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Clinical and biochemical characteristics of individuals with low cholesterol syndromes: a comparison between Familial Hypobetalipoproteinemia (FHBL1) and Familial combined hypolipidemia (FHBL2)

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CLINICAL AND BIOCHEMICAL CHARACTERISTICS OF INDIVIDUALS WITH LOW CHOLESTEROL SYNDROMES: A COMPARISON BETWEEN FAMILIAL HYPOBETALIPOPROTEINEMIA (FHBL1) AND FAMILIAL COMBINED HYPOLIPIDEMIA (FHBL2).

Running title: Clinical and biochemical features in *APOB*-related FHBL1 and *ANGPTL3*-related FHBL2.

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ABSTRACT

Background. The most frequent monogenic causes of low plasma cholesterol are familial hypobetalipoproteinemia (FHBL1), due to truncating mutations in apolipoprotein B coding gene (*APOB*), and familial combined hypolipidemia (FHBL2) due to loss-of-function (LOF) mutations in *ANGPTL3* gene. **Objective.** A direct comparison of lipid phenotypes of these two conditions has never been carried out. In addition, while an increased prevalence of liver steatosis in FHBL1 has been consistently reported, the hepatic consequences of FHBL2 are not well established. **Methods.** We investigated 350 subjects, 67 heterozygous carriers of *APOB* mutations, 63 carriers of **the p.S17* mutation** in *ANGPTL3* (57 heterozygotes and 6 homozygotes) and 220 non-carrier normolipemic controls. Prevalence and degree of hepatic steatosis was assessed by ultrasonography.

Results. A steady decrease of low-density lipoprotein cholesterol (LDL-C) level was observed from heterozygous to homozygous FHBL2 and to FHBL1 individuals, with the lowest levels in heterozygous FHBL1 carrying truncating mutations in exons 1-25 of *APOB* (*P* for trend <0.001). Plasma triglycerides levels were similar in heterozygous FHBL1 and homozygous FHBL2 individuals,

but higher in heterozygous FHBL2. The lowest high-density lipoprotein cholesterol (HDL-C) levels were detected in homozygous FHBL2 (P for trend < 0.001). Compared to controls, prevalence and severity of hepatic steatosis was increased in heterozygous FHBL1 ($p < 0.001$), but unchanged in FHBL2 individuals.

Conclusion. Truncating *APOB* mutations showed the more striking LDL-C lowering effect as compared to **p.S17* mutation** in *ANGPTL3*. Reduced HDL-C levels were the unique lipid characteristic associated to FHBL2. Mutations impairing liver synthesis or secretion of apolipoprotein B are crucial to increase the risk of liver steatosis.

Keywords. Low cholesterol syndromes, Familial Hypobetalipoproteinaemia, Familial Combined Hypolipidemia, *APOB* gene, *ANGPTL3* gene, hepatic steatosis, HDL cholesterol

INTRODUCTION

Hypobetalipoproteinemia (HBL) is defined as total cholesterol (TC) and low-density cholesterol (LDL-C) levels below the 5th percentile of the general population adjusted for age, gender and race [1]. It is also typically characterized by reduced plasma concentration of apolipoprotein B (apoB), the structural apoprotein of LDL and very low-density lipoprotein (VLDL) particles [2]. In addition to secondary causes, HBL may be attributed to inherited disorders, which consist of a group of rare diseases classified depending upon the gene involved and the mode of inheritance, together with the severity of mutation [3-4].

Familial hypobetalipoproteinaemia (FHBL1, OMIM #615558) is the most frequent monogenic form of HBL and occurs with dominant inheritance. It has been linked to heterozygous mutations in the *APOB* coding gene (*APOB*-related FHBL1) [4], which in most cases interfere with the complete translation of apoB mRNA. This, in turn, causes the production of truncated proteins of various lengths, ranging from apoB-2 to apoB-89, (i.e. from 2% to 89% of apoB-100 size, according to a centile nomenclature) [5]. Mutants apoB may or may not be detectable in plasma. Only the truncated apoB with a size above that of apoB-29/30, due to mutations located in the *APOB* gene region spanning from exon 26 to exon 29, are detectable in plasma [5-6]. Truncated apoB, below that size, mostly due to mutations located in the first 25 exons of the *APOB* gene, are not secreted and, consequently, not detectable in plasma. In addition, it has been demonstrated that the length of apoB protein affects lipoprotein size and composition as there is a 13% reduction in the lipoprotein core volume, for every 10% decrease in apoB length [7-8].

The metabolic consequence of apoB truncations is the impairment of synthesis and export of VLDL by the liver and the increased fractional catabolic rate of apoB-containing lipoproteins (VLDL and LDL) [9]. In particular, the impaired export of VLDL is responsible for the accumulation of neutral lipids, namely triglycerides (TGs), in hepatocytes so that subjects with *APOB*-related FHBL1 are

highly prone to develop hepatic steatosis [10-11]. Indeed, the prevalence of hepatic steatosis has been reported to be as twice as high in FHBL1 individuals as compared to normolipemic controls [12]. More recently, the detection of loss-of-function mutations (LOF) in *ANGPTL3* gene revealed the existence of a new HBL phenotype, denominated familial combined hypolipidemia (FHBL2, OMIM #605019). It is caused by homozygous or compound heterozygous mutations in the *ANGPTL3* gene that lead to the absence or the marked reduction of ANGPTL3 protein in the plasma. ANGPTL3 protein is involved in the regulation of extracellular lipases and the disruption of its production increases lipolysis, enhancing clearance of lipoproteins and decreasing circulating lipid concentrations [13]. As a consequence, FHBL2 status is characterized by markedly reduced levels of all apoB-containing lipoproteins (VLDL and LDL) as well as apolipoprotein AI-containing plasma lipoproteins [high-density lipoprotein, (HDL)] [14-15]. It has been reported that FHBL2 may be also caused by reduced liver production of VLDL, even though the mechanism underlying this effect is, at present, unknown [16].

While several cases of FHBL1 and FHBL2 have been described, a direct comparison of their lipid phenotypes has never been carried out. In addition, despite individuals with FHBL2 **having** reduced plasma levels of apoB-containing lipoproteins and lower hepatic production of VLDL comparable with *APOB* mutation carriers, no evidences of association of FHBL2-status with liver abnormalities were ever reported [15,17].

Therefore, the aim of the present study is to perform a direct comparison between FHBL1 and FHBL2 by evaluating their lipid, clinical and biochemical characteristics, with particular reference to the association with hepatic steatosis. To evaluate genotype-phenotype relation, the lipid comparison analysis has been also carried out according to *APOB* and *ANGPTL3* mutation type. We think that the comparison of these two distinct low-cholesterol phenotype may be helpful in shedding additional lights on the metabolic mechanisms underlying FHBL2. Moreover, the identification of differences between these two disorders may be useful to set a phenotype-oriented screening for the differential diagnosis of these primary HBL syndromes.

METHODS

Study population

The cohort of hypocholesterolemic subjects was composed by 67 FHBL1 heterozygous subjects among which 28 were un-related and 39 belonged to 10 families (10 proband and 29 related subjects) and by 63 FHBL2 subjects among which 18 were un-related and 45 belonged to 15 families (including 15 proband and 30 related subjects). Two hundred twenty normocholesterolemic individuals, **80 of whom were relatives**, served as controls.

FHBL1 subjects have been recruited at the Department of Biomedicine, Internal Medicine and Medical Specialties of University of Palermo and at the Department of Life Sciences at University of

Modena and Reggio Emilia, Italy. As shown in **Table 1**, FHBL1 subjects were heterozygous carriers of 31 different mutations in *APOB* gene. Twenty nine mutations were non-sense, 12 of which (41.3%) were predicted to give rise to truncated apoB of size $\leq$ apoB-30. Two were missense variations causing non-conservative amino acid changes in exon 3 and 11 of *APOB*, respectively.

FHBL2 subjects have been identified during a screening in the small town of Campodimele, whom genetic and clinical characteristics have been already reported elsewhere [15, 17, 18]. All were carriers of the p.S17* mutation in exon 1 of *ANGPTL3* gene (57 heterozygotes and 6 homozygotes) [15, 17]. Control subjects have been selected among individuals identified in Campodimele and not carrying any known mutation in *APOB* and *ANGPTL3* genes (n=145), and among healthy blood donors recruited at the Transfusion Medicine Unit, Policlinico Umberto I, in Rome, Italy (n=75).

In FHBL2, medical history (with particular reference to liver, endocrine and cardiovascular diseases), coronary risk factors and current medications (including statin, antihypertensive, vitamins and supplements) were recorded using a structured questionnaire as previously reported [15]. Alcohol consumption was assessed using a semi quantitative scale (0, abstainers; 1, less than two glasses per day; and 2, more than two glasses per day) [15, 17, 18]. Similarly, controls from both Campodimele and blood donors cohorts were evaluated for liver, endocrine as well as current drugs use by the same structured questionnaire [19]. In controls recruited among healthy blood donors, alcohol consumption was recorded by the Alcohol Use Disorder Identification Test (AUDIT) [19]. Unfortunately, information about current medications and concomitant diseases were not available for FHBL1 subjects.

Written informed consent was obtained from all enrolled subjects and the Institutional Human Investigation Committees of each participating institution approved the study. All subjects were measured for weight, standing height, BMI and waist circumference (WC, cm) using standard procedures. In FHBL1 group we collected information on age in 46/67 subjects, BMI measurements in 17/67 and no information on WC.

Laboratory measurements

Blood samples were collected early in the morning after overnight fasting. Aliquots of plasma were used for determination of the following laboratory parameters: plasma lipids, alanine aminotransferase (ALT), aspartate aminotransferase (AST), ApoB and ApoA1 according to standard procedures [17]. LDL-C levels were calculated according to the Friedewald's formula [20] and non-HDL-C by subtracting HDL-C to TC. Values of ApoB and ApoA1 were available in 47 FHBL1 subjects and 145 controls, respectively. No data on liver function tests were available in subjects with FHBL1. **Elevated liver transaminases were defined as AST and/or ALT levels above >40 U/L and $\geq$ 35 U/L respectively.**

Genetic analysis

Genetic analysis of *APOB* and *ANGPTL3* genes was performed by a combination of PCR amplification and direct sequencing, as previously described [7, 15, 17].

A mutation score was developed to further classify FHBL1 according to the functional effect of *APOB* mutations as predicted by truncated apoB sizes. As described above, the apoB size ~ 29-30% appears to be the minimum length of apoB required for lipoprotein assembly [5-7]. Truncated apoB, whose size is below that of apoB-29/30, are not detectable in plasma, as they are not secreted. They are mostly due to mutations located in the first 25 exons of *APOB* [7]. Based upon these criteria, we **further** divided the study population in six subgroups: 1) normolipemic controls, 2) heterozygous FHBL2 carriers **of the p.S17* mutation in *ANGPTL3* gene**, 3) homozygous FHBL2 carriers **of the p.S17* mutation in *ANGPTL3* gene**, 4) FHBL1 heterozygous carriers of mutations located in exon 26-29 of *APOB*, 5) FHBL1 heterozygous carriers of truncating and missense mutations located in exons 1 to 25 of *APOB* gene.

Evaluation of hepatic steatosis

The presence of hepatic steatosis was determined by ultrasound examination following the procedures used at each collaborating sites. Radiologists were blinded to the disease state of the subjects and used an ultra-sonographer equipped with 2-6-MHz convex transducers. Even though no effort to standardizing ultrasonographic procedures between sites was carried out, similar criteria were used to define and classify steatosis [17]. In particular, steatosis was defined by an appearance of hyperechoic liver parenchyma with tightly packed fine echoes and posterior beam attenuation. The severity were defined according to the Hamaguchi's criteria, which involves semi-quantitative scoring of three findings (bright liver an hepatorenal echo contrast, echo penetration into deep portion of the liver, and blurring of intrahepatic vessel structure). The sum of the three scores provides an index of the severity of the steatosis (0, absent; 1–2, mild; 3–4, moderate; 5–6, severe) [21]. The severity score was determined in all **controls and FHBL2 individuals** and in 42 FHBL1 subjects (62.7%).

Statistical analysis

All statistical analyses were performed with SPSS/WIN program (version 20.0; SPSS Inc., Chicago, IL). **All continuous variables were tested for normal distribution using the Kolmogorov Smirnov test. In case of normally distributed data, results were presented as mean $\pm$ standard deviation (SD), whereas non-normally distributed data were expressed as median and interquartile ranges. Categorical variables were expressed as a percentage.** Continuous variables were **compared** using Student's test and categorical variables by **chi-squared**, when appropriate. Differences between groups were assessed by the Mann–Whitney U test non-parametric tests. Differences in lipid levels between groups were tested for significance by using univariate analysis including age, gender and BMI as covariates when appropriate. Bivariate correlation tests

(Pearson or Spearman when appropriate) was employed to evaluate the relationship between anthropometric and biochemical variables. Multinomial logistic regression analysis (stepwise model) was performed to identify predictors of FHBL1 and FHBL2 phenotypes. Three models were developed, one including controls and FHBL1, one including controls and FHBL2 heterozygous carriers and the third controls and FHBL2 homozygous carriers as dependent variables. Plasma lipids and presence/absence of hepatic steatosis were included as covariates. Overall P-value < 0.05 was considered statistically significant.

RESULTS

The comparison of clinical and biochemical characteristics of study groups are reported in **Table 2**. Groups were comparable for gender distribution, but homozygous FHBL2 subjects were significantly older and more obese compared to the other groups. In comparison with controls, a steady decrease of LDL-C plasma levels were observed from heterozygous to homozygous FHBL2 and to FHBL1 subjects (106.2 ± 33.6 mg/dl vs. 56.5 ± 10.5 mg/dl vs. 40.6 ± 16.0 mg/dl respectively; $P < 0.001$). This was paralleled by a decrease of non-HDL-C and plasma levels of apoB, which reached the lowest levels in FHBL1.

No subjects in FHBL2 and control group showed history of viral hepatitis, clinically overt renal diseases and changes in body weights (data not shown). By protocol, controls were not taking lipid modifying medications or vitamin supplements. The overall prevalence of hypertension and diabetes was not different between FHBL2 and controls. The prevalence of moderate and heavy drinkers were significantly higher in FHBL2 than in control subjects ($P < 0.001$).

Heterozygous FHBL1 and homozygous FHBL2 showed markedly and comparably reduced TG levels ($P < 0.001$), while heterozygous FHBL2 displayed TG levels not statistically different from controls. Plasma HDL-C levels were not different in FHBL1 subjects compared to controls, while they were significantly reduced in FHBL2, reaching the lowest levels in homozygous FHBL2 individuals.

It is noteworthy that no difference between groups in plasma levels of ApoA1 was detected. These results persisted even after adjustments for age, gender and BMI considering only subjects with complete anthropometric measurements. In FHBL1 heterozygotes, plasma lipid and lipoprotein levels did not correlate with age; in heterozygous FHBL2, but not in homozygous FHBL2, age was significantly associated with TC ($r = 0.30$; $p = 0.021$), TG ($r = 0.39$; $p = 0.002$), but not with LDL-C or HDL-C.

Figure 1 shows the comparisons of plasma LDL-C and TG concentrations among groups according to *APOB* and *ANGPTL3* mutation severity score. As depicted in **Panel A**, a significant steady decrease of LDL-C among groups was observed. However, while LDL-C levels did not significantly differ between homozygous **carriers of the p.S17* mutation in *ANGPTL3* gene** and FHBL1 subjects carrying mutations in *APOB* exon 26-29, they were markedly reduced in FHBL1 carriers of mutations

in *APOB* 1-25 exons. Conversely, TG levels in FHBL1 patients carrying *APOB* mutations either in exons 26-29 or in exons 1-25 were comparable to **those** found in homozygous FHBL2 **p.S17* mutation carriers** and significantly lower than heterozygous FHBL2 **p.S17* mutation carriers** (**Panel B**). When the analysis was restricted to the subgroup of individuals with measured plasma levels of apoB, we observed a similar trend with the lowest apoB values in FHBL1 carriers of mutations in *APOB* 1-25 exons (data not shown).

One aim of our study was to compare the prevalence of hepatic steatosis in the different groups of HBL subjects. The results of this analysis are shown in **Figure 2**. Compared to controls, the highest prevalence of hepatic steatosis was observed in FHBL1 individuals (85.07%, $P<0.001$) while both heterozygous (43.8%) and homozygous FHBL2 (50%) showed comparable prevalence ($P=0.7$) (**Panel A**). Similarly, the distribution of steatosis severity was significantly different between groups ($P=0.001$) (**Panel B**). The proportion of subjects with severe hepatic steatosis was significantly higher in FHBL1 patients (26.2%) as compared with heterozygous FHBL2 (7%; $P=0.001$) and controls (6.8%; $P<0.001$). Even though homozygous FHBL2 **carriers of the p.S17* LOF mutation** appeared to have more severe steatosis as compared to controls, the difference was not statistically significant ($P=0.7$).

To evaluate whether consanguinity might have inflated the prevalence of NAFLD, the comparison between groups were repeated after exclusion of relatives. We found that the prevalence of NAFLD was still higher in FHBL1 as compared to FHBL2 and control subjects (88.9 % vs 50 % vs 34.3 %). In respect to the prevalence of elevated liver transaminases, 4.9% of controls showed elevated AST and 1.8% elevated ALT. None of FHBL2 subjects presented elevation of AST and 7% presented elevation of ALT. No comparable figures were obtained in FHBL1, as no data on liver transaminases were obtained in these subjects.

Finally, we employed a multinomial logistic regression analysis to determine the best clinical and biochemical predictors of FHBL1 and FHBL2 disorders. **As well as expected** the analysis showed that reduced level of non-HDL-C were associated with an increased risk of FHBL1 ($P<0.001$) as well as FHBL2 ($P<0.001$), whereas the presence of hepatic steatosis appeared to be the discriminatory factor of FHBL1 (**OR, 27.7; 95%CI, 3.7-206.7; $P=0.001$**). HDL-C levels seemed to be the unique predictor of FHBL2 status ($P<0.001$).

DISCUSSION

The major finding of present study was that FHBL1 and homozygous **FHBL2 carrying p.S17* LOF** mutation showed the most striking reduction of plasma LDL-C. However, when we further sub-classified FHBL1 subjects according to the functional severity of apoB truncations, we found that heterozygous carriers of truncating mutations located on exon 1-25 of *APOB* gene showed even lower levels of LDL-C. Indeed, in these latter individuals LDL-C levels were respectively 74% lower

than those in normolipemic controls. **Conversely, homozygous carriers of the p.S17* mutation in the *ANGPTL3* gene showed a less severe hypocholesterolemic phenotype**, being their LDL-C levels reduced by 56%, a figure comparable to that caused by long apoB truncations (namely due to mutations ranging from exon 26 to exon 29). **Interestingly, FHBL2 subjects did not show change in the liver fat accumulation.** Conversely, in agreement with previous reports [4, 22, 23], FHBL1 showed two-fold higher prevalence of liver steatosis as well as a more severe degree of fat accumulation. Overall, these data illustrate that truncating mutations in *APOB* gene, in particular those determining shorter apoB truncations, produced a cholesterol-lowering effect that is more pronounced than that caused by complete *ANGPTL3* deficiency. This difference may be, at least in part, explained by the observation that the production rate of LDL particles strictly depends on the size of apoB truncation: the shorter is the truncation, the lower is the production rate [7, 8, 24, 25]. This effect may be directly related to the reduced residence time of VLDL-apoB (the precursor of LDL) in individuals carrying apoB truncations. It has been, in fact, reported that in these individuals the fractional catabolic rate of VLDL-apoB was more than 5-fold higher as compared to normolipemic controls [26]. **Our data appear to be in contrast with a recent study in a murine model reporting that ASO-mediated inhibition of apoB synthesis, but not MTP, resulted in the accumulation of lipid in the ER, causing ER stress that stimulated ER autophagy, thereby preventing steatosis [27].** Despite the elegance of this study, we think that mouse models suffer from a number of limitations that make the results unlikely to be applicable to humans. Notably, clinical trial using mipomersen (a 20-nucleotide, second-generation, anti-sense oligonucleotide that inhibits human apolipoprotein B) have shown to increase hepatic steatosis, as assessed by MRI. This effect was confirmed when hepatic steatosis was determined by hepatic biopsy [28]. **Anyway, additional studies are necessary to elucidate this important point.**

The mechanism underlying low LDL-C in the absence of *ANGPTL3* is less clear. It has been reported that FHBL2 individuals carrying two inactivating alleles showed raised removal rate of LDL from plasma, but the factors explaining this mechanism has not been determined yet [29]. More recently, we have reported that complete *ANGPTL3* deficiency blunts postprandial lipaemia [30]. This effect has been interpreted as a consequence of increases lipoprotein lipase (LPL) activity due to the loss of inhibiting action of *ANGPTL3* on LPL activity. Therefore, the higher delipidation rate of TG-rich lipoproteins (chylomicrons and VLDL), accelerates their conversion into remnants and, thereby, their removal from the liver [30, 31]. As a consequence, less remnants particle is converted into LDL, thus contributing to hypocholesterolemia.

On the other hand, it must be noted that plasma TG levels resulted comparably reduced in heterozygous FHBL1 and homozygous FHBL2, and this effect appears to be independent from the severity of *APOB* mutations. As it is known that apoB truncation significantly impairs packaging of TGs into VLDL particles and, thereby, their secretion into the blood, this finding indicates that these two hypocholesterolemic syndromes may have in common a reduced secretion of VLDL particles by the liver. To this regard, it is noteworthy that *in vivo* turnover study demonstrated reduced VLDL-apo

B synthesis in FHBL2 subjects [13]. This is consistent with our previous observation that VLDL particles of ANGPTL3-deficient subjects are smaller in size and show a 4.3-fold reduction in the TG/apoB-100 ratio [32]. However, the present observation that FHBL2 subjects **carrying p.S17* LOF mutation in ANGPTL3 gene**, in contrast to FHBL1, did not show an increased prevalence of hepatic steatosis strongly suggest that these subjects may have a compensatory reduced synthesis of TG by hepatocytes. The underlying molecular mechanism causing reduced TG synthesis in FHBL2 is obscure. We have provided indirect evidence that complete ANGPTL3 deficiency is associated with a smaller postprandial expansion of the free fatty acids pool (FFA) and a decreased influx of dietary-derived FFA into the liver [30]. Thus, it is plausible to envision a scenario where the lack of ANGPTL3 decreases the availability of FFA to hepatocytes and this, in turn, may reduce TG synthesis. Certainly, additional investigations are required to clarify this issue.

A marked reduction of HDL-C levels was the unique lipid trait associated to ANGPTL3 deficiency. The low HDL cholesterol trait **may** due to the loss of inhibiting action of ANGPTL3 on endothelial lipase (EL) activity in FHBL2 subjects, even more than on LPL [29, 33-34]. Our results are in agreement with Noto *et al* [35], which demonstrated that *APOB* mutations were likely to be found in HBL subjects with HDL-C > 2nd decile, whereas *ANGPTL3* mutations were highly prevalent in HBL subjects in whom the very low TC levels (< 2nd percentile) were associated with low levels of HDL-C (< 2nd decile). Interestingly, the mean percentage reduction of HDL-C levels described in carriers of two mutant alleles of the ANGPTL3 gene with respect to the lipid levels of non-carrier family members was equal to 55% [35].

Overall, our data may have some clinical implications. Indeed, the different phenotypic expression of FHBL1 and FHBL2 might be helpful in designing a phenotype-oriented screening for the differential diagnosis of these two primary HBL syndromes. We propose to use TC and/or apoB measurements as the first steps to suppose the diagnosis of HBL. Subsequently, the assessment of HDL-C and hepatic steatosis can be used as reliable discriminatory criteria to direct the differential diagnosis of FHBL1 or FHBL2 syndrome.

The present study has limitations that must be discussed. It is noteworthy that the absolute prevalence of hepatic steatosis in our FHBL1 group was strikingly higher compared to data reported in the literature [5, 6, 8, 12]. A possible explanation of this discrepancy may be related to the fact that we have included in the FHBL1 group several affected relatives. However, carrying a separate analysis in related and not-related FHBL1 subjects we obtained similar results. Also control group showed a slightly higher prevalence of NAFLD compared to the data reported in the Italian population [36]. However, when we have excluded from the analysis control relatives, the prevalence lowered to 34%, which is well within the figures reported for the general population [37]. The lack of some biochemical data as well as information on medications, prevalence of diabetes, renal disease, use of alcohol and history of viral hepatitis in FHBL1 subjects did not permit a fully evaluation of steatogenic risk factors in this group. Moreover, we recognize that ultrasound has limits in detecting fatty liver and the intrahepatic

triglyceride (IHTG) content was not determined by using more accurate methods (e.g. magnetic resonance). Finally, it must be recognized that FHBL2 subjects were carrying the same p.S17* mutation and therefore our observation cannot be directly extrapolated to FHBL2 individuals carrying different mutations in the *ANGPTL3* gene. Nevertheless, to our knowledge, this is the first study that investigates a largest cohort of FHBL1 and FHBL2 subjects and points light on differences in the expression of their clinical phenotypes, essential to simplify the diagnosis, treatment and appropriate follow up of subjects affected by these, apparently similar, low cholesterol genetic disorders.

In conclusion, our data indicate that truncating *APOB* mutations showed the more striking LDL-C lowering effect as compared to p.S17* mutation in *ANGPTL3* gene. Reduced HDL-C levels were the unique lipid characteristic associated to FHBL2. In contrast to FHBL1, p.S17* mutation in *ANGPTL3* gene does not appear to increase the risk of hepatic steatosis.

Author contributions: Marcello Arca, Patrizia Tarugi and Maurizio Averna designed the study, reviewed all analyses, interpreted the data and had the final approval of the version to be submitted. Alessia Di Costanzo acquired data, performed and revised all analyses, interpreted the data and prepared the manuscript. Davide Noto and Angelo Baldassare Cefalù recruited subjects, performed clinical evaluations and revised the article critically. Ilana Minicocci, Luca Polito, Enza Di Leo and Rossella Spina performed the biochemical and genetic analysis. Vito Cantisani carried out the ultrasound examinations. Laura D'Erasmo implemented data analysis and revised the draft paper.

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Tables

Table 1. List of mutations in *APOB* gene identified in FHBL1 patients

Location	Nucleotide change	Protein Change	Predicted mutant APOB form	Origin
Ex 3	c.172G>C	Ala31Pro	A31P	Italy
IVS 4	c.384 -1G>C	R101fsD153X	(B-3.35)	Turkey
Ex 10	c.1182delC	Leu368fsLeu371X	(B-8.15)	Italy
Ex 10	c.1315C>T	Arg412X	(B-9.06)	Italy
Ex 11	c.1468C>T	Arg463Trp	R463W	Italy
Ex 13	c.1621C>T	Gln514X	(B-11.30)	Italy
Ex 15	c.2085delA	Gly667fsLeu707X	(B-15.56)	Italy
Ex 18	c.2786insC	Ser901fsGlu931X	(B-20.5)	Italy
Ex 19	c.2876delA	Glu931fsLeu946X	(B-20.83)	Italy
Ex 19	c.2889G>A	Trp936X	(B-20.61)	Italy
IVS 23	c.3697-1G>C	Val1205fsThr1221X	(B-26.89)	Italy
IVS 24	c.3843-2A>G	Ser1254fsArg126	(B-27.77)	Italy
Ex 25	c.3917delC	Ser1279fsLeu1283X	(B-28.25)	Italy
Ex 25	c.3997C>T	Arg1306X	(B-28.77)	Italy
IVS 25	c.4217-1G>T	Gly1379_Ser1772fsVal1381X	(B-30.40)	Italy
Ex 26	c.4471-4472delAG	Phe1463fsAla1470X	(B-32.38)	Italy
Ex 26	c.4818C>A	Tyr1579X	(B-34.8)	Italy
Ex 26	c.5263-5266del4	Thr1727fsSer1729X	(B-38.09) ^a	Italy
Ex 26	c.5291insCT	Ser1736fsLys1775X	(B-39.11)	Italy
Ex 26	c.5350-5363del14	Thr1756fsThr1768X	(B-38.95)	Italy
Ex 26	c.6634delC	Ser2184fsVal2193X	(B-48.32)	Italy/Spain
Ex 26	c.6718 A>T	Lys2213X	(B-49.4)	Italy
Ex 26	c.7505C>A	Ser2475X	(B-54.5)	Italy
Ex 26	c.7537C>T	Arg2486X	(B-54.78)	Italy
Ex 26	c.9466insA	Lys3128fsAsp3143X	(B-69.27)	Spain
Ex 26	c.10386delT	Lys3434fsTyr3435X	(B-75.7)	Italy
Ex 26	c.10641_10642del2(CT)	Val3477fsSer3487X	(B-76.85)	Italy
Ex 26	c.11095A>T	Arg3672X	(B-80.93)	Italy
Ex 26	c.11433insT	Phe3784fsGlu3785X	(B-83.42)	Italy
Ex 26	c.11482delC	Leu3800fsLeu3813X	(B-84.04)	Italy
Ex 28	c.11905delG	Gln3941fsVal3979X	(B-87) ^b	Italy

^a Previously reported as APOB-37 by Young et al. (ref. [38]).^b Previously reported as apoB-87_{Padova} by Gabelli et al. (ref. [39]).

Nucleotide number is according to Knott et al. Nucleic Acids Res 1986;14:7501-7503. The reference sequence used was NM 000384.2, with the A of the ATG translation initiation codon numbered +1 and the methionine numbered as amino acid -27.

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Table 2. Clinical and laboratory characteristics of study subjects according to genotypes

	Controls	FHBL2 Het	FHBL2 Hom	FHBL1 Het						
	0	1	2	3	0 vs.1	0 vs.2	0 vs.3	1 vs.2	1 vs.3	2 vs. 3
N	220	57	6	67						
Age (years)	50.9±15.9	49.5±20	66.3±12.1	47.0±16.3	ns	0.021	ns	0.049	ns	0.008
Gender (M/F)(%)	57.3/42.7	52.6/47.4	50/50	56.1/43.9	ns	ns	ns	ns	ns	ns
BMI (kg/m²)	26.2 (23.9-29.1)	27.4 (25.3-29.9)	31.2(28.3-34.8)	27.2(25.0-32.0)	0.03	0.026	ns	ns	ns	ns
WC (cm)	92.3±11.4	95.1±12.9	103.8±13.3	-	ns	0.016	-	ns	-	-
Alcohol consumption (%)										
Moderate	74 (33.6)	28 (49.1)	1 (16.6)	-	<0.001	ns	-	ns	-	-
Heavy	23 (17)	14 (24.5)	2(33.3)	-	<0.001	ns	-	ns	-	-
Hypertension, n (%)	88 (40.4)	20 (35.1)	3 (50)	-	ns	ns	-	ns	-	-
Diabetes mellitus, n(%)	13 (15.9)	5 (8.8)	0	-	ns	ns	-	ns	-	-
TC (mg/dl)	210±41.6	178.7 ±41.6	91.5±15.3	106.4±23.9	<0.001	<0.001	<0.001	<0.001	<0.001	ns
TG (mg/dl)	102 (73-136.7)	86 (61.5-142)	47.5 (38-51.2)	42.5 (30.0-62.2)	ns	<0.001	<0.001	0.002	<0.001	ns
HDL-C (mg/dl)	59.8±14.7	52.0±13.2	25.8 ±7.0	55.9±20.0	<0.001	<0.001	ns	<0.001	ns	0.001
LDL-C (mg/dl)	127.1±36.2	106.2±33.6	56.5±10.5	40.6±16.5	<0.001	<0.001	<0.001	0.001	<0.001	0.024
Non-HDL-C (mg/dl)	150±40.6	126±21.0	65.6±11.9	49.0±21.0	<0.001	<0.001	<0.001	<0.001	<0.001	ns
ApoA1 (mg/dl)	137 (124-163)	134 (111-162)	167 (159-175)	135 (117-146)	ns	ns	ns	ns	ns	ns
ApoB (mg/dl)	89.0 (77.5-105)	64.0(33.0-98.0)	31.0(22.0-40.0)	26.0(20.0-30.0)	<0.001	<0.001	<0.001	ns	<0.001	ns
ALT (U/l)	15 (12-20)	17 (12-23)	21 (15.5-27.5)	-	ns	ns	-	ns	-	-
AST (U/l)	21 (18-26)	19 (16-22)	27 (16.5-33)	-	0.001	ns	-	ns	-	-

Controls include normal subjects, FHBL2 heterozygotes include subjects carrying c.50 CC>GA (p.S17*) mutation in *ANGPTL3* gene with heterozygous genotype, FHBL2 homozygotes include subjects carrying c.50 CC>GA (p.S17*) mutation in *ANGPTL3* with homozygous genotype, FHBL1 heterozygotes include subjects carrying *APOB* truncating mutations with heterozygous genotype and missense mutations.

FHBL1, Familial Hypobetalipoproteinaemia, FHBL2, Familial Combined Hypolipidemia, BMI, Body Mass Index, WC, Waist Circumference, TC, total cholesterol, TG, triglycerides, HDL, high density lipoprotein, LDL, low density lipoprotein, FBG, fasting blood glucose, APOB, Apolipoprotein B, APOA1, Apolipoprotein A1, ALT, alanine aminotransferase, AST, aspartate aminotransferase.

In FHBL1 group were collected information on age distribution in 46/67 subjects, BMI measurements in 17/67, plasma ApoB and ApoA1 concentration in 47/67 subjects. In control group plasma ApoB and ApoA1 concentration were available for 145/220 subjects, respectively. Data are expressed as percentage, mean ($\pm$ SD) and median (25°-75°percentiles) as appropriate.

A

B

Figure 1. Plasma LDL-C (Panel A) and Triglycerides (Panel B) concentrations among hypocholesterolaemic groups according to *APOB* and *ANGPTL3* severity mutation score.

Control group includes normal subjects; *Het FHBL2* group includes heterozygous subjects for c.50 CC>GA (p.S17*) mutation in *ANGPTL3* gene; *Homo FHBL2* group includes homozygous subjects for c.50 CC>GA (p.S17*) mutation in *ANGPTL3* gene; *Het Ex 26-29 FHBL1* group includes heterozygous subjects for *APOB* truncating mutations located in a genomic region spanning from EX 26 to EX 29 and secreted

protein longer than apoB-29/30; *Het Ex 1-25 FHBL1* group includes heterozygous subjects carrying *APOB* truncating and missense mutations located in a genomic region spanning from EX 1 to EX 25 and not detectable in plasma and not secreted protein shorter than apoB-29/30.

ANGPTL3, angiopoietin-like protein 3; APOB, apolipoprotein B; EX, exon; FHBL1, Familial Hypobetalipoproteinaemia; FHBL2, Familial Combined Hypolipidemia; Het, heterozygous subjects; Homo, homozygous subjects; LDL, low density lipoprotein; TG, triglycerides.

Data were reported as mean ($\pm$ SD) in Panel A and median (95% CI) in Panel B. Only significant *P*-values were shown.

Figure 2. Prevalence (Panel A) and severity (Panel B) of hepatic steatosis according to groups and genotypes

Control group includes normal subjects (n=220), *Het FHBL1* group includes heterozygous subjects for *APOB* truncating and missense mutations (n=67), *Het FHBL2* group includes heterozygous subjects for c.50 CC>GA (p.S17*) mutation in *ANGPTL3* gene (n=57), *Homo FHBL2* group includes homozygous subjects for c.50 CC>GA (p.S17*) mutation in *ANGPTL3* gene (n=6).

Severity of hepatic steatosis was expressed as absent (0 Hamaguchi score), mild (1 to 2 Hamaguchi's scores), moderate (3 to 4 Hamaguchi's scores) or severe (5 to 6 Hamaguchi's scores).

ANGPTL3, angiopoietin-like protein 3; APOB, apolipoprotein B; FHBL1, Familial Hypobetalipoproteinaemia, FHBL2, Familial Combined Hypolipidemia, Het, heterozygous subjects, Homo, homozygous subjects, HS, Hamaguchi score.

Only significant *P*-values were shown.

Highlights

- We compared *APOB*-related (FHBL1) to *ANGPTL3*-related (FHBL2) low cholesterol syndromes
- FHBL1 subjects showed the more pronounced LDL-C reduction
- Low HDL-C levels was the unique lipid trait associated to the FHBL2 status
- No increased prevalence of hepatic steatosis was detected in FHBL2
- Low HDL-C and hepatic steatosis may differentially diagnose FHBL1 from FHBL2