

Clinical characteristics and plasma lipids in subjects with familial combined hypolipidemia: a pooled analysis[§]

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Abstract Angiopoietin-like 3 (ANGPTL3) regulates lipoprotein metabolism by modulating extracellular lipases. Loss-of function mutations in *ANGPTL3* gene cause familial combined hypolipidemia (FHBL2). The mode of inheritance and hepatic and vascular consequences of FHBL2 have not been fully elucidated. To get further insights on these aspects, we reevaluated the clinical and the biochemical characteristics of all reported cases of FHBL2. One hundred fifteen FHBL2 individuals carrying 13 different mutations in the *ANGPTL3* gene (14 homozygotes, 8 compound heterozygotes, and 93 heterozygotes) and 402 controls were considered. Carriers of two mutant alleles had undetectable plasma levels of ANGPTL3 protein, whereas heterozygotes showed a reduction ranging from 34% to 88%, according to genotype. Compared with controls, homozygotes as well as heterozygotes showed a significant reduction of all plasma lipoproteins, while no difference in lipoprotein(a) [Lp(a)] levels was detected between groups. The prevalence of fatty liver was not different in FHBL2 subjects compared with controls. Notably, diabetes mellitus and cardiovascular disease were absent among homozygotes. FHBL2 trait is inherited in a codominant manner, and the lipid-lowering effect of two *ANGPTL3* mutant alleles was more than four times larger than that of one mutant allele. No changes in Lp(a) were detected in FHBL2. Furthermore, our analysis confirmed that FHBL2 is not associated with adverse clinical

sequelae.^{¶¶} The possibility that FHBL2 confers lower risk of diabetes and cardiovascular disease warrants more detailed investigation.—Minicocci, I., S. Santini, V. Cantisani, N. Stitzel, S. Kathiresan, J. A. Arroyo, G. Martí, L. Pisciotta, D. Noto, A. B. Cefalù, M. Maranghi, G. Labbadia, G. Pigna, F. Pannozzo, F. Ceci, E. Ciociola, S. Bertolini, S. Calandra, P. Tarugi, M. Averna, and M. Arca. **Clinical characteristics and plasma lipids in subjects with familial combined hypolipidemia: a pooled analysis.** *J. Lipid Res.* 2013. 54: 3481–3490.

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Familial hypobetalipoproteinemia includes a heterogeneous group of inherited disorders of lipid metabolism typically characterized by very low levels (below the 5th percentile of age- and sex-specific values) of plasma low density lipoprotein cholesterol (LDL-C) and/or apoB (1, 2). Beside the very rare recessive disorder abetalipoproteinemia (ABL, OMIM # 200100) caused by mutation in the gene coding for the microsomal triglyceride transfer protein (*MTP*), the best-characterized cases are those with

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Abbreviations: ANGPTL3, angiopoietin-like 3; BMI, body mass index; FBG, fasting blood glucose; FHBL, familial hypobetalipoproteinemia; FHBL2, familial combined hypolipidemia; FLI, fatty liver index; γ -GT, γ -glutamyltranspeptidase; GFR, glomerular filtration rate; Lp(a), lipoprotein(a); TG, triglyceride.

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familial hypobetalipoproteinemia (FHBL, OMIM #107730). FHBL shows a dominant mode of inheritance with variable (from moderate to severe) degree of LDL-C or apoB reduction (3). Most of FHBL cases have been linked to mutations in the apoB gene (*APOB*) that lead to defects in secretion of VLDL in the liver (4). However, loss-of-function mutations in the gene coding for the proprotein convertase subtilisin kexin type 9 (*PCSK9*), a protein involved in the posttranslational regulation of the LDL receptor (LDLR), have been also reported to cause FHBL (5). Most patients with FHBL have no symptoms, although this disorder has been associated with hepatic steatosis, fat malabsorption, and, in the most severe forms, variable neurological manifestations (6).

Familial combined hypolipidemia (FHBL2, OMIM #605019) is a recently described low-cholesterol syndrome characterized by a peculiar lipid profile in which VLDL, LDL, and HDLs are comprehensively reduced (7). It is caused by mutations in the Angiopoietin-like 3 gene (*ANGPTL3*) that lead to the absence or the marked reduction of ANGPTL3 protein in the plasma (8–11).

In humans, *ANGPTL3* is located on chromosome 1 (1p31.1-p22.3) and encodes a 460 amino acid protein consisting of a signal peptide, an N-terminal segment containing coiled-coil domains, and a C-terminal fibrinogen-like domain, which are connected with a linker region (12). *ANGPTL3* is secreted almost exclusively by the liver, and its primary function is to act as a partial inhibitor of lipoprotein lipase and endothelial lipase, two enzymes involved in the lipolytic degradation of VLDL and HDL particles, respectively (13–17). Therefore, it is thought that *ANGPTL3* deficiency causes an overactivation of these lipases, leading to an accelerated catabolism of VLDL and HDL (16). The impact of *ANGPTL3* deficiency on LDL-C levels is more difficult to explain. An *in vivo* lipoprotein kinetic study in a family with FHBL2 showed a reduced production of VLDL and an increased LDL catabolism, but the mechanisms causing these defects are not well understood (18).

Since the first report (7), several additional cases of FHBL2 have been described. However several questions related to the lipid phenotypes of this disorder remain unclarified. First, the mode of inheritance of FHBL2 has not been firmly defined, as both recessive and codominant transmission have been reported. Musunuru et al. (7) proposed that inactivating *ANGPTL3* mutations show a codominant effect on LDL-C and plasma triglycerides (TGs) and a recessive effect on HDL-C, as only homozygous carriers presented significantly lower plasma concentration of this lipoprotein. Conversely, another report describing a larger cohort of FHBL2 subjects concluded that both TG and HDL-C levels are affected in a codominant manner by *ANGPTL3* mutations, while LDL-C was inherited as a recessive trait (11). Even though these discrepancies may be due to differences in studied cohorts, there is the possibility that they could be related to functional differences in FHBL2-causing alleles. Second, it is unknown whether *ANGPTL3* deficiency also affects plasma levels of lipoprotein (a) [Lp(a)]. Lp(a) is a LDL-like

lipoprotein, and previous studies have documented that its concentration may be modulated by levels of LDL-C in the plasma (19). A study investigating Lp(a) concentrations in kindred with FHBL, a cognate syndrome also characterized by low LDL-C levels, showed that Lp(a) levels are not changed comparing affected with unaffected individuals (20). Nevertheless, there is evidence that inhibition of apoB synthesis may affect plasma level of Lp(a) (21). Therefore, the investigation of Lp(a) in FHBL2 might provide further insight into LDL metabolism in these subjects.

It is well established that in *APOB*-linked FHBL severe fatty liver is present with a prevalence rate of approximately 20–30% (6). However, whether FHBL2 individuals have also increased risk of liver steatosis is unknown. An additional aspect that remains to be clarified is whether changes in glucose metabolism are part of the clinical phenotype associated with FHBL2. In fact, Minicocci et al. (11) reported lower fasting glucose and the lack of diabetes mellitus among homozygous carriers of inactivating mutation in the *ANGPTL3*, but the consistency of these findings remain to be confirmed. Finally, it has been suggested that the deficiency of *ANGPTL3* may be protective against atherosclerosis (22). In a previous report, homozygous FHBL2 subjects did not show evidence of history of cardiovascular disease, but the low number of cases investigated precluded any definitive conclusion (11).

Therefore, to get additional insight on these unclarified aspects of FHBL2, we designed this collaborative study aimed at collecting all available information in reported FHBL2 individuals with the following goals: 1) to more firmly establish the lipid expression of FHBL2, especially in heterozygous individuals; 2) to assess the clinical characteristics associated with FHBL2, with particular relevance to the presence of hepatic steatosis and the history of cardiovascular disease; 3) to compare plasma concentrations of *ANGPTL3* in subjects carrying different mutations in the *ANGPTL3* gene; and 4) to evaluate plasma levels of Lp(a) in homozygous and heterozygous FHBL2 carriers.

MATERIALS AND METHODS

Data sources and study selection

For the electronic searches, published studies were found through PubMed at the National Library of Medicine (<http://ncbi.nlm.nih.gov/entrez/query>) and in Medline databases by using the following queries “familial combined hypolipidemia” and “*ANGPTL3* mutation” and “familial hypocholesterolemia.” The literature search was done on studies up to December 2012 with availability of an English-language abstract and without country restriction. This search yielded five hits (7–11). Two authors (I.M. and M.A.) reviewed all articles independently either to determine the eligibility criteria or to examine the appropriateness of the research issue. The inclusion criteria were the availability of demographic, plasma lipid, and genetic data. None of articles was excluded. Afterwards, corresponding authors were invited to provide published and unpublished data of study subjects according to a prespecified protocol. All authors agreed to confer available data.

Study characteristics

Musunuru et al. (7) reported a family whose members had hypobetalipoproteinemia in which linkage analysis had ruled out *APOB* as the causative gene. Subjects reported by Pisciotta et al. (8) have been selected from a group of 150 individuals with primary hypobetalipoproteinemia who had been referred to the Lipid Clinics of the University Hospitals in Genova, Parma, Milan, and Modena over the last two decades. The selection criteria included low LDL-C and apoB associated with a marked reduction of HDL-C (all below the 5th percentile) and apoAI in the absence of mutations in the *APOB*, *MTP*, *PCSK9*, *APOA1*, *LCAT*, and *ABCA1* genes. Noto et al. (9) searched for FHBL2 subjects in a sample of 390 Italian and 523 American individuals with a total cholesterol below the 5th percentile of the relative population distributions. Martín-Campos et al. (10) selected unrelated subjects with biochemical features of FHBL based on a persistent age- and sex-specific cutoff point below the 5th percentile level of LDL-C and apoB for the Spanish population, in the absence of mutations in the *APOB* gene. Finally, Minicocci et al. (11) identified in Campodimele, a small town located in the province of Latina (Italy), a group of individuals with primary hypobetalipoproteinemia (LDL-C below the 5th age- and gender-specific percentile for the general Italian population) not carrying mutations in the *APOB*, *MTP*, or *PCSK9* gene.

In all studies the screening procedures were comparable. Subjects were examined in the morning and demographic information, medical history (with particular reference to liver and cardiovascular diseases), coronary artery disease (CAD) risk factors, and current medications (including vitamins and supplements) were recorded using a structured questionnaire. Measurements included height and weight and systolic and diastolic blood pressures. Fasting blood samples for laboratory determinations were also obtained. In one study (11), alcohol consumption was assessed using a semiquantitative scale (0 = abstainers, 1 = less than 2 glasses/day, and 2 = more than 2 glasses/day). All subjects were classified as hypertensive based upon the recording of blood pressure >140/90 mmHg and/or the use of antihypertensive medication, and/or affected by diabetes mellitus if they showed fasting blood glucose greater than 126 mg/dl and/or were using hypoglycemic medications. Details of data provided by each site are reported in the supplementary Table I. All participating studies were approved by the appropriate institutional ethical committees.

Genetic analyses

Musunuru et al. (7) analyzed the *ANGPTL3* gene by exome-sequencing technique, while the other authors performed genetic analyses by a combination of PCR amplification and direct sequencing of coding regions and intron-exon boundaries of *ANGPTL3* gene, as described (8–11). ApoE genotypes were determined in 68 FHBL2 subjects and 319 controls by using a previously described procedure (23).

Evaluation of hepatic steatosis

The association between FHBL2 and hepatic steatosis was evaluated by two different approaches. In all subjects we estimated fatty liver index (FLI), which is an algorithm based on body mass index (BMI), waist circumference (WC), TG, and γ -glutamyl transferase (γ -GT) and might serve as a simple and accurate predictor of hepatic steatosis in the general population (24). The FLI was calculated as reported and elevated FLI was defined as greater than 60 (24). In addition, the presence of hepatic steatosis was determined by ultrasound in 167 subjects belonging to the Campodimele's cohort. Liver examination was performed by a single radiologist blinded to the disease state of the subjects using

an Accuvix V 20 ultrasonographer (equipped with 2-6-MHz convex transducers) (Samsung Medison, Italy) and following a previously reported procedure (25, 26). Steatosis was defined by an appearance of hyperechoic liver parenchyma with tightly packed fine echoes and posterior beam attenuation (25).

Laboratory determinations

Plasma lipids, apoB, and apoAI were measured by standard techniques. LDL-C values were calculated by the Friedewald's formula. The assessment of Lp(a) was performed in 284 subjects (supplementary Table I). Measurements at Musunuru's, Pisciotta's, and Martín-Campos's sites were carried out by employing a nephelometric procedure (Siemens AG Healthcare, Munich, Germany), while those at Minicocci's site by using a particle enhanced turbidimetric immunoassay (Tina-quant Lipoprotein(a) (Latex)-Roche Diagnostics GmbH, Mannheim, Germany) adapted for the Roche/Hitachi COBAS CE 6000 Analyzer (27). ANGPTL3 plasma concentration was assessed in 177 subjects by ELISA method using an in-house-developed assay (n = 170) (11) and a commercially available kit (ADIPOGEN, Adipogen Inc., Korea) (n = 7). Serum samples were diluted 50-fold and measured in triplicate. Repeated measurements were made in homozygotes after 10-fold dilution of serum samples. The detection limit of both assays was 0.5 ng/ml; intra- and interassay coefficients of variation were less than 10% for the in-house assay and less than 7% for the commercially available assay, respectively.

Statistical analysis

All statistical analyses were performed with SPSS/WIN program (version 18.0; SPSS Inc., Chicago, IL). Descriptive statistics such as means, standard deviation (SD), and ranges were undertaken for all the variables. Continuous variables were compared by the Mann-Whitney U test, whereas the categorical variables were compared by χ^2 or Fisher's exact test. Bonferroni's correction was performed for multiple comparisons. Correlations were estimated by Pearson's test. Differences in lipid levels between mutation carriers and noncarriers were tested for significance by using ANOVA including age, sex, BMI, and site as covariates. The effect of number of mutant alleles on plasma lipids was also evaluated by comparing lipid residuals between carriers and noncarriers. To calculate residuals, age-, sex-, and BMI-adjusted coefficients for all lipid variables were calculated in the screened population by linear regression. The obtained equation model was used to calculate the expected lipid values for each individual, and residuals were obtained by subtracting expected from observed values. A two-sided $P < 0.05$ was considered statistically significant.

RESULTS

The study cohort was composed by 517 subjects, including 14 homozygous, 8 compound heterozygous, and 93 heterozygous carriers of mutations in the *ANGPTL3*, and 402 noncarrier controls. In this cohort were included 31 nuclear families that comprised 149 relatives (87 first-degree, 47 second-degree, and 15 third-degree relatives) and 45 spouses. Among FHBL2 individuals, 13 different mutations in the *ANGPTL3* gene were identified: 2 were nonsense (p.S17*, p.E129*), 7 were frameshift mutations introducing stop codons (p.I19Lfs*22, p.S122Kfs*3, p.K123Sfs*8, p.N147*, p.G400Vfs*5) in which two different nucleotide deletions (c.361-365delAACTC and c.363-367delCTCAA)

resulted in the same truncated protein (p.N121Kfs*3); one was an amino acid deletions (p.E96del) and 3 were missense mutations (p.G56V, p.F295L, p.R332Q). They were mostly located within the N-terminal portion of ANGPTL3 protein, with the exception of p.F295L, p.R332Q, and p.G400Vfs*5 mutations, which were located in the fibrinogen-like domain of the protein. The functional impact of missense variations was evaluated by using PolyPhen-2, and all missense mutations were annotated as “probably damaging.” The distribution of different genotypes in FHBL2 carriers is detailed in supplementary Table II.

The comparison of demographic and clinical characteristics of carriers and noncarriers of *ANGPTL3* mutations is reported in **Table 1**. No significant differences were observed between groups in gender and age distribution as well as in the prevalence of major coronary risk factors.

Homozygous carriers showed a tendency toward lower plasma fasting glucose, but this difference did not reach the statistical significance when compared with the other groups. No case of diabetes mellitus was reported among FHBL2 homozygous subjects. A significant reduction of plasma creatinine levels was observed in homozygotes when compared with either heterozygotes ($P < 0.01$) or noncarriers ($P < 0.05$), but this did not translate into a statistically significant difference in the calculated glomerular filtration rate (GFR). The overall prevalence of cardiovascular disease in FHBL2 was not different compared with that in noncarrier subjects (4.3% versus 3.7%; $P = 0.76$). However, it is noteworthy that no homozygous carrier reported history of cardiovascular disease. The prevalence of fatty liver estimated by FLI was not significantly different between the groups, and this corresponded

TABLE 1. Demographic and clinical characteristic of FHBL2 carriers and noncarriers controls

Variables	FHBL2 (n = 115)		
	Homozygotes /Compound Heterozygotes (n = 22)	Heterozygotes (n = 93)	Noncarriers (n = 402)
Demographic characteristics			
Age, in years (range)	51.7 ± 20.6 (12–88)	46.1 ± 20.9 (10–89)	50.4 ± 19.9 (9–96)
Sex, n (M/F)	9/13	46/47	187/215
Menopause, n (%)	5 (22.7)	16 (17.2)	108 (26.7)
BMI, (kg/m ²)	27.3 ± 5.1 n = 18	27.1 ± 5.0 n = 88	27.1 ± 4.8 n = 382
Family history of CVD, n (%)	3 (13.6)	24 (25.0)	104 (25.7)
Family history of diabetes mellitus, n (%)	4 (18.1)	27 (29.0)	127 (31.4)
Smokers, n (%)	5 (22.7)	20 (21.5)	81 (20.0)
Systolic blood pressure (mmHg)	131.2 ± 24.2	128.56 ± 20.8	129.0 ± 19.35
Diastolic blood pressure (mmHg)	79.7 ± 7.6	79.9 ± 10.2	80.6 ± 10.1
Alcohol consumption, n (%)			
Moderate	6 (27.3)	43 (46.2)	229 (56.5)
Heavy	0	3 (3.2)	6 (1.5)
Disease status, n (%)			
Hypertension	3 (13.6)	20 (21.5)	85 (21.1)
Diabetes mellitus	0	5 (5.3)	36 (9.0)
CVD	0	5 (5.4)	15 (3.7)
Cholelithiasis	0	1 (1.0)	17 (4.2)
Chronic hepatitis	0	1 (1.0)	4 (1.0)
Liver cirrhosis	0	1 (1.0)	0
Hepatic steatosis (FLI)	2 (9)	19 (20.4)	65 (16.2)
Pancreatitis	0	1 (1.0)	0
Current medication, n (%)			
Statin	0	3 (3.2)	40 (9.9)
Aspirin	1 (4.5)	5 (5.4)	31 (7.7)
Antihypertensive	5 (22.7)	16 (17.2)	86 (21.2)
Laboratory measurements			
FBG (mmol/l)	4.9 ± 0.7 n = 16	5.4 ± 1.5 n = 83	5.4 ± 1.3 n = 383
Creatinine (μmol/l)	79.6 ± 18.6* [#] n = 12	96.4 ± 16.8 n = 59	93.7 ± 17.7 n = 320
eGFR	74.6 (61.7–148.1) n = 10	77.9 (62.6–86.9) n = 58	73.5 (53.4–94.9) n = 303
ALT (U/l)	26.7 ± 6.8 n = 12	27.8 ± 6.9 n = 71	29.2 ± 10.0 n = 377
AST (U/l)	29.6 ± 10.8 n = 12	28.6 ± 15.7 n = 71	28.6 ± 13.8 n = 71
γ-GT (U/l)	35.0 ± 13.8 n = 6	30.5 ± 24.3 n = 65	33.5 ± 27.2 n = 201

Data are reported as number and percentage or mean ± SD. Frequency of menopause was calculated over the female sample; definition of hypertension is reported in Materials and Methods. eGFR was calculated using the Cockcroft-Gault formula; values are reported as median (interquartile ranges). * $P < 0.05$ for comparison between noncarriers versus homozygotes carriers; [#] $P < 0.01$ for comparison between homozygotes versus heterozygotes carriers. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CVD, ischemic cardiovascular disease.

to the lack of differences in liver transaminases and γ -GT across the groups. None of homozygous subjects reported positive history of liver disease. In addition, a liver ultrasound examination was performed in 64 p.S17* mutation carriers and in 103 noncarriers. In this subgroup, carriers showed on average higher BMI than noncarriers, but the difference was not significant ($28.0 \pm 5.2 \text{ kg/m}^2$ versus $27.0 \pm 4.6 \text{ kg/m}^2$; $P = 0.1$). Overall, we did not observe increased prevalence of liver steatosis in the FHBL2 group compared with the control group (43.7% versus 44.7%, $P = 0.70$). In addition, no association was detected between the severity of steatosis and the FHBL2 status (Fig. 1). One homozygous FHBL2 subject was classified as having severe steatosis (16.7%) as compared with 7 subjects in the non-carrier group (5.8%), but she was severely obese, showing a BMI of 41.0 kg/m^2 .

Plasma lipids and lipoproteins in *ANGPTL3* mutation carriers

The comparison of plasma lipid levels in carriers and noncarriers of *ANGPTL3* mutations was first performed within family members ($n = 225$) after adjustment for age, gender, and BMI (Table 2). Compared with noncarriers, homozygotes as well as heterozygotes showed a significant reduction of all plasma lipoproteins and apoproteins. When the analyses were performed in the entire cohort ($n = 517$), similar results were obtained (Table 2). No significant differences between groups were observed in plasma levels of Lp(a). Changes of plasma lipid in FHBL2 and controls were further investigated by analysis of lipid residues according to genotype number of mutant *ANGPTL3* alleles (Fig. 2). This analysis indicated that the presence of two mutant alleles significantly reduced LDL-C levels by 67.2%, TG by 71.2%, HDL-C by 39.0%, apoB by 48.4%, and apoAI by 95.1% (all $P < 0.001$). The presence of a single mutant allele reduced LDL-C by 8.6% ($P = 0.007$),

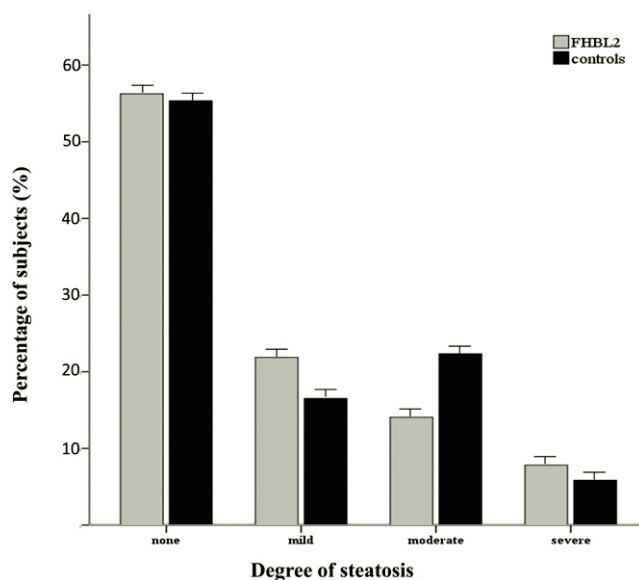


Fig. 1. Grade of liver steatosis in FHBL2 subjects carrying the *ANGPTL3* p.S17* mutation and noncarrier controls.

TG by 21.1% ($P = 0.005$), HDL-C by 16.8% ($P < 0.001$), apoB by 7.2% ($P < 0.008$), and apoAI by 13.1% ($P = 0.001$). Compared with men, heterozygous women showed higher HDL-C ($54.6 \pm 14.1 \text{ mg/dl}$ versus $47.0 \pm 13.9 \text{ mg/dl}$; $P = 0.006$) and lower TG ($67.9 \pm 30.7 \text{ mg/dl}$ versus $87.8 \pm 46.2 \text{ mg/dl}$; $P < 0.05$), while no significant gender-related difference in LDL-C or apoB level was observed. Moreover, in heterozygotes, age was significantly associated with LDL-C ($r = 0.18$; $P < 0.0001$), TG ($r = 0.11$; $P = 0.012$), HDL-C ($r = 0.12$; $P = 0.006$), apoB ($r = 0.27$; $P < 0.0001$), and apoAI ($r = 0.32$; $P < 0.0001$), as well as BMI with LDL-C ($r = 0.15$, $P = 0.001$), TG ($r = 0.26$; $P < 0.0001$), HDL-C ($r = -0.17$; $P < 0.0001$) and apoB ($r = 0.27$; $P < 0.0001$) but not with apoAI ($r = 0.01$; not significant). As expected, TG showed strong inverse correlation with HDL-C ($r = 0.33$; $P < 0.0001$). No significant difference in the distribution of apoE genotypes was observed between FHBL2 subjects and controls (Table 2).

The lipid values as well as the demographic characteristics of carriers of different *ANGPTL3* mutant alleles are also shown in supplementary Table II. The lowest plasma lipid concentrations were seen among carriers of p.S17*/p.E129* and p.N147*/p.F295L genotypes, even though the low number of individuals in each group prevents drawing any definitive conclusions.

The cohort enrolled by Minicocci et al (11), was remeasured four years after the first screening. Therefore, this allowed us to evaluate the time-trend changes of plasma lipids in mutations carriers and noncarriers. To this purpose, plasma lipids of 199 subjects (6 FHBL2 homozygotes and 50 heterozygotes) and 143 noncarriers tested in the years 2007 and 2011 were compared (Fig. 3). While homozygotes showed stable lipid profile over time, heterozygotes had a significant increase of TG ($P < 0.001$) and apoB ($P < 0.05$) concentrations. Notably, noncarrier subjects presented a significant increase of LDL-C and TG levels ($P < 0.001$) and a reduction of HDL-C ($P < 0.01$). It is worth mentioning that during follow-up, a significant average 7% increase ($P < 0.05$) of BMI was observed in all subjects. However, despite these changes, in heterozygotes plasma levels of TG ($P = 0.004$), LDL-C ($P < 0.001$), and HDL-C ($P = 0.043$) remained significantly lower than those in noncarrier controls.

Serum concentration of *ANGPTL3* protein according to genotypes

Measurements of plasma levels of *ANGPTL3* were available in carriers of p.S17*, p.I19Lfs*22, p.E96del, p.S122Kfs*3, and p.G400Vfs*5 mutations. All homozygotes or compound heterozygotes ($n = 12$) showed undetectable plasma levels of *ANGPTL3*, thus clearly suggesting that all mutations cause null alleles (Fig. 4). To evaluate whether different alleles might have a variable effect on protein expression, plasma levels of *ANGPTL3* were compared between carriers of the different *ANGPTL3* gene variants (Fig. 4). Compared with noncarriers, we observed markedly ($P < 0.0001$) reduced plasma levels of *ANGPTL3* in heterozygous carriers of p.S17* (-39.3%) and p.S122Kfs*3 (-87.9%). Also carriers of mutations p.I19Lfs*22, and

TABLE 2. Lipid, lipoprotein profile, and apoE genotypes in FHBL2 carriers and noncarrier controls identified in families and in the whole cohort

Variables	Family Study (n = 225)			All Subjects (n = 517)		
	FHBL2 (n = 98)			FHBL2 (n = 115)		
	Homozygotes/Compound Heterozygotes	Heterozygotes	Noncarriers (n = 127)	Homozygotes/Compound Heterozygotes	Heterozygotes	Noncarriers (n = 402)
TC (mmol/l)	1.9 ± 0.5 ^{ci} (1.3–2.8) (n = 19)	4.2 ± 0.9 ^f (2.1–6.2) (n = 79)	4.9 ± 1.0 (3.2–9.5) (n = 127)	1.9 ± 0.5 ^{ci} (1.3–2.8) (n = 22)	4.2 ± 1.0 ^f (1.6–6.2) (n = 93)	4.9 ± 0.9 (3.1–9.5) (n = 402)
HDL-C (mmol/l)	0.6 ± 1.3 ^{ci} (0.3–1.2) (n = 19)	1.3 ± 0.3 ^f (0.7–2.2) (n = 79)	1.5 ± 0.4 (0.8–2.4) (n = 127)	0.6 ± 0.2 ^{ci} (0.3–1.2) (n = 22)	1.3 ± 0.4 ^f (0.6–2.0) (n = 93)	1.6 ± 0.4 (0.8–2.8) (n = 402)
TG (mmol/l)	0.4 ± 0.1 ^{ci} (0.2–0.7) (n = 19)	0.8 ± 0.4 ^f (0.2–2.1) (n = 79)	1.1 ± 0.7 (0.4–4.3) (n = 127)	0.4 ± 0.2 ^{ci} (0.2–0.8) (n = 22)	0.9 ± 0.5 ^f (0.2–2.2) (n = 93)	1.1 ± 0.7 (0.3–4.3) (n = 402)
LDL-C (mmol/l)	1.3 ± 0.6 ^{ci} (0.5–1.4) (n = 19)	2.4 ± 0.8 ^f (0.7–4.4) (n = 79)	2.8 ± 0.9 (1.5–7.3) (n = 127)	1.1 ± 0.4 ^{ci} (0.5–1.4) (n = 22)	2.5 ± 0.8 ^f (0.5–4.4) (n = 93)	2.8 ± 0.9 (1.0–7.3) (n = 402)
ApoB (g/l)	0.5 ± 0.1 ^{ci} (0.3–0.7) (n = 19)	0.8 ± 0.2 (0.4–1.3) (n = 79)	0.9 ± 0.2 (0.5–1.6) (n = 126)	0.5 ± 0.1 ^{ci} (0.3–0.7) (n = 20)	0.8 ± 0.2 ^f (0.3–1.3) (n = 92)	0.9 ± 0.2 (0.5–1.8) (n = 398)
ApoAI (g/l)	0.7 ± 0.2 ^{ci} (0.4–1.1) (n = 18)	1.5 ± 0.3 (0.7–2.2) (n = 79)	1.6 ± 0.3 (1.0–2.4) (n = 114)	0.7 ± 0.2 ^{ci} (0.4–1.1) (n = 18)	1.5 ± 0.4 ^f (0.7–2.2) (n = 92)	1.6 ± 0.3 (0.9–2.4) (n = 302)
Lp(a) (μmol/l)	0.2 (0.1–2.1) (n = 13)	0.5 (0.2–0.9) (n = 70)	0.6 (0.2–1.9) (n = 85)	0.2 (0.2–1.2) (n = 13)	0.5 (0.2–0.9) (n = 78)	1.0 (0.1–2.1) (n = 193)
ApoE genotype, n (%)						
ε2 / ε2	—	1 (2.0)	—	—	1 (1.7)	—
ε3 / ε3	8 (80.0)	35 (71.4)	71 (84.6)	8 (80.0)	42 (72.4)	284 (89.0)
ε4 / ε4	—	—	—	—	1 (1.7)	—
ε2 / ε3	1 (10.0)	9 (18.4)	6 (7.1)	1 (10.0)	9 (15.5)	14 (4.4)
ε2 / ε4	—	—	1 (1.2)	—	—	1 (0.3)
ε3 / ε4	1 (10.0)	4 (8.2)	6 (7.1)	1 (10.0)	5 (8.6)	20 (6.3)

ApoE genotypes have been determined in 68 FHBL2 and in 319 noncarrier subjects. Data are reported as mean ± SD (minimum to maximum values). Lp(a) values are reported as median (interquartile ranges). Statistical comparisons were adjusted for age, gender, BMI, sites, and family. TC, total cholesterol; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; ApoB, apolipoprotein B; ApoAI, apolipoprotein AI, Lp(a), lipoprotein (a).

^a*P* < 0.05, ^b*P* ≤ 0.01, ^c*P* < 0.001 for comparison between noncarriers and homozygotes carriers.

^d*P* < 0.05, ^e*P* < 0.01, ^f*P* < 0.001 for comparison between noncarriers and heterozygotes carriers.

^g*P* < 0.001 for comparison between homozygotes and heterozygotes carriers.

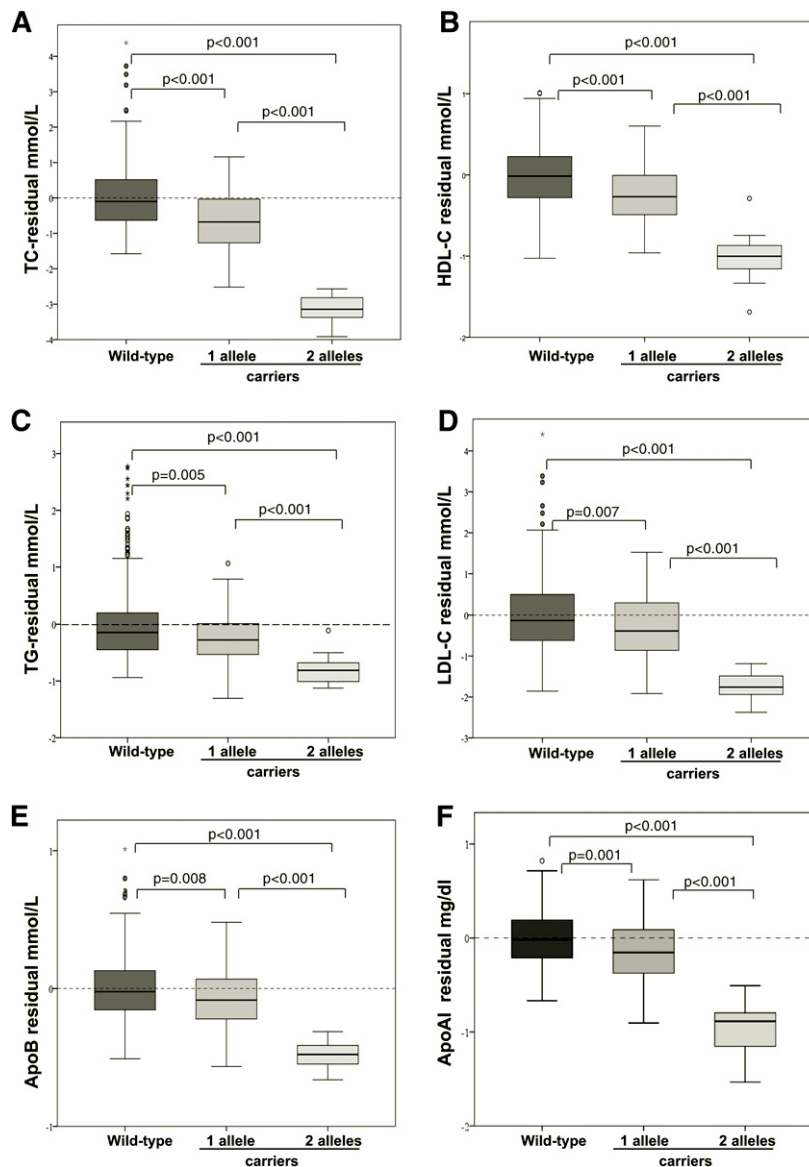


Fig. 2. Adjusted plasma lipid residuals, according to the number of mutant *ANGPTL3* alleles. Shown are the levels of TC- (A), HDL-C- (B), TG- (C), LDL-C- (D), apoB- (E), and apoAI- (F) residuals according to the *ANGPTL3* genotype. The box plots give the median levels (middle horizontal line in each box), the interquartile ranges (delineated by the top and bottom of each box), and outliers falling below the 5th percentile or above the 95th percentile (points below or above the vertical lines, respectively).

p.G400Vfs*5 showed lower plasma levels of *ANGPTL3*, although the difference did not reach the statistical significance. Surprisingly, the p.E96del variant was associated with plasma *ANGPTL3* concentration in the upper level of normal.

DISCUSSION

We analyzed a large set of individuals affected with FHBL2 and controls to get additional insights into the phenotypic expression of FHBL2. We find that individuals carrying one mutant *ANGPTL3* allele had a significant reduction of all apoB- and apoAI-containing lipoproteins, thus indicating that inactivating *ANGPTL3* mutations affect the lipid

phenotype in a codominant manner. However, we noted that there was not a linear gene-dosage-dependent effect of *ANGPTL3* mutations, as two mutant alleles reduced plasma lipids ranging from 48% to 95%, whereas one mutant allele reduced them from 7% to 20% depending on the lipoprotein class. It is noteworthy that despite changes in BMI, homozygotes (but also heterozygotes) showed persistently lower plasma when compared with noncarrier controls.

In our collection, the vast majority of FHBL2-causing mutations (78%) predicted a truncated *ANGPTL3* protein, and only three caused missense alleles. To explore the genotype-phenotype relationship in terms of impact of different mutant alleles on plasma concentration of *ANGPTL3* protein, we compared the plasma levels of *ANGPTL3* in heterozygous carriers of some *ANGPTL3*

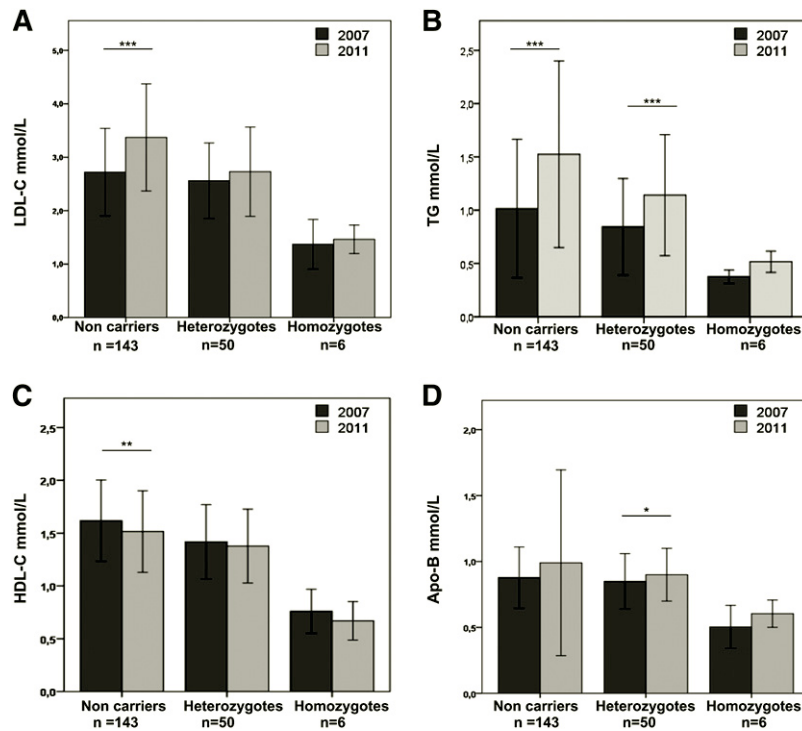


Fig. 3. Time-trend changes of plasma lipids in ANGPTL3 mutations carriers and noncarriers. LDL-C (A), TG (B), HDL-C (C), and apoB (D). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for comparison between 2007 and 2011 examinations.

mutations. Although these comparisons might be hampered by the small number of measured individuals and by the difference in the methods used, we detected some relevant variations in the functional effect of the different *ANGPTL3*

variants. In fact, mutations predicting a truncated protein (p.S17*, p.G400Vfs*5, and p.S122Kfs*3) had the most pronounced effect in reducing plasma levels of ANGPTL3. Conversely, the p.E96del variant was associated with plasma

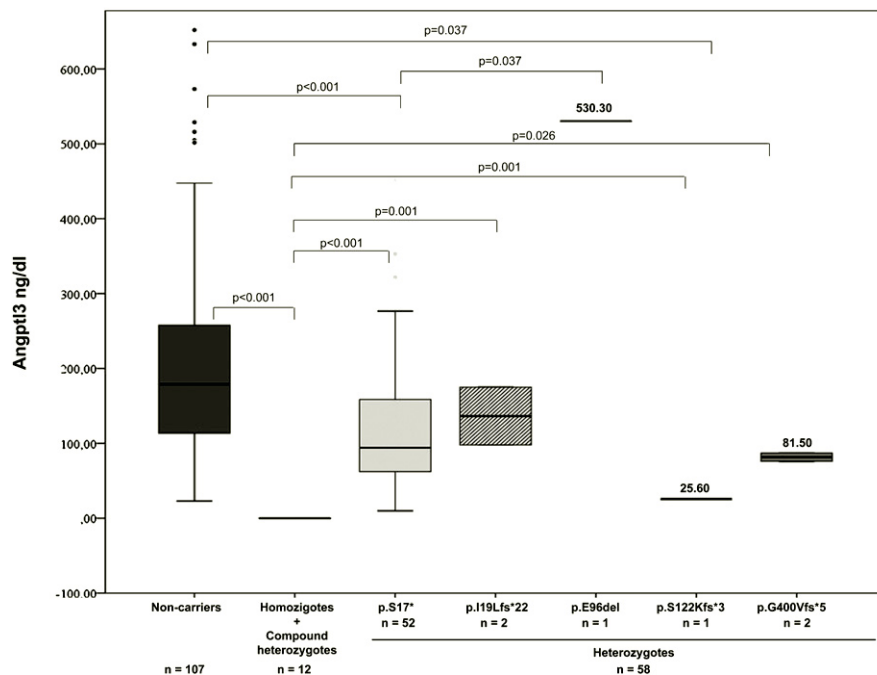


Fig. 4. Serum ANGPTL3 levels according to carriers of the different *ANGPTL3* gene variants. Shown are the mean plasma levels of ANGPTL3 according to the genotype. The box plots give the median levels (middle horizontal line in each box), the interquartile ranges (delineated by the top and bottom of each box), and outliers falling below the 5th percentile or above the 95th percentile (points below or above the vertical lines, respectively).

ANGPTL3 levels in the upper level of normal, thus questioning its functional role. The observation that mutations predicting protein truncation were associated with a complete absence of ANGPTL3 in the plasma, when present in a homozygous state, strongly argues that they may severely impair the synthesis or secretion of ANGPTL3 from hepatocytes. Conversely, no information about the functional role of the three missense *ANGPTL3* mutations (p.G56V, p.F295L, and p.R332Q) can be derived from the present data. Nonetheless, it must be noted that these mutations involved highly conserved amino acids and turned out to be probably damaging to in silico analysis. It may be interesting to report that nearby mutations have been demonstrated in vitro to abolish the capacity of ANGPTL3 to inhibit lipoprotein lipase activity (p.K63T) or the secretion of ANGPTL3 from cells (p.F295L and p.R332Q) (28). Only the direct expression of these missense variants could clarify their functional impact.

In the present cohort of FHBL2 subjects, we did not observe any increase in the prevalence of fatty liver as estimated by FLI or by raised serum transaminase levels. This conclusion was further supported by the results of the ultrasound examination of a FHBL2 subgroup, in which no significant association was detected between the prevalence and degree of steatosis and the FHBL2-affected status. It must be noted that the prevalence of fatty liver disease in noncarriers (44%) was similar to that reported by ultrasounds in overweight Italian population samples (29, 30) making them reliable controls. Therefore, the hepatic consequence of FHBL2 appears to be strikingly different from that of *APOB*-linked FHBL in which association with increased prevalence of liver steatosis has been consistently reported (6, 31). The fact that FHBL2 subjects do not show excessive liver fat infiltration despite reduced secretion of VLDL (18) might suggest the presence of a compensatory reduced synthesis of TG by hepatocytes (32). It has been demonstrated that mice lacking ANGPTL3 have lower plasma FFA (33) and that recombinant ANGPTL3 produces an elevation of both FFAs and glycerol in cultured adipocytes (34). Therefore, we could speculate that in FHBL2 there is a lower flow of FFA into the liver that provides lesser substrates for the synthesis of TG and their assembly with apoB into nascent VLDL particles. Further investigations will be needed to directly demonstrate this mechanism.

A still open question related to the lipid phenotype of FHBL2 is its impact on plasma concentration of Lp(a). Here we found that, compared with nonaffected subjects, FHBL2 individuals did not have different Lp(a) levels and that the very low levels of apoB seen in homozygotes was not associated with significant variation in Lp(a). Any possible interpretation of this finding should be cautious. In fact, we did not evaluate the apo(a) genotypes, which are the strongest determinants of plasma levels of Lp(a) (35), thus leaving the possibility that the lack of difference in Lp(a) levels might be due to a different distribution of apo(a) isoforms between groups. Nevertheless, our data might indicate that the lack of ANGPTL3 does not have significant impact on Lp(a) metabolism. To better interpret this findings, direct measurements of plasma levels of

apo(a) and the accurate estimation of the proportion of apo(a) complexed to LDL in FHBL2 is warranted.

Given the striking reduction of plasma HDL-C and the demonstration that sera obtained from FHBL2 have reduced HDL-mediated cholesterol efflux capacity in vitro (8), it may be of interest to explore the association between FHBL2 and atherosclerosis. Even though systematic search for asymptomatic vascular damage was not performed, no increased prevalence of clinically overt manifestations of atherosclerotic cardiovascular disease was detected in the present cohort of FHBL2 individuals. This observation might be interpreted on the basis that the life-long exposure to low levels of VLDL and LDL may counterbalance the putative “proatherogenic” effect related to low HDL-C. Unfortunately, the small size of the present cohort precludes drawing any definitive conclusion about the association between ANGPTL3 deficiency and atherosclerosis, an issue that merits additional, more detailed investigation.

Finally, extending our previous observation (11), we found that none of homozygotes was affected by diabetes mellitus. It seem interesting to consider this observation in the light of results by Romeo et al. (28) that reported in a population study higher prevalence of nonsynonymous (probably inactivating) variations in the *ANGPTL3* genes among subjects in the lowest quartile of blood glucose levels. Moreover, we recently reported that FHBL2 homozygotes showed lower plasma levels of insulin and lower degree of insulin-resistance as estimated HOMA-IR index (36). If further confirmed in additional investigations, these findings could highlight a more comprehensive role of ANGPTL3 in regulating not only lipid but also glucose metabolism.

The present study has some limitations. Although this represents the largest cohort of FHBL2 subjects collected so far, the number of affected individuals, mainly homozygous carriers, remains too small to firmly establish the impact of the FHBL2 on cardiovascular risk. More importantly the different sources of data might have introduced some bias in estimates. However we tried to compensate for this by adjusting comparisons for sources of the studies.

In conclusion, our results demonstrate that loss-of-function mutations in the *ANGPTL3* gene causing FHBL2 affect plasma levels of apoB- and apoAI-containing lipoproteins in a codominant manner and that the quantitative lipid-lowering effect of the presence of two mutant alleles was almost four times greater than that of one mutant allele. The deficiency of ANGPTL3 does not affect plasma levels of Lp(a). The present analysis indicates the lack of association between FHBL2 and fatty liver disease, but an apparent lower risk of diabetes mellitus and cardiovascular disease was noticed in FHBL2 homozygotes. More detailed investigations are needed to further explore these clinical aspects associated with FHBL2. **■**

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