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# PCSK9 Dominant Negative Mutant Results in Increased LDL Catabolic Rate and Familial Hypobetalipoproteinemia

Bertrand Cariou, Khadija Ouguerram, Yassine Zair, Raphael Guerois, Cédric Langhi, Sanae Kourimate, Isabelle Benoit, Cédric Le May, Constance Gayet, Khaldia Belabbas, Fabienne Dufernez, Maud Chétiveaux, Patrizia Tarugi, Michel Krempf, Pascale Benlian, Philippe Costet

**Objective**—Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a central player in the regulation of cholesterol homeostasis, increasing the low-density lipoprotein (LDL) receptor degradation. Our study aimed at exploring the pathogenic consequences in vivo and in vitro of a PCSK9 prodomain mutation found in a family with hypobetalipoproteinemia (FHBL).

**Methods and Results**—A white 49-year-old diabetic man had profound FHBL (LDLC: 16 mg/dL) whereas his daughter and sister displayed a milder phenotype (LDLC 44 mg/dL and 57 mg/dL, respectively), all otherwise healthy with a normal liver function. A monoallelic *PCSK9* double-mutant R104C/V114A cosegregated with FHBL, with no mutation found at other FHBL-causing loci. A dose-effect was also found in FHBL relatives for plasma APOB and PCSK9 (very-low to undetectable in proband,  $\approx 50\%$  decreased in sister and daughter) and LDL catabolic rate (256% and 88% increased in proband and daughter). Transient transfection in hepatocytes showed severely impaired processing and secretion of the double mutant which acted as a dominant negative over secretion of wild-type PCSK9.

**Conclusion**—These results show that heterozygous PCSK9 missense mutations may associate with profound hypobetalipoproteinemia and constitute the first direct evidence in human that decrease of plasma LDLC concentrations associated to PCSK9 LOF mutations are attributable to an increased clearance rate of LDL. (*Arterioscler Thromb Vasc Biol.* 2009;29:2191-2197.)

**Key Words:** PCSK9 ■ LDL ■ mutation ■ hypobetalipoproteinemia

Hypobetalipoproteinemia (HBL) refers to a heterogeneous group of monogenic disorders characterized by very low plasma concentrations of low-density lipoprotein cholesterol (LDLC) and apolipoprotein B (apoB) (ie, <5 percentile of the distribution in the population; for review see<sup>1</sup>). HBL includes 3 inherited disorders: (1) familial hypobetalipoproteinemia (FHBL; OMIM 107730), (2) abetalipoproteinemia (ABL; OMIM 200100), and (3) chylomicron retention disease (CRD; OMIM 246700). The frequency of subjects with heterozygous FHBL has been estimated to be 1:500/1:1000.<sup>2</sup> FHBL heterozygotes are often asymptomatic or express mild clinical manifestations such as fatty liver disease and intestinal fat malabsorption.<sup>3</sup> FHBL are often caused by *APOB* gene mutations.<sup>1,4</sup> However, a substantial number of FHBL (varying to 36%<sup>1</sup> to 56%<sup>5</sup> in the literature) do not harbor apoB mutations.

In the last 5 years, proprotein convertase subtilisin/kexin 9 (PCSK9) has emerged as a crucial modulator of cholesterol

metabolism.<sup>6</sup> PCSK9 is primarily expressed in the liver and the intestine. PCSK9 inhibits the LDL receptor (LDLR) pathway in a posttranscriptional manner.<sup>6</sup> In human, PCSK9 was initially reported as the third gene causing autosomal dominant hypercholesterolemia, in addition to LDLR and *APOB* mutations.<sup>7</sup> Indeed, *PCSK9* gain-of-function (GOF) mutations lead to increased plasma LDLC levels and premature atherosclerosis.<sup>7,8</sup> In contrast, *PCSK9* loss-of-function (LOF) mutations are associated with low LDLC levels and protection against coronary diseases.<sup>9,10</sup> To date, only 2 individuals fully deficient in PCSK9 have been identified with LOF mutations causing very low plasma LDLC (14 mg/dL and 15 mg/dL).<sup>11,12</sup> Although *PCSK9* truncating mutations are more prevalent in FHBL subjects of African ancestry, LOF missense mutations associated with lowered plasma LDLC in the general population were reported on all continents.<sup>6</sup>

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In this study, we identified and characterized a novel PCSK9 LOF double mutant that acts as a dominant negative. By performing lipoprotein kinetics, we demonstrated for the first time that FHBL linked to PCSK9 deficiency is attributable to an increase of LDL clearance in human.

## Subjects and Methods

For a complete "Subject and Methods" section please see the supplemental materials (available online at <http://atvb.ahajournals.org>).

### Subjects

The experimental protocol was approved by the ethic committee of the Nantes University Hospital and written consents were obtained from each volunteer before inclusion in the study (Protocol referenced as No. 15/06 - BRD 06/3-E), including members of the proband's family and healthy normolipidemic controls studied by the same compartmental method.<sup>13</sup>

## Results

### Clinical Findings in the FHBL Proband and Relatives

The proband is a 49-year-old French white man who was initially hospitalized for the rapid-onset of an insulin-requiring diabetes-mellitus. He exhibited extremely low plasma LDLC levels (7 mg/dL) on admission and also at distance of diabetes onset (16 mg/dL). Abdominal ultrasonography showed a moderate liver steatosis. However, hepatic enzymes levels and liver function tests were not altered. There was neither history of diarrhea nor eye or neurological abnormalities related to any vitamin deficiency. To date, the etiology of the diabetes of the proband remains uncertain. HbA1C was initially increased at 11.5%. On admission, ketonuria (+++) was detected, evocative of a transient insulin-deficient state. However, islet-related autoantibodies (anti-GAD and anti-IA2) were negative, probably excluding autoimmune type 1 diabetes. There was neither argument for endocrinopathy nor pancreas exocrine disease, with a normal abdominal computed tomography. He was free of microvascular and macrovascular diabetes-related complications. The patient was transiently treated with a basal-bolus insulin therapy regimen, and he is currently controlled (HbA1C=6.5%) under sitagliptin (100 mg/j) only. In accordance with a persistent endogenous insulin secretion, fasting plasma C-peptide levels were in the normal range. Proband's mother was deceased at 66 from dementia, whereas his father was healthy at age 79. His grandparents died at the age of 79, 87, 91, and 94 years, suggesting familial longevity.

Clinical and biological parameters were explored in fasted volunteer family members of the proband (II.3; Table 1). Circulating PCSK9 was undetectable in the proband. Both his sister (II.2) and his daughter (III.1) had much less plasma PCSK9 concentrations than the noncarrier II.4 and II.1 (respectively, 100 and 125 ng/mL versus 216 and 435 ng/mL). For comparison, PCSK9 circulating concentration was determined in 10 normolipemic fasted controls (5 women and 5 men, 25.7±4.7 year old, total cholesterol: 173±3 mg/dL, LDLC: 96±2 mg/dL, HDLC: 62±1 mg/dL, TG: 72±2 mg/dL). Mean PCSK9 concentration was 238±80 ng/mL, representing approximately twice as much as deter-

**Table 1. Clinical and Biological Characteristics in Carriers of the R104C/V114A PCSK9 Mutant and in Their Relatives**

	Individuals				
	II.1	II.2*	II.3*	II.4	III.1*
Age, y	51	37	49	48	29
Sex	M	F	M	F	F
BMI, kg/m <sup>2</sup>	nd	nd	28.8	21.0	22.9
Total serum cholesterol, mg/dl	167	152	100	235	143
LDLC, mg/dl	93	57	16	140	58
HDLC, mg/dl	57	81	75	82	83
Triglycerides, mg/dl	84	71	49	68	71
PCSK9, ng/ml	435	100	Und.	216	125
ApoA1, mg/dl	141	218	188	203	226
ApoB, mg/dl	72	52	25	87	44
ApoB/apoA1	0.51	0.24	0.13	0.43	0.19
ApoC2, mg/l (n:14–54)	nd	nd	62	44	48
ApoC3, mg/l (n:66–171)	nd	nd	155	89	135
ApoE, mg/l (n:25–53)	nd	nd	34	51	35
Lp(a), g/l	nd	nd	<0.12	<0.12	<0.12
Fasting plasma glucose, mmol/l	5.4	4.8	7.0	4.5	5.2
HbA1c, %	5.4	4.8	5.6	ND	ND
ASAT (multiple of the upper limit of the normal range)	0.67	0.51	0.93	0.88	0.74
ALAT (multiple of the upper limit of the normal range)	0.85	0.37	0.74	0.64	0.66

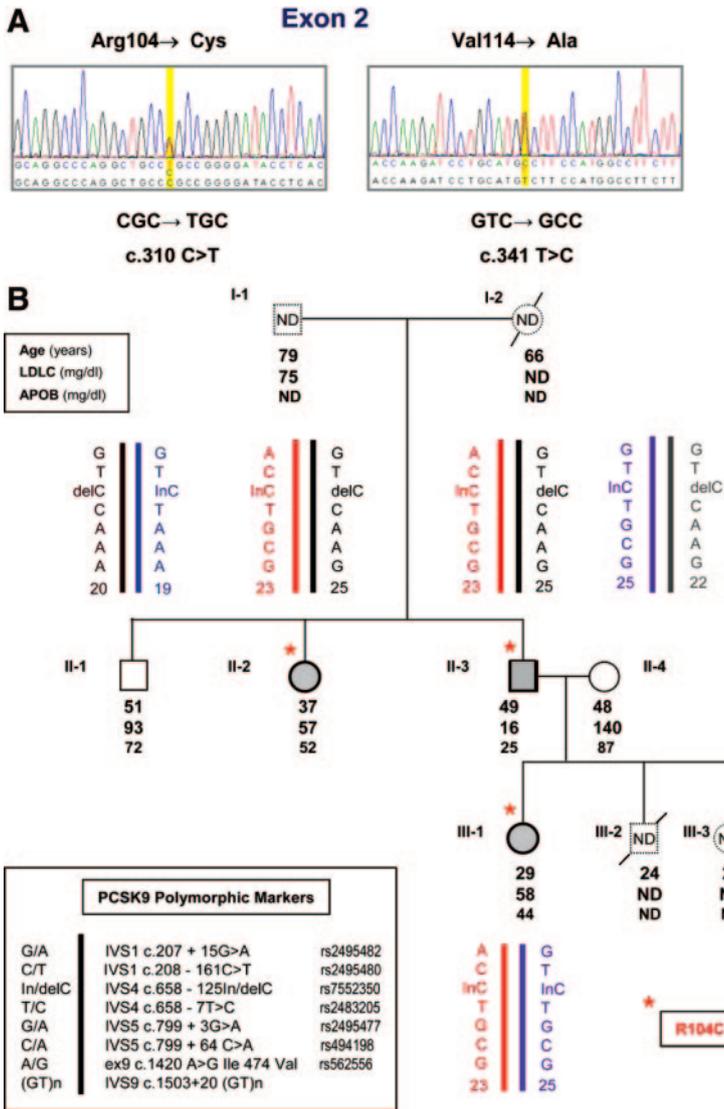
BMI indicates body mass index; Apo, apolipoprotein; LDLC, low density lipoprotein-associated cholesterol; HDLC, high density lipoprotein-associated cholesterol; Lp(a), lipoprotein a; HbA1c, glycated haemoglobin A1c; ASAT, aspartate amino transferase; ALAT, alanine amino transferase; Und, undetected; nd, not determined. \*Carrier of the R104C/V114A amino acid substitution.

mined for II.2 and III.1. More information on proband's relatives is presented as supplemental Results.

A fast protein liquid chromatography performed on plasma from mutation-carriers II.3 and III.1 is presented as supplemental Figure I and confirms low level of LDLC in these subjects. Gel-electrophoresis analysis of apoB-rich lipoprotein fractions isolated by ultracentrifugation did not reveal any abnormal apoB isoform in the proband and his daughter.

### Genotypic Findings in the Proband and Family

The proband was heterozygous for two PCSK9 missense mutations R104C and V114A in exon 2 (Figure 1A). Both mutations were absent from 600 and 300 chromosomes from French hypercholesterolemic patients and normolipidemic blood donors, respectively. Cosegregation analysis (Figure 1B) revealed that FHBL relatives II.2 and III.1 were also heterozygous for both mutations, whereas normolipidemic relatives were noncarriers of these mutations. Haplotyping informative single nucleotide polymorphism (SNP) and mic-



**Figure 1.** Genotypic findings and cosegregation analysis in the HBL family. A, DNA sequence chromatogram (Gensearch output) centered on PCSK9 mutations found in FHBL proband. Exon 2 reference sequence is shown in black letters (bottom line), patient sequence is colored according to PHRED base-calling (top line). Alternate bases found in patient at specific positions are shown in between. Positions with high probability of mutation are highlighted in yellow. B, Segregation analysis of PCSK9 exon 2 mutations (indicated by \*) and informative polymorphic markers, with HBL in the family. HBL subjects are represented by filled symbols, normolipidemic subjects by open symbols. Subjects represented by dotted-line symbols filled with “ND” were not available for genetic studies. Age (years) and plasma LDLC, apoB concentrations (mg/dL) is indicated below symbols (ND indicates not determined). Single nucleotide polymorphisms (SNPs) are described according to coding sequence numbering, with corresponding accession number in dbSNP. The number (n) of short tandem repeats in intron 9 (GT)<sub>n</sub> is indicated within haplotypes (above or below individual symbols). Haplotype segregating with exon 2 allelic mutations and HBL is colored in red.

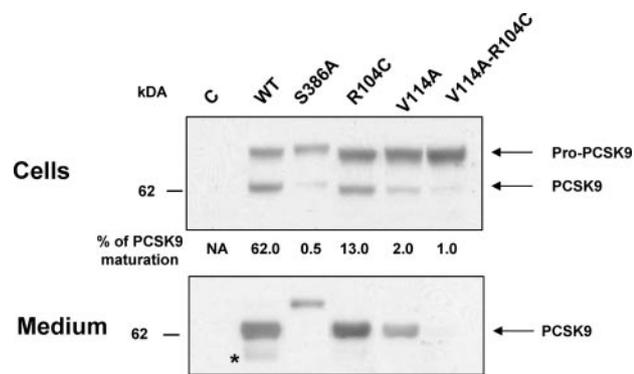
rosatellite at the PCSK9 locus confirmed that both mutations were allelic and therefore adjacent on the same DNA strand. The proband’s normolipidemic spouse (II.4) and HBL daughter (III.1) were respectively homozygous and heterozygous carriers of a rare conservative L483V variation (c.1447C>G, in exon 9). The PCSK9 proximal promoter and 5’-UTR DNA sequences matched the reference wild-type genomic sequence. Extensive analysis of the APOB coding and splicing sequences in the family did not reveal any pathogenic mutation causing FHBL. Analysis of common polymorphisms excluded segregation of any APOB haplotype with FHBL in the family (see supplemental Figure II). Extensive DNA sequencing of similar regions and proximal promoter at the LDLR locus was normal. All family members were carriers of the E3E3 genotype of APOE.

**Computed Estimation of Mutation Functionality**

Computed estimation of functional changes induced by the R104C and V114A mutations, although discrepant when each mutation was considered alone, suggested a potential deleterious effect induced by the double mutant (see supplemental

Methods). Indeed, the presence of 2 adjacent DNA changes would generate a mutant protein harboring 2 amino acid substitutions within a 10-aa stretch of the PCSK9 prodomain, which is highly conserved in 3 primate species (see supplemental Figure III). In contrast, the L483V conservative genetic variation found in the normolipidemic spouse (II.4) and FHBL daughter (III.1) was consistently estimated as neutral by 4 algorithms.

The X-ray structure of PCSK9 (protein data bank code: 3BPS) can be used to map the positions of the mutations R104C and V114A and shed light on their potential impact (supplemental Figure IVA). PCSK9 is composed of 3 domains, the N-terminal domain corresponding to the prodomain known to inhibit the proteolytic activity of the enzyme, the catalytic domain itself, and a C-terminal domain adopting a 3 6-stranded β-sheet adipokine resistin-like structure. Both point mutations are located in the prodomain, remote from the catalytic site of the enzyme. R104 is rather solvent exposed, whereas V114 is part of the hydrophobic core of the prodomain suggesting that this mutation may decrease the stability of the prodomain.



**Figure 2.** PCSK9 processing is abolished by the double R104C/V114A mutation. Human immortalized hepatocytes were transfected with the plasmid pcDNA3.1 empty (control) or coding for wild-type PCSK9 (WT), the loss-of-function variants S386A, R104C, V114A, or the double mutant R104C/V114A. Proteins were extracted from cell lysates or media and analyzed by Western blot using an anti-V5 antibody. Within cell lysates, the intensity of each band (proPCSK9 and PCSK9) was determined by densitometry and the percentage of maturation was calculated for each variant independently of each other, as the contribution of the lower band (PCSK9) to the sum of the signals obtained for both bands (proPCSK9 and PCSK9). A star indicates the band corresponding to the cleavage of PCSK9 by furin and PC5/6A.<sup>17</sup>

It has been demonstrated that the final structure of the catalytic domain of subtilisin-like proteases is dependent on the folding process guided by their prodomain. Notably, a previous study showed that a conservative mutation from Ile to Val (I-48V) in the hydrophobic core of the prodomain of *Bacillus subtilis* subtilisin could change the structure and specificity of the active folded enzyme by altering the folding process.<sup>14</sup> A remarkable feature of the V114A mutation is that it affects the same region of the prodomain hydrophobic core as the I-48V mutation in the bacterial subtilisin (supplemental Figure IVB).

### Processing and Secretion of PCSK9 R104C/V114A

To assess the effect of R104C and V114 amino acid substitutions, alone or in combination, on PCSK9 processing and secretion, immortalized human hepatocytes (IHH) were transiently transfected with the corresponding expression vectors flagged with a V5 epitope. In parallel, wild-type PCSK9 (WT-PCSK9) and the catalytically inactive S386A mutant<sup>15</sup> were also transfected in IHH cells (Figure 2). In lysates from cells expressing WT-PCSK9, 2 bands of 73 and 64 kDa were detected, corresponding to the pro-PCSK9 and the mature cleaved PCSK9 protein, respectively. The relative amounts of pro- and mature PCSK9 for each variant were determined by Western blot, independently of each other in order to avoid any bias that could be due to the level of expression of the constructs. Mutant R104C appeared to have decreased autocatalytic activity, whereas the autocatalytic cleavage of the V114A mutant was dramatically abolished when compared with that observed in cells expressing WT-PCSK9 (percentage of PCSK9 maturation at 62%, 13% and 2% in WT, R104C and V114A, respectively). Moreover, combination of both amino acid substitutions R104C and V114A totally abolished autocatalytic cleavage, in a similar extent to that of

the catalytically inactive S386A mutant. Secreted mature WT-PCSK9 was detected in cell culture medium by Western blot analysis using anti-V5 antibody (Figure 2, lower). As previously described,<sup>16</sup> cells expressing the S386A mutant appeared to secrete the uncleaved form of PCSK9 in the medium. A lower band appeared in the cells transfected with WT-PCSK9, potentially corresponding to the previously described cleavage product by furin and PC5/6A at RFHR<sub>218</sub>↓.<sup>17</sup> Compared with WT-PCSK9, amino acid substitution R104C slightly reduced and the V114A strongly impaired the secretion of the protein. Strikingly, the combination of both V114A and R104C resulted in no immunodetectable PCSK9 in the media, suggesting an additive effect. Altogether, these results indicate that the combination of both R104C and V114A amino acid substitutions, observed in heterozygous carriers, is associated with a severe defect of processing and secretion of PCSK9 in vitro.

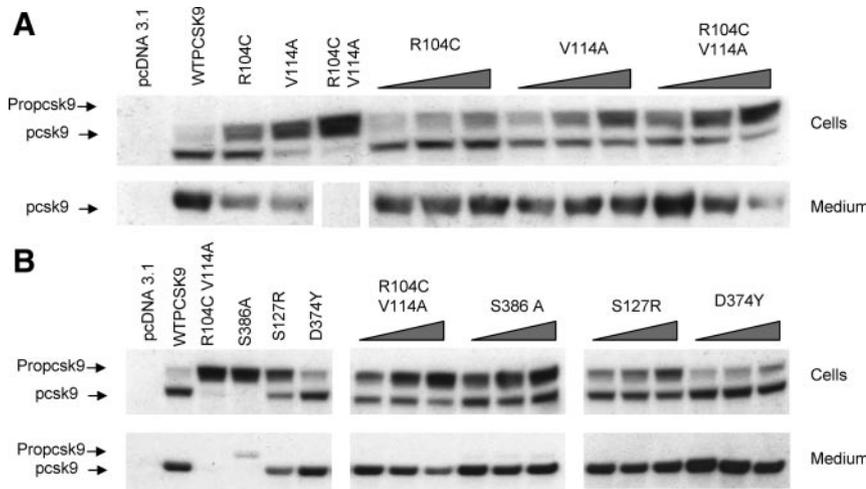
### PCSK9 R104C/V114A Acts as a Dominant Negative

Because the proband had undetectable levels of circulating PCSK9 and both mutations were found on a single allele, we hypothesized that R104C/V114A might exhibit dominant negative activity over the wild-type allele. We coexpressed WT-PCSK9 with increasing amounts of R104C, V114A, or R104C/V114A carrying the same V5 tag to avoid any bias attributable to the nature of the tag (Figure 3A). Single R104C and V114A mutations had no effect on WT-PCSK9 processing or secretion. The double mutant, however, obviously impaired PCSK9 protein secretion. The effect was patent when R104C/V114A expression represented as little as 25% that of WT-PCSK9 and was more pronounced when both were transfected in equal quantity (last lane). Next, we verified whether other PCSK9 variants would affect WT-PCSK9 secretion. Figure 3B presents the results obtained with the LOF mutant S386A and the GOF mutants S127R and D374Y. None of these variants seemed to affect PCSK9 secretion, suggesting that our findings are specific to the R104C/V114A mutant.

### PCSK9 R104C/V114A Increases LDL Clearance In Vivo

There is no demonstration yet that *PCSK9* LOF mutations increase LDL catabolism in human. To show that *PCSK9* R104C/V114A double mutant could alter LDL metabolism in human, in vivo kinetics of apoB100-containing lipoproteins using a 14-hour primed constant infusion of [2H] leucine were conducted in the proband (II.3) and his daughter (III.1), as well as in healthy control subjects (n=11, 34±12 years old; body mass index, 26±4.4 kg/m). The proband's sister, II.2, was not available for further investigation. Time course of enrichments in VLDL, IDL, and LDL apoB100 for the carriers and controls are shown as supplemental Figure VI. Model fitted lines and experimental points showed close agreement. Kinetic parameters are shown in Table 2.

For the proband, the apoB100 production is lower compared to controls rate (−25%; Table 2). This low apoB100 production is related to a lower proportion of apoB100 secreted in VLDL. Importantly, the fractional catabolic rate



**Figure 3.** PCSK9R104C/V114A acts as a dominant negative. Human immortalized hepatocytes were transfected with wild-type PCSK9 or mutants coding for PCSK9 variants R104C, V114A, R104C V114A, S127R, S386A, D374Y. Lanes 1 to 5 (A) or 1 to 6 (B) correspond to cells transfected with 5  $\mu$ g/well empty pcDNA3.1 or 2.5  $\mu$ g/well of WT PCSK9 or indicated variants +2.5  $\mu$ g/well empty pcDNA3.1. Dark colored triangles indicate lanes corresponding to cells transfected with constant amount of WTPCSK9 (2.5  $\mu$ g/well) with indicated variants at 0.62, 1.25, and 2.5  $\mu$ g/well from the left top to the right of the panel and adjustable doses of empty pcDNA3.1 for a total of 5  $\mu$ g/well of plasmid. After protein extraction, equal amounts were analyzed by Western blots with anti-V5 epitope antibodies.

(FCR) of these lipoproteins is higher than controls particularly in LDL (+256% for LDL compared with controls). The higher FCR in VLDL and IDL is related to a higher direct removal of VLDL (0.395 versus  $0.05 \pm 0.02$  hour) and a higher conversion rate of IDL (0.51 versus  $0.31 \pm 0.10$  hour), respectively.

For the proband’s daughter III.1, the apoB100 total production rate was higher than controls (+88%). However, the proportion of apoB100 secreted in VLDL, IDL, and LDL was similar to controls (respectively, 72%, 9%, and 17%), suggesting a higher production rate but a normal distribution of apoB100 on each lipoprotein class. This subject also displayed a higher FCR for VLDL, IDL, and LDL (+88%). Once again, this higher FCR is related to a higher direct removal (0.22 hour versus  $0.05 \pm 0.02$  hour for VLDL and 0.92 hour versus  $0.20 \pm 0.15$  hour for IDL).

**Discussion**

We report a French FHBL family with several individuals exhibiting low plasma LDLC, apoB, and PCSK9 concentrations, potential familial longevity, and normal liver function. Mutation carriers were heterozygous for R104C and V114A amino acid substitutions in PCSK9 prodomain. In the present case, family analysis revealed that both mutations segregated on a single-allele in subjects with a phenotype compatible with a mutation at a FHBL disease-causing locus. Both mutations were previously reported independently, as part of population screening by PCSK9 gene sequencing in healthy

individuals. The R104C was reported as a heterozygous rare allele (frequency <0.005) in a single hypercholesterolemic individual living in the area of Osaka, Japan.<sup>18</sup> The V114A mutation was also found heterozygous and rare (frequency <0.005) in a single hypocholesterolemic blood donor originating from Sicily, Italy.<sup>19</sup> A similar picture of very rare allele frequency was found in the French population because none of these mutations were found in both hypercholesterolemic and normolipidemic individuals. We also looked for potential mutations at other known FHBL-causing loci and found no evidence for any genetic complementation on LDL metabolism. In addition, the clinical presentation of a late dominant FHBL with very limited clinical consequences (ie, absence of impaired neurological development, of severe intestinal malabsorption, or severe liver steatosis) made complete deficiency rather unlikely at the *APOB* or *Microsomal triglyceride transfer protein* loci.

We used stable isotope tracer methodology to study in vivo kinetics of apoB100 in 2 subjects carrying the double mutation R104C/V114A. Our study demonstrates for the first time an accelerated LDL catabolism in patients affected by a PCSK9 LOF mutation. Taking into account previous studies in PCSK9<sup>-/-</sup> mice,<sup>20</sup> it is very probable that the increased FCRs in human are related to the overexpression of the hepatic LDLR. Indeed it was shown that not only LDL but also VLDL can be recaptured by the liver via the LDLR.<sup>21</sup> The increased apoB100-rich lipoprotein catabolism in PCSK9 LOF mutation carriers mirrors the deficient lipopro-

**Table 2. Lipoprotein Kinetic Parameters in Carriers of PCSK9 R104C/V114A Mutation and Controls**

	TPR	VLDL			IDL			LDL			
		PR	FCR	CR	PR	PRd	FCR	CR	PR	PRd	FCR
II.3	0.45	0.22	0.71	0.32	0.24	0.07	0.79	0.51	0.24	0.09	0.089
III.1	1.13	0.82	0.87	0.65	0.67	0.10	1.22	0.30	0.34	0.19	0.047
Controls											
Mean	0.60	0.43	0.38	0.33	0.47	0.06	0.51	0.31	0.43	0.11	0.025
SD	0.31	0.15	0.18	0.15	0.14	0.04	0.17	0.17	0.13	0.11	0.008

TPR indicates total production rate (in mg/kg/h); PR, production rate; PRd, direct production rate; FCR, fractional catabolic rate (in h-1); CR, conversion rate (in h-1).

Cf. supplemental Methods and supplemental Figure III for more explanation on the model used for rate constant calculations.

tein catabolism we previously reported in patients with the GOF mutation S127R.<sup>13</sup> However, the picture is not the same for the apoB100 production. We had found an apoB100 production twice that of FH patients in patients with the S127R mutation,<sup>13</sup> suggesting that PCSK9 could be involved in the packaging and plasma delivery of VLDL. Interestingly, circulating PCSK9 plasma concentration was correlated with plasma triglycerides in 2 recent studies.<sup>22,23</sup> However, in the present case, the daughter presented with increased apoB100 production compared with controls. The fact that estrogens increase apoB100 production while androgens attenuate this effect<sup>24,25</sup> supports possible effect of gender on variability of lipoprotein kinetics.

FHBL carriers of the R104C/V114A mutation had lowered to undetectable fasting plasma PCSK9 concentrations whereas noncarriers displayed no difference compared with levels found in unrelated fasted normolipidemic subjects. Our data fit with several recent reports, even though there is a large heterogeneity in the literature concerning PCSK9 plasma concentration range and absolute values.<sup>22,23,26,27</sup> Of note, its association with gender is not consistent.<sup>22,23</sup> Transient transfections of human hepatocytes by recombinant vectors showed that R104C somehow impaired PCSK9 cleavage, but V114A appeared even more deleterious on processing and overall secretion of PCSK9.

There was an obvious difference in the phenotype displayed by the proband with profound FHBL exhibiting about 250% increase in LDL catabolic rate and virtual absence of PCSK9 in plasma, and a phenotype of mild FHBL displayed by his sister and daughter also heterozygous for the same PCSK9 mutation but with appreciable PCSK9 plasma concentrations. Independent measurements were made at a distance from each other and confirmed this trait. A first explanation for lower LDLC in the proband comes from the apoB production that is increased in the daughter, as discussed above. The FHBL phenotype in the proband appears similar to that reported by Zhao et al (ie, 14 mg/dL) in a healthy compound heterozygote for 2 PCSK9 mutations ( $\Delta$ R97 and Y142X) or by Hooper et al in a homozygote for the C679X mutation resulting in the absence of PCSK9 in the bloodstream.<sup>11,12</sup> Therefore, profound FHBL cannot be explained solely by the presence of a single heterozygous LOF PCSK9 missense mutation, in the family described herein. We cannot exclude another unidentified mutation at the *PCSK9* locus, impairing PCSK9 expression. However this hypothesis is rather unlikely for several reasons. First, the severely affected proband and his mildly affected sister displayed exactly identical *PCSK9* genotype and parental haplotypes, ruling out the en face *PCSK9* allele for bearing another disease-causing mutation. Second, the daughter and sister both displaying a similar phenotype of mild FHBL had different en face *PCSK9* alleles, with 1 clearly inherited from proband's normolipidemic spouse, consistent with them being heterozygous carriers of a FHBL disease-causing mutation. Third, all relatives displayed heterozygosity for different *PCSK9* haplotypes spanning a large portion of the *PCSK9* locus, ruling out for very large gene rearrangement causing apparent haplo-insufficiency. Therefore the hypothesis of any mutation altering *PCSK9* expression (proximal promoter,

cryptic intronic, flanking, etc) present on the en face parental allele within the *PCSK9* gene appears rather unlikely in the proband.

Witzum et al identified an increased clearance rate of glycated LDL in diabetic patients that could be related to the presence of antibodies directed toward glycated proteins in their plasma, suggesting additional immune-mediated clearance of LDL in the proband.<sup>28</sup> However, we believe that this hypothesis is unlikely. Indeed, we and others have observed with kinetic studies a substantial decrease of LDL catabolism (20 to 30%) in diabetic patients.<sup>29,30</sup> Furthermore the proband's diabetes was well controlled under diet only at the time of the present kinetics, as illustrated by his level of HbA1c (5.6%). Since then, despite a very good glycaemic control (HbA1c <6.5%), his LDLC has remained in the very low concentration range.

Another plausible explanation for discrepant FHBL phenotype between proband and relatives is that the R104C/V114A mutant not only is a LOF mutation but could act as a dominant negative impairing wild-type allele PCSK9 function or secretion. This might explain why the intact PCSK9 allele is apparently silent in the proband, whereas its expression remains partially preserved in his sister and daughter. Indeed, we found in vitro evidence of such an effect from liver cells transiently cotransfected with several PCSK9 mutants together with wild-type PCSK9 allele. Only the R104C/V114A double mutant displayed a dominant negative dosage effect on wild-type PCSK9 secretion. There are numerous examples in human disease that dominant negative mutations may display highly variable disease penetrance and expressivity in heterozygous carriers of the same mutation. For example, patients with the same dominant negative mutations of PPAR $\gamma$ —a nuclear receptor involved in carbohydrate and lipid metabolism targeted by thiazolidinediones—display a variable phenotype of partial lipodystrophy, insulin resistance, and metabolic syndrome.<sup>31</sup> Nevertheless, we cannot isolate the dominant negative effect from the other factors influencing LDLC beside the LDLR pathway (like apoB production) and determine to which extent it really contributes to the phenotype.

Thus the R104C/V114A is the first example of a LOF PCSK9 missense mutation displaying dominant negative activity, which could account for the variable FHBL phenotype observed in this family. This is also the first demonstration that PCSK9 LOF mutations are accompanied by increased LDL catabolic rate in human, providing an additional argument for developing inhibitors of PCSK9 to treat hypercholesterolemia.

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# SUPPLEMENTAL MATERIAL

## Supplemental Methods

### BIOCHEMISTRY

#### Plasma marker analyzes.

Biochemical analysis was performed by the Biochemical Analysis Facility of Nantes University Hospital. PCSK9 concentration in plasma from overnight-fasted individuals, collected in an EDTA tube, was determined in duplicates using an ELISA kit targeting human PCSK9, according to the manufacturer recommendations (CycLex Co, Japan). The ELISA kit employs a quantitative sandwich enzyme immunoassay technique.

#### Fast Protein Liquid Chromatography.

Lipoproteins were isolated by FPLC (Fast Protein Liquid Chromatography). Two hundred microliters of plasma were injected into a MV-7 multi-injection loop and separation was performed on 2 superose 6 HR 10/30 columns in series with an elution flow rate of 0.35 mL/min; 0.5 mL was collected for each fraction and entire profile was completed within 105 minutes. The system was controlled by FPLC DIRECTOR software (Amersham Pharmacia Biotech Inc). Cholesterol and triglycerides levels were measured on FPLC fractions using commercially available enzymatic kits (Biomérieux, Marcy l'Etoile, France).

### MOLECULAR GENETICS

DNA was extracted from blood leucocytes by a phase exchange method (Puregene, Gentra systems, Minneapolis, USA) in family members and in 150 healthy blood donors from the Etablissement Français du Sang (Paris, Saint Antoine). Genomic sequences of *PCSK9* (<http://www.ncbi.nlm.nih.gov>, RefSeq# NC\_000001.9, GeneID 255738), *LDLR* (NCBI

Refseq # NC\_000019.8, GeneID 3949) and *APOB* (GenBank accession number NM\_000384), were PCR-amplified and sequenced as previously described<sup>1,2</sup>.

Proximal promoter, exonic and perixonic regions were sequenced on forward and reverse strands, by the Big-Dye terminator cycle-sequencing protocol on a 16-capillary DNA sequencer (ABI 3130, Applied Biosystems, France). For mutation detection, electronic DNA sequence tracks were read using software Seqscape™ (Applied Biosystems). For *APOB* mutation identification amino acid sequence changes in apoB are described according to the National Center for Biotechnology Information reference sequence (NP\_000375.2, GI:105990532). *APOE* polymorphism (alleles E2, E3 and E4; dbSNP rs429358 and rs7412, <http://www.ncbi.nlm.nih.gov/SNP/>) was assessed by allele-specific hybridization of PCR products on nitrocellulose strips (InnoGenetics, Belgium). Quality control analyses provided consistent genotyping results from three independent blood samples obtained in family members. A panel of informative Short Tandem Repeats (STR) from 6 independent human chromosomes (Amplif/STR COfiler, Applied Biosystems) was used to check for genetic relatedness and for quality control purposes.

## **BIOINFORMATICS**

The Gensearch® software (v3.5, Phenosystems, Belgium) was used to analyze raw sequence data for mutation detection and functionality qualification against HUGO criteria and public genomic databases (<http://www.phenosystems.com>). The algorithm uses base caller “Phred” ([www.phrap.com/Phred](http://www.phrap.com/Phred)) and incorporates computed criteria for DNA variation (substitutions or In/Del) qualification as possible or probable heterozygous or homozygous mutations, after filtering for known DNA polymorphisms. These criteria derive from computed comparisons of peak areas and relative positions along the sequence track, with genomic reference sequences and with wild-type sequence tracks. In addition, the software

provides links with open-access tools to public databases for genomic comparisons, structure and ontology analyses. In the present study, the BLAT and PhastCons (<http://genome.ucsc.edu/>) tools were used for estimation of evolutionary sequence conservation and genetic polymorphism analysis. Protein sequence alignments (NCBI accession#: NP\_777596 (human/ Homo Sapiens Sapiens); NP\_001104592 (Chimpanzee/Pan Troglodytes); NP\_001106130 (Rhesus Monkey/Macaca Mulatta); NP\_705793 (Mouse/Mus Musculus), were performed with BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Several amino acid substitution prediction algorithms based on protein sequence comparisons and estimated functional changes from amino-acid structural differences, were used: POLYPHEN <http://genetics.bwh.harvard.edu/pph/>; PMut <http://mmb2.pcb.ub.es:8080/PMut>; SNPs3D <http://www.snps3d.org>; SIFT <http://blocks.fhrc.org/sift>. Estimation of potential functionality of an amino acid change was considered as valid, if at least 3 algorithms provided consistent predictions.

## **MOLECULAR AND CELLULAR BIOLOGY**

### **Expression constructs for PCSK9-WT and mutant forms of PCSK9**

Wild type human PCSK9 (genebank AX207686) with a V5 epitope flag was inserted into pcDNA3.1 and used as a backbone in order to produce various mutants (Genscript, Piscataway, NJ, USA).

### **Western blot analysis of transfected IHH**

Immortalized human hepatocytes (IHH) <sup>3</sup> were cultured on collagen-coated flasks in William's E medium in the presence of a 10% fetal calf serum (FCS). They were seeded (1 X 10<sup>6</sup> cells/well) in 6-well plates. Expression plasmids (2.5 µg/well) were transiently transfected

into IHH cells with lipofectamine (5 $\mu$ L/well) in Dulbecco's modified Eagle Medium (DMEM) with 4.5 mM glucose, according to the manufacturer's protocol (InVitrogen). For co-transfection experiments, a total quantity of 5 $\mu$ g/well was used using empty pcDNA3.1 to reach this value. After 4.5 h, transfection medium was replaced with William's E medium without FCS for 24 h. Cells were scraped and homogenized in 1x phosphate-buffered saline containing 0.25% sodium-deoxycholate, 1% Triton X-100, and a protease inhibitor mixture (Roche Diagnostic). Proteins were analysed by western blot (70  $\mu$ g per lane) as described elsewhere <sup>4</sup>, using a mouse anti-V5 monoclonal antibody (InVitrogen). For secretion analysis, proteins from 500  $\mu$ l of cell culture media were precipitated with acetone and 30  $\mu$ g of proteins used for Western blot.

## **KINETICS OF LIPOPROTEINS**

The endogenous labelling of apoB100 was carried out by constant infusion of [2H<sup>3</sup>]-leucine in subjects fasted overnight for 12 h prior to the study, and who remained fasting during the entire procedure. Each subject received intravenously a prime of 10  $\mu$ mol.kg<sup>-1</sup> of tracer immediately followed by a constant tracer infusion (10  $\mu$ mol.kg<sup>-1</sup>.h<sup>-1</sup>) for 14 h. Venous blood samples were drawn into EDTA tubes (Venoject, Paris, France) at baseline and at 15, 30, 45 min, 1, 1.5, 2, 2.5 h, and then hourly until 14 h. Sodium azide, an inhibitor of bacterial growth, and Pefabloc SC (Interchim, Montluçon, France), a protease inhibitor, were added to blood samples at a final concentration of 1.5 and 0.5 mmol/L, respectively.

### **• *Isolation of apoB100 and measurement of enrichment.***

Lipoproteins were separated by ultracentrifugation and apoB100 was isolated by sodium dodecylsulfate polyacrylamide gel electrophoresis. The amino acids obtained by hydrolysis were esterified and derivatized and analyzed by electron-impact gas chromatography-mass spectrometry (5891A gas chromatograph connected with a 5971A

quadrupole mass spectrometer). The isotopic ratio was determined by selected ion-monitoring at m/z of 282 and 285. Calculations of apoB100 kinetic parameters were based on the tracer-to-tracee mass ratio<sup>5</sup>.

• *Measurements of lipids and apoB100*

Cholesterol and triacylglycerol concentrations were measured using commercially available enzymatic kits (Boehringer Mannheim GmbH, Mannheim, Germany) at three different sampling times. The percentage recovery of cholesterol, triacylglycerol and apoB100 after FPLC separation was higher than 85%. ApoB100 concentrations were obtained in lipoprotein fractions by combining selective precipitation and mass spectrometry (Beghin, 2000).

• *Modeling*

Kinetic analysis of tracer-to-tracee ratios was achieved using computer software for simulation, analysis and modeling (SAAMII, Resource Facility for Kinetic Analysis, SAAM Institute, Seattle, WA). The model used (**supplemental figure V**) and the measure of the kinetic parameters were previously described<sup>6</sup>. This model takes into account the heterogeneity of VLDLs, e.g. large (VLDL1) and small VLDL (VLDL2) and the shunt between VLDL and LDL. In this model, a forcing function, corresponding to the time course of plasma leucine enrichment, was used to drive the appearance of leucine tracer into apoB100 during the process of synthesis. Methods provided identified values  $\pm$  standard deviation obtained by iterative least squares fitting for individual kinetic parameters. Standard deviations were less than 30% for most of the parameters (data not shown). The use of more complex models did not provide significant improvement in the fitting from F test and Akaike information criterion<sup>7</sup>. For comparison with controls, the VLDL1 and VLDL2 data were presented as VLDL conversion rate (CR) and VLDL fractional catabolic rate (FCR), which represents the sum of conversion rate and direct removal rate. The VLDL conversion rate was

calculated as VLDL2 delipidation flux divided by total VLDL mass. The VLDL direct removal was calculated as a sum of VLDL1 and VLDL2 direct removal divided by total VLDL mass. The apoB100 production rate (PR) in mg/kg/h represents the product of FCR and pool size of apoB100 in lipoprotein fractions. Pools of apoB100 in VLDL, IDL, LDL were calculated by multiplying the apoB100 concentration by 0.045 (L/kg) assuming a plasma volume of 4.5% of body weight <sup>8</sup>.

## STATISTICS

Results are representative of 2 or more experiments. Error bars represent means  $\pm$  SD. A Student t test was used to estimate statistical significance.

## Supplemental Results

### Clinical and biological parameters of the proband's family members

Clinical and biological parameters were explored in volunteer family members of the proband (II.3) (**Table 1**). Of note, his son (III.2) was victim of an accidental death and one daughter (III.3) was not available for further investigations. One of his daughters (III.1, LDLC: 44 mg/dl) and his sister (II.2, LDLC: 57 mg/dl) had also FHBL. His wife (II.4, LDLC: 140 mg/dl) and his brother (II-1, LDLC: 93 mg/dl) were normolipidemic. Except for the proband (II.3), fasting plasma glucose was strictly normal in FHBL relatives (II.2: 4.8 mmol/l, III.1: 4.7 mmol/l). As expected, FHBL subjects had also low plasma apoB concentrations. Consequently, FHBL subjects exhibited highly cardioprotective apoB/apoA1 ratio (<0.25). The proband appeared to have low fasted plasma triglycerides levels for a type 2 diabetic patient, while both his sister (II.2) and his daughter (III.1) had near normal levels. In addition,

there was no striking difference in plasma levels of other apolipoproteins (i.e. apoC2, apoC3, apoE, Lp[a]), between FHBL subjects and normolipidemic relatives.

### **Fast Protein Liquid Chromatography (FPLC)**

Plasma from mutation-carriers II.3 and III.1 and from two healthy volunteers, a 36 year old male with a LDLC of 133 mg/dl and a 25 year-old women with a LDLC of 77 mg/dl were analysed by FPLC (**supplemental figure I**). Subjects fasted for 12 h and cholesterol and triglycerides were measured in the various fractions. As expected, controls exhibited peaks of LDLC higher than that of HDLC but this was not the case for FHLB mutation-carriers (**supplemental figure I, upper panel**). Of note, the peak of HDL for III.1 was shifted to the left, suggesting larger particles. This was probably due to a larger content of triglycerides in the HDLs of III.1, (**supplemental figure I, lower panel**).

### **Independent analysis of amino acid sequence conservation across animal species**

The independent analysis of amino acid sequence conservation across animal species suggested a potential functional effect induced in human, by the single allele double R104C/V114A mutation (**supplemental figure III**). Indeed, the presence of two adjacent DNA changes would generate a mutant protein harbouring 2 amino acid substitutions within a 10 amino acid stretch of the PCSK9 prodomain which is highly conserved in 3 primate species, with lower level of amino acid sequence conservation in other mammals (8 species). In contrast, when the R104C or the V114A mutations were considered alone, amino-acid substitution prediction algorithms failed to provide consistent estimations of functionality, both mutations being alternately predicted as probably damaging, possibly deleterious or neutral. The only valid result was obtained for the L483V genetic variant, being consistently estimated as neutral by the four algorithms.

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## Supplemental Figure Legend

### Figure I: Fast-protein liquid chromatography of R104C/V114A carriers

Fast-protein liquid chromatography (FPLC) profile of plasma samples from the proband (II-3, black square) and his daughter (III-1, black circle), carrying the R104C/V114A amino acid substitutions, and a 36-year old male (empty square) and 24-year old female (empty circle) controls. Cholesterol (upper panel) and triglycerides (lower panel) concentrations in the eluted fractions are indicated on the y axis.

### Figure II: Segregation Analysis of *APOB* Polymorphic Markers in the Family.

HBL subjects are represented by filled symbols, normolipidemic subjects by open symbols. Subjects represented by dotted-line symbols filled with “ND”, were not available for genetic studies. Age (years) and plasma LDLC (mg/dl) is indicated below symbols (ND: not determined). Single nucleotide polymorphisms (SNPs) are described according to coding sequence numbering.

### Figure III: Amino acid sequence alignment across species

Amino acid sequence alignment of PCSK9 N-terminal domain for human (*homo sapiens*), primates (Chimpanzee: *Pan troglodytes*, and Rhesus monkey: *Macaca mulatta*) and mouse (*Mus musculus*). Residues corresponding to the prodomain are coloured in blue. Residues non-identical to human sequence are coloured in red, and marked by a red “\*” below sequences; identical residues are marked by a “-“. R104 and V114 residues are highlighted in green and indicated by an arrow.

**Figure IV. R104C and V114A amino acid substitutions**

A) Ribbon representation of the complex between PCSK9 and EGF-A domain of LDLR using the PDB code 3BPS<sup>9</sup>. PCSK9 pro-domain is coloured from blue to red from the N- to the C-terminus. The catalytic domain is coloured in gray, the EGF-A domain of is coloured in yellow. The locations of various mutants are shown as sticks. The C-terminal domain of PCSK9 is not shown. B) Comparison between the structures of the Bacillus subtilis subtilisin (blue ribbon) and of the human PCSK9. The location of the I-48V mutant is shown as pink sticks. Images were produced using PYMOL

**Figure V: Model used for the lipoprotein kinetic analyses**

This model takes into account the heterogeneity of VLDLs.

**Figure VI:** Time course of enrichment of VLDL (x), IDL (●), and LDL (■) apolipoprotein B100 in a representative control subject and in the proband (II.3) and his daughter (III.1) subjects during [2H<sup>3</sup>]-leucine perfusion.

Figure I

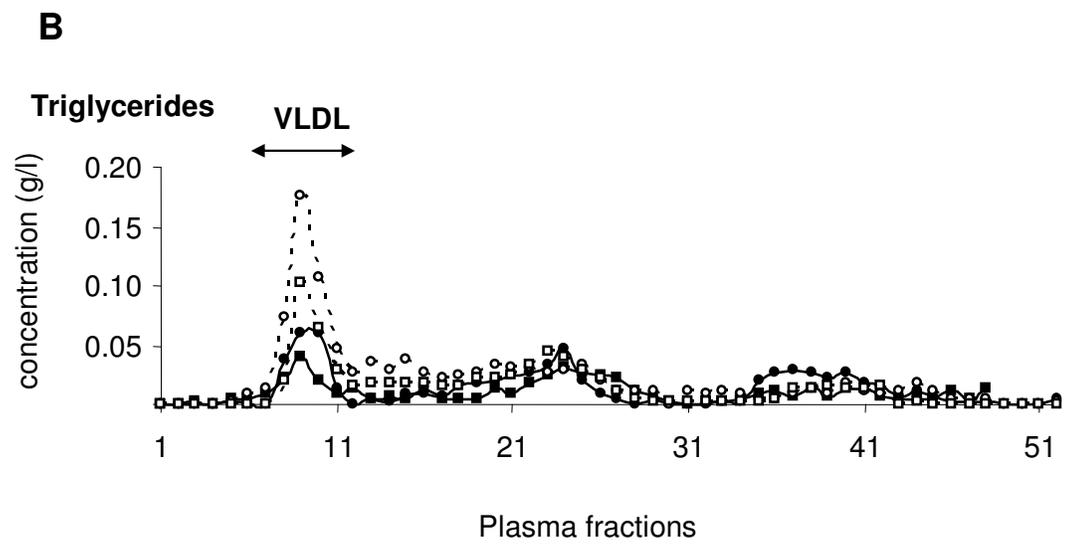
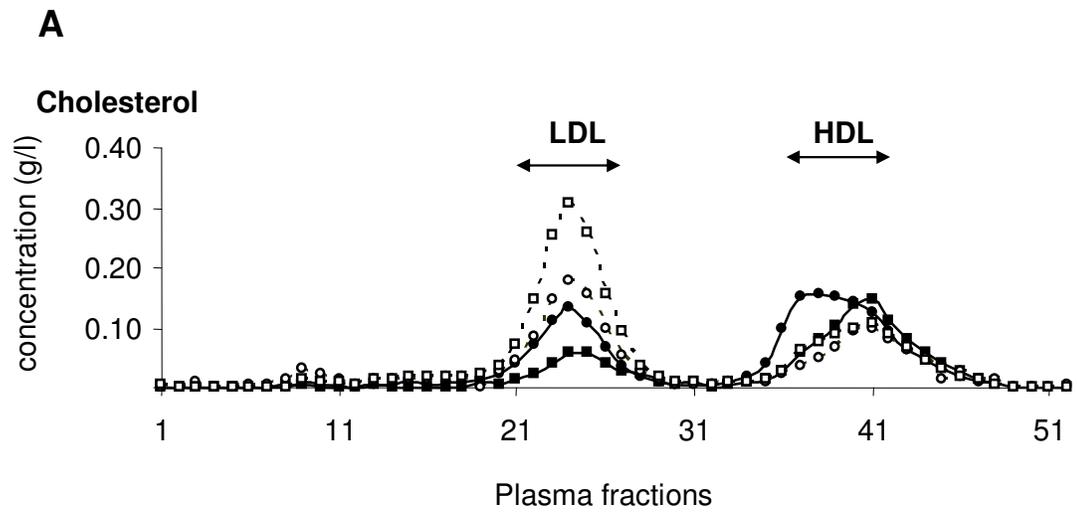


Figure II

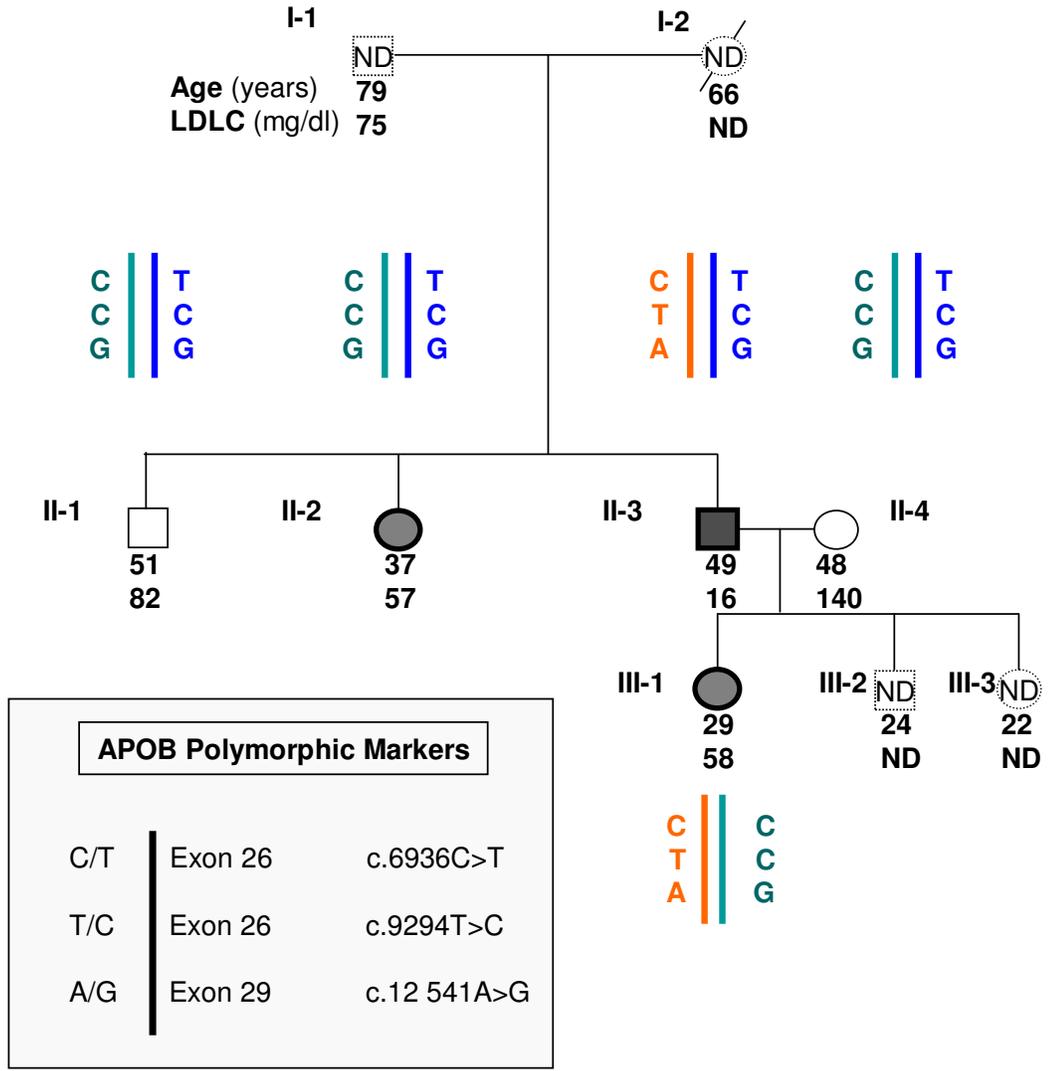


Figure III

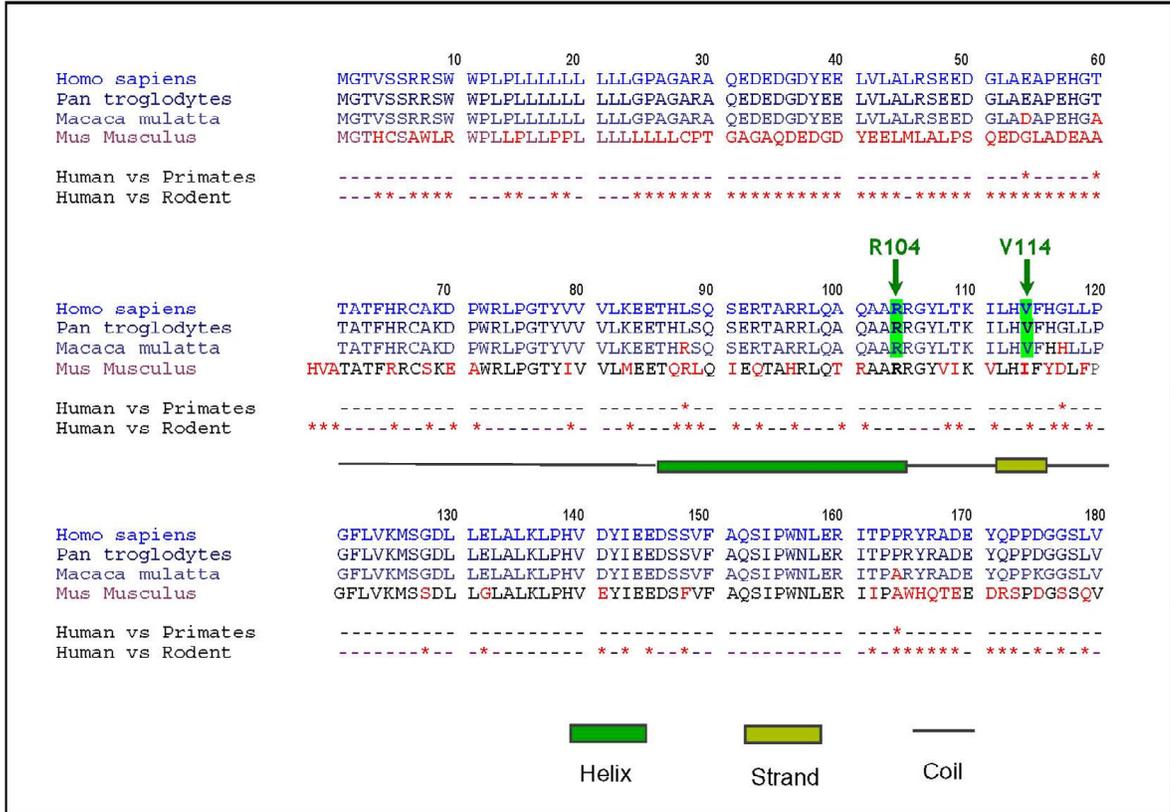
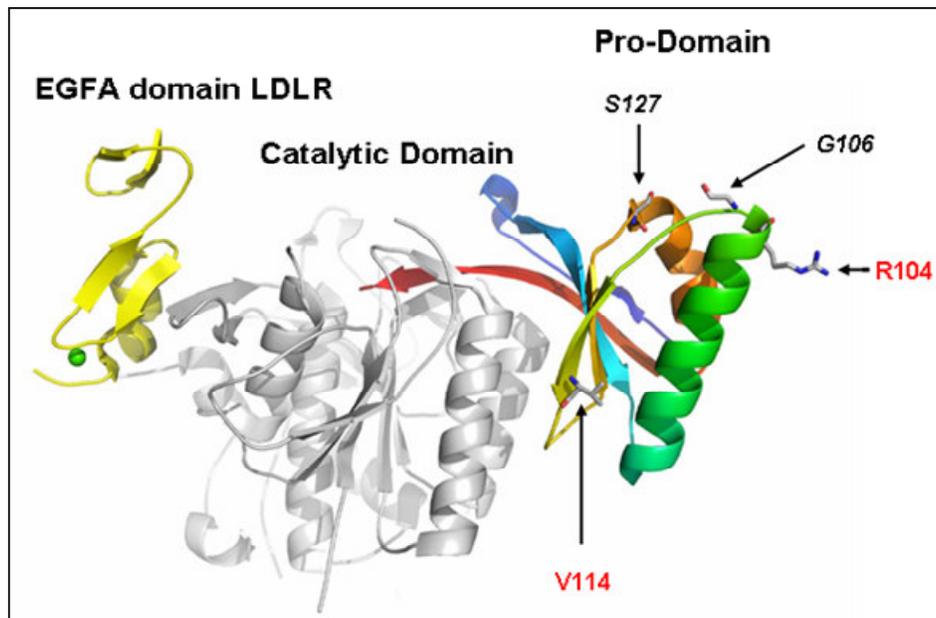


Figure IV

A



B

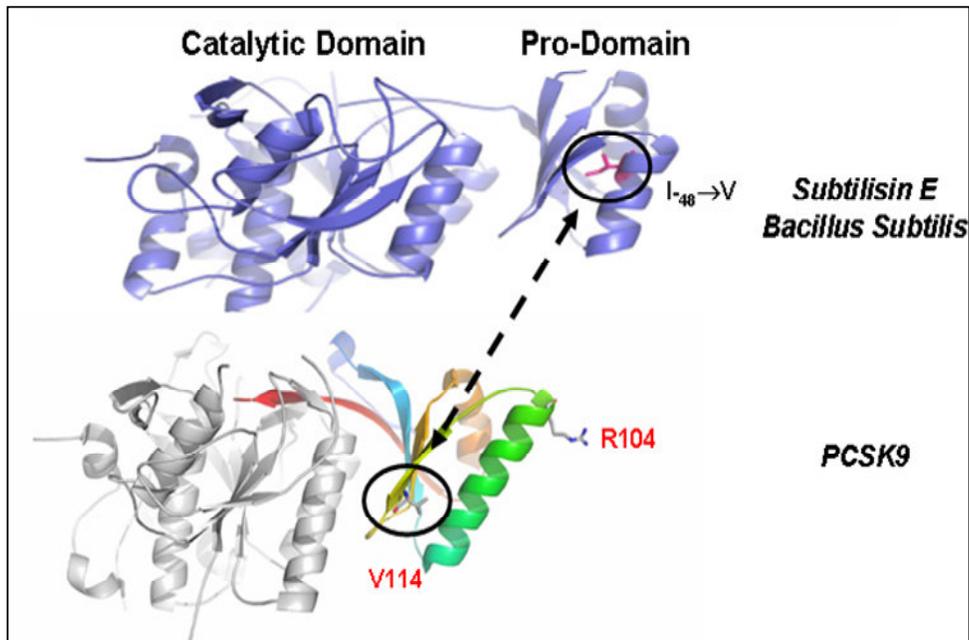


Figure V

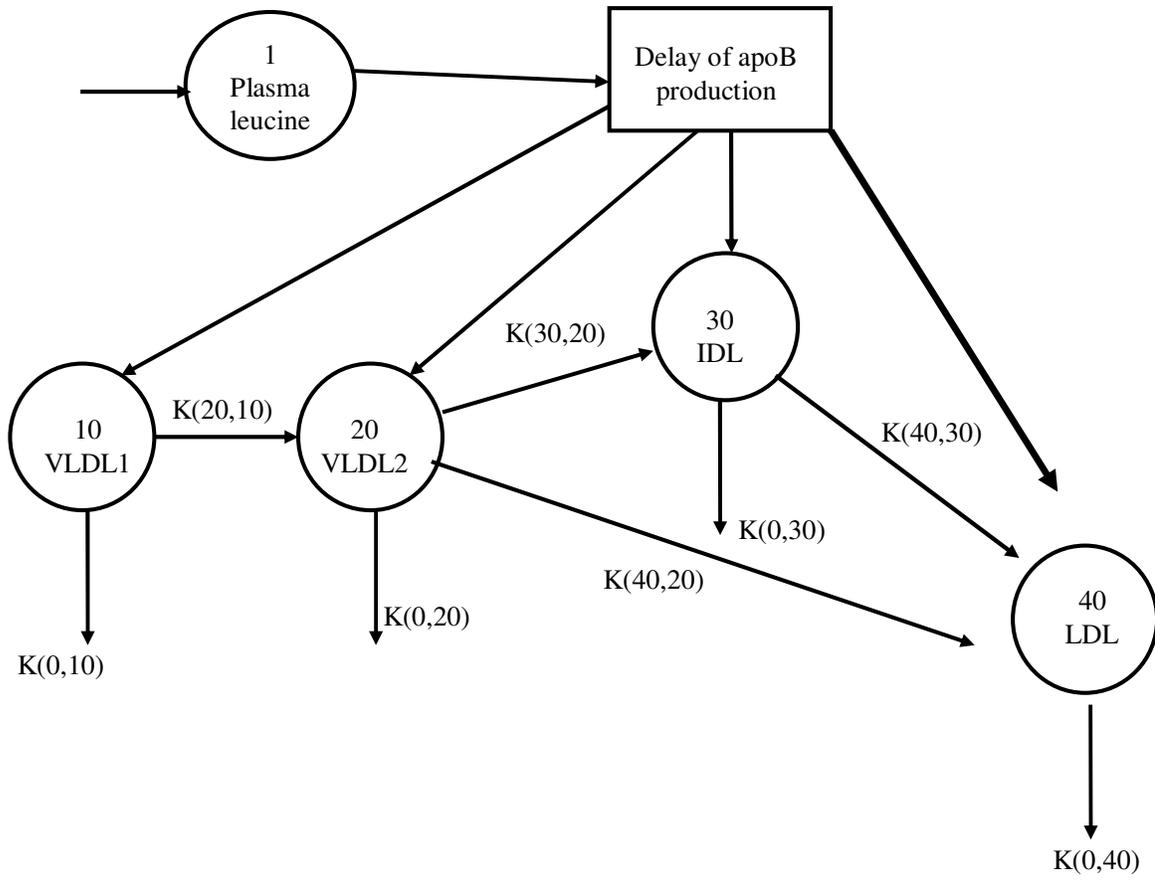


Figure VI

