The recruitment of the HC126 family was initiated through the female proband II-2, and expanded to 27 individuals over 3 generations, thus exploring 23 meioses (Figure 1A). Several cardiovascular accidents were reported in the previous generations and the affected proband's brother II-7 underwent a double bypass at age 43. Family members II-5 and II-15 were scored as "unknown" for linkage analyses because their lipid values without medication were not available. One hundred thousand simulations were performed to evaluate the power and the relevance of a genome wide linkage scan in HC126. The av.ELOD score was 2.1, with a max.ELOD score of 4.8, therefore indicating that the statistically significant threshold of 3 could be reached. The genome-wide scan provided a maximum LOD score of 2.55 (θ=0) for rs718087 on chromosome 19 (Supp. Table S1). Fine regional microsatellite markers provided positive two-point LOD scores : D19S900, D19S903, D19S574, and D19S217 (Supp. Table S1). The 19q13.31–13.32 multipoint analysis gave a maximum LOD score of 3.15 between rs1988065 and rs3513657 (19:43,374,601–45,439,163) (Supp. Table S1, Supp. Figure S1). Regional haplotype construction allowed the identification of a common region for affected members from D19S417 to D19S908 (Figure 1A). Phenotypically unclassified patient II-5 did not carry the familial-disease haplotype, as well as the two affected members I-3 and II-14 who highly probably display a phenocopy form of ADH and suggesting the coexistence of another lipid abnormality in the family, which remains to be identified.

To identify the disease gene, we combined two sequencing strategies. For the first strategy, an inventory of all the genes at 19:43,374,601–45,439,163 was drawn up from online data (Supp. Table S2). We investigated the *APOE/APOC1/APOC4/APOC2* gene cluster, as well as the two hepatic control regions (HCR-1,-2), in HC126-II-7, HC126-III-1 and the c. 500_502delTCC (p.Leu167del, formerly called Δ149Leu) variation in the *APOE* gene

(RefSeq NM_000041.2) was found (Supp. Figure S2). For the second strategy, whole exome sequencing was performed for three affected members of HC126 (III-2, III-7 and III-8), to possibly identify a mutation within another regional gene that had not been investigated in the first strategy. Four variants were found, the c.437G>A (p.Arg146Gln) in the *CCDC123* gene, the c.1151A>G (p.Gln384Arg) in the *RHPN2* gene, the c.657G>C (p.Asn219Lys) in the *CEACAM8* gene and the c.500_502delTCC (p.Leu167del) in the *APOE* gene. The two first variants were shown to be frequent genomic variants (//snp.gs.washington.edu/EVS/, // www.1000genomes.org), excluding them from being a possible disease-causing mutation. The *CEACAM8* variant is rare, 3/10755 in the Exome Variant Server and not reported by the "1000 genome" project. However, tissue expression profile and reports of alterations of this gene's expression in acute lymphoblastic leukemias (Lasa A et al. 2008) excluded this variant as a possible ADH disease-causing mutation. Finally, the p.Leu167del in the *APOE* gene was the only possible disease-causing mutation within the candidate locus.

To confirm the involvement of the *APOE* gene in ADH, we screened the *APOE* p.Leu167del through a direct fluorescent PCR method in a control group and we sequenced the whole *APOE* gene in other ADH or hyperlipidemic probands. The *APOE* p.Leu167del was not detected in over 220 controls, in agreement with Faivre et al. who had investigated 50 French controls, and indicating that this apo E variation is not a frequent polymorphism. Furthermore, we sequenced the *APOE* gene in a sample of 153 non-*LDLR*/-*APOB*/-*PCSK9* probands (55 French ADH and 98 Norwegian hyperlipidemics). Within the French ADH probands, the p.Leu167del variation, and p.Arg269Gly (c.805C>G) were found for family HC512 and HC298, respectively (Figure 1, Supp. Figure S2). The p.Arg269Gly apo E mutant has been previously reported in a proband with type IIb hyperlipoproteinemia, differing from ADH by elevated plasma triglyceride levels (Wardell MR et al. 1991). Within the Norwegian hyperlipidemic cohort, p.Arg235Trp (c.703C>T) was found in a 56 years old man with 9.4, 2.7, 6.4 and 1.8 mmol/L of total cholesterol, triglycerides, LDL and HDL, respectively, and the E3E4 apo E isoformes (Supp. Figure S2). The two missense variations, p.Arg269Gly and p.Arg235Trp, were absent in over 500 French controls, indicating that they are not frequent. Their predicted effect was "not tolerated" with SIFT or, "possibly damaging" and "probably damaging", with Polyphen and Polyphen-2, respectively, providing relevant effects on the protein activity. The identification of these other *APOE* mutations for ADH probands is a major finding that confirms the involvement of apo E alterations in the pathogenesis of ADH. To evaluate the possible effect of the p.Leu167del mutation on apo E function, we performed homology modeling of the human wild type apo E protein and of the deleted mutant. Leu167 of apoE is not a conserved amino acid, but is the fourth residue in a 6 amino acid motif of which positions 1, 2, 3 and 6 are very well conserved from *Xenopus tropicalis* to *Homo sapiens* (Supp. Figure S3). It is thus very likely that a deletion of an amino acid in this motif would alter the general structure of this highly conserved part of apo E. Because human apo E is 76.4% identical in amino acid sequence with mouse apo E, we used the crystal structure 1YA9 to realize the predicted model shown in Figure 1B. The p.Leu167del variation interrupts one α helix in a group of four helices stabilized by a leucine zipper. Disruption of one helix in the group very probably alters the interaction with the three others. The electrostatic surface charges are altered in apo E p.Leu167del, that also likely influences interaction with lipids and affinity of apo E to its receptors. Nguyen et al. performed a competitive receptor binding test *in vitro* with apo E isolated from VLDL of one p.Leu167del carrier and observed no significant binding defect. However, their proband was heterozygous for apo E p.Leu167del, thus, the effect of the deleted mutant could not be observed alone. Thus, a LDL receptor-binding defect could not directly be excluded under the hypothesis of a compensatory increased activity of the normal apoE in the presence of the deleted mutant. The other mutations, p.Arg235Trp and p.Arg269Gly, are at the origin of a loss of charge in the first and second α helices of the C-

terminal domain that very probably change each helix's properties, interaction with lipoproteins, and receptor binding.

Total apo E levels were measured for p.Leu167del carriers, HC126-II-2 (apo E = 4.09 mg/dL, under rosuvastatin), HC126-III-2 (apo E = 4.03 mg/dL), and were both in the normal range (2.30–6.30 mg/dL in French controls). Apo E distribution in each lipoprotein fraction showed a higher proportion in HDL (77% vs 63–69%), and a lower proportion in LDL (12% vs 18–25%) (Supp. Table S3). However, these data need to be confirmed because they are based on the study of only two patients, including one under a lipid lowering therapy.

In order to evaluate if LDL bearing the apo E p.Leu167del display an altered catabolism *in vivo*, we performed kinetic studies of apo B-100-containing lipoproteins in HC126-II-3 after a 2-month wash out of any hypolipidemic treatment (Table 1). After this period, the lipid values for this patient were: 3.7, 10.5, 8.1 and 0.7 mmol/L for triglycerids, total-, LDL- and HDL-cholesterol respectively. VLDL and IDL kinetic parameters were in accordance with the high triglyceride level only observed for this measurement, and never before in this patient, showing similarities with a type III hyperlipoproteinemia (E2E2 genotype) such as the dramatically increased VLDL and IDL pool due to a decreased conversion rate (Ooi EM et al. 2010). Interestingly, the increased VLDL and IDL pool for the HC126 patient was also due to an increased production rate, that was not observed for E2E2 patients, but already reported for patients carrying a *PCSK9* gene mutation (Ouguerram K et al. 2003). LDL kinetic parameters were similar to those from FH patients (mutation in the *LDLR* gene) with an increased LDL pool, which was the consequence of both an increase in LDL production rate and a decrease in LDL catabolism (Table 1). Despite the decreased conversion of VLDL to IDL, the very important VLDL production implies that a higher than normal proportion of lipoprotein follows the metabolic cascade leading to an increased apo B-100 flux into the next lipoprotein fractions. Accordingly, IDL production is increased and, combined to the low IDL catabolism, leads to an increased LDL production. Finally, there is an increased LDL pool due to the combination of this abnormal metabolic cascade and decreased LDL catabolism. LDL kinetic parameters of apo E mutant carrier were similar to those from FH patients strengthening the very probable effect of LDL bearing an apo E mutant on their catabolism. Two hypotheses may be put forward to explain the increased LDL pool observed. The first one is a decreased affinity of the apo E mutant to the LDL receptor as suggested by our *in silico* structural prediction and *in vitro* lipoprotein composition analysis. The second hypothesis is that the increased LDL production is related to the increased apo B-100 flux from VLDL as suggested here by *in vivo* kinetic studies. Altogether, both hypotheses are in agreement with a decreased catabolism of LDL bearing apo E p.Leu167del and thus explaining the hyperLDLemia observed in the family. However, these data need to be confirmed because they are based on the study of only one patient.

No splenomegaly or thrombocytopenia were present in the 12 *APOE* p.Leu167del carriers of the HC126 family nor in the proband of HC512 indicating that these subjects did not present the sea-blue histiocytosis disease. It seems thus reasonable to hypothetisize that sea-blue histiocytosis develops only for carriers of two mutations, the *APOE*-p.Leu167del and another mutation in another gene. The hyperLDLemia observed in the HC126 family would thus reveal the pure effect of the p.Leu167del mutation. The digenic mechanism hypothesis could explain the only two reports in the literature of the association between the p.Leu167del in the *APOE* gene and sea-blue histiocytosis (Nguyen TT et al. 2000, Faivre L et al. 2005).

The *APOE* p.Leu167del mutation was reported in subjects with familial combined hyperlipidemia (FCHL, type III dyslipoproteinemia) (Solanas-Barca M et al. 2012) and in the HC126 family reported here with a *bona fide* ADH. This overlap between the FCHL and

ADH phenotype has also been reported for mutations in the *LDLR* gene (Civiera et al. 2008). Hypertriglyceridemia can sometimes be observed in ADH subjects, mainly because of the many common genetic and environmental factors contributing to triglyceride elevation (Talmud PJ 2001). Mutations in the *LDLR* or *APOE* gene may amplify the effect of these factors, and thus, according to the number or the nature of these factors, could be associated with an overlapping phenotype between FCHL and ADH.

In conclusion, our results show that screening of the *APOE* gene is warranted in the setting of molecular diagnosis of ADH along with the *LDLR*, *APOB* and *PCSK9* genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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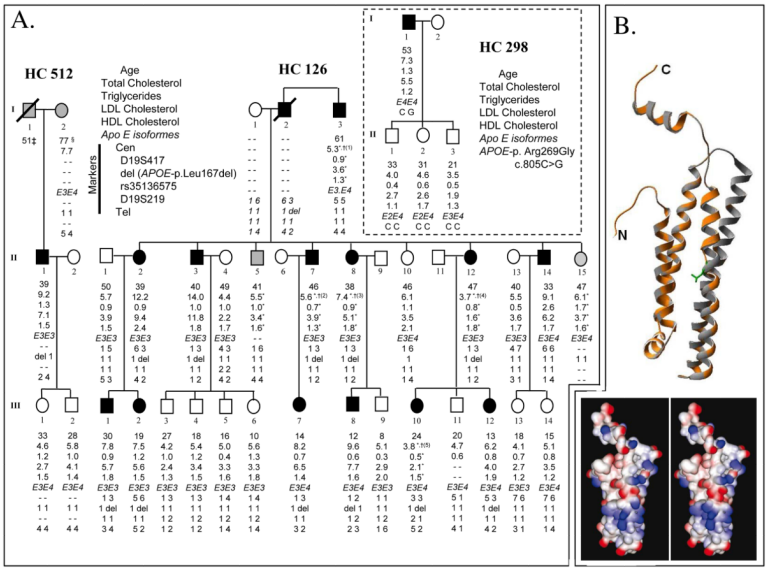

Figure 1. A. Lipid values and regional haplotype of the 19q13.31–13.32 locus in the HC126, HC298 and HC512 families

Blackened symbols represent affected subjects, white for unaffected subjects while individuals with an undetermined status are in grey. Genotypes are provided in straight, while deduced genotypes are provided in italics. Lipid values are given in mmol/L. * Values under hypocholesterolemic drug therapy. † Total cholesterol without drug : (1) 8.8 mmol/L at 55 years old, (2) 7.98 mmol/L at 43, (3) 14.8 mmol/L at 30, (4) 11.2 mmol/L at 45, (5) 7.98.8 mmol/L at 16. ‡ Died from myocardial infarction at 51 years old. § Presented myocardial infarction at 77 years old. **B. *In silico* analysis of the putative impact of the p.Leu167del mutation.** Top: Model obtained. In orange: structural prediction of pLeu167del mutation. In grey: human wild type apo E structural prediction. Leu166 and 167 are shown in green. Bottom: Electrostatic potential of wild-type apo E and p.Leu167del mutant. Charge distribution is represented as a gradient in which positive potentials are drawn in blue and negative in red.