Atherosclerosis in the HIV and non-HIV setting: detecting and modifying cardiovascular risk
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Hepatic and Cardiovascular Consequences of Familial Hypobetalipoproteinemia

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Barbara A. Hutten
Eric de Groot
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Abstract

Objective: Individuals with familial hypobetalipoproteinemia (FHBL) have been reported to be prone to fatty liver disease (FLD). Conversely, the profound reduction of LDL-cholesterol in this disorder might decrease cardiovascular risk. In the present study, we assessed hepatic steatosis as well as non-invasive surrogate markers for cardiovascular disease (CVD) in subjects with FHBL and in matched controls.

Methods: Hepatic steatosis was assessed by abdominal ultrasonography. Carotid intima-media thickness (IMT) and distal common carotid arterial wall stiffness as surrogate markers for CVD risk were measured using high-resolution B-mode ultrasonography.

Results: Whereas transaminase levels were only modestly elevated, both prevalence (54% versus 29%; p=0.01) and severity of steatosis were significantly higher in FHBL individuals compared to controls. In spite of similar IMT measurements, arterial stiffness was significantly lower in FHBL (p=0.03) compared to controls. Additionally, the increase in arterial stiffness as seen in the presence of traditional risk factors was attenuated, suggesting that very low levels of apoB-containing lipoproteins can negate the adverse effects of other risk factors on the vasculature.

Conclusions: FHBL is characterized by an increased prevalence and severity of fatty liver disease. The observed decreased level of arterial wall stiffness, most pronounced in the presence of non-lipid risk factors, is indicative of cardiovascular protection in these subjects.
Introduction

Familial hypobetalipoproteinemia (FHBL) is a hereditary disorder of lipoprotein metabolism characterized by very low levels of apolipoprotein (apo) B-100. Plasma levels below the fifth percentile are distinctive for this condition which inherits as an autosomal dominant trait. The prevalence in the general population is estimated to vary from 0.1% to 1.9%. Genetic causes of hypobetalipoproteinemia include FHBL, abetalipoproteinemia and chylomicon remnant disease (OMIM numbers 601519, 246700 and 200100, respectively). A small percentage of FHBL can be explained by mutations in the gene encoding apolipoprotein B-100 (APOB). These include nonsense, frame-shift, and splicing mutations. Recently, it was reported that a missense mutation in the APOB gene can also lead to FHBL. APOB gene mutations lead to truncated forms of apoB and are characterized by slower hepatic secretion as well as more rapid plasma clearance compared to wild-type apoB-100 particles. Since apoB is the main constituent of such lipoproteins, including very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) and low-density lipoprotein (LDL), FHBL subjects are characterized by exceptionally low levels of these pro-atherogenic particles from birth onwards. Whereas subjects with heterozygous FHBL are generally asymptomatic, two potential implications have been attributed to this particular condition. First, the impairment of hepatic VLDL-TG secretion in FHBL may contribute to fat accumulation in the liver. Potential consequences of fat accumulation are highlighted by the occurrence of hepatic steatosis in subjects with non-alcoholic steatohepatitis (NASH), in whom progression towards cirrhosis has been observed. Indeed, case reports and smaller studies have reported a relationship between fatty liver disease (FLD) and FHBL. This is best illustrated by two recent studies by Schonfeld et al who showed that hepatic fat percentage, as assessed by magnetic resonance spectroscopy (MRS), was significantly increased in subjects with FHBL as compared to healthy controls. Nevertheless, the natural course of this potential fat accumulation in FHBL is as yet unknown. Second, FHBL offers a unique opportunity to evaluate the impact of life-long exposure to unusually low levels of apoB-containing, atherogenic lipoproteins. In this respect, FHBL subjects might be regarded as a natural model for ‘intensive lipid-lowering therapy’. In fact, the potential success of intensive lipid-lowering therapy has recently been reinforced by data from the REVERSAL and PROVE-IT study, showing a more pronounced reduction of cardiovascular disease risk upon intensive lowering of LDL-C levels.

In the present study we evaluated the impact of FHBL on hepatic steatosis as well as on surrogate markers for cardiovascular disease. Here we present the results of these investigations.
Methods

Subjects and protocols
For a detailed description of methods please visit http://atvb.ahajournals.org.
Eighty-two subjects were enrolled on study, 41 with FHBL and 41 healthy controls, matched for sex and body mass index (table 1). Autosomal codominant inheritance was a necessary characteristic for the clinical diagnosis of FHBL. FHBL subjects were identified from a group of individuals who were referred to our Lipid Clinic because of extreme low LDL-levels. These subjects were characterized by direct sequencing of the entire apoB gene, as published previously.17 Secondary causes for low LDL-C levels, i.e. (strict) vegetarian diet, or generalized diseases such as cancer, were excluded. The controls consisted of unaffected family members as well as unrelated volunteers. In the FHBL group, 4 subjects had diabetes mellitus (DM) compared to none in the control group. Since DM is strongly associated with both liver steatosis18-21 and carotid IMT/arterial stiffness22-25 we repeated the analyses after excluding the 4 diabetic subjects in the FHBL-group (FHBL minus DM) to assess whether possible differences between groups were obscured by the presence of DM.

Liver ultrasound
In all subjects ultrasound examination of the liver was performed by a single radiologist, blinded to the disease state of the subjects. The extent of hepatic fatty infiltration was classified according to previously published criteria.26

Carotid Ultrasound
B-mode ultrasound intima-media thickness (IMT) measurements were performed in the far walls of the carotid arteries and M-mode arterial stiffness was measured bilaterally in the common carotid arteries.

Statistical analysis
Statistical analyses were performed using linear or logistic regression analyses with generalized estimating equations in the SAS procedure GENMOD to account for correlations within families. Analyses were performed using SAS software (release 8.02 SAS Institute Inc, Cary, NC, USA). A p-value <0.05 was considered significant.
Clinical characteristics of FHBL subjects and controls are presented in Table 1. Forty-one subjects who met the clinical criteria of FHBL (apoB and LDL-C < fifth percentile adjusted by age, gender and race) participated in the study. Thirty-three of these subjects had mutations in the apoB gene, characteristic of FHBL. These genetically affected subjects were recruited from 8 families with different apoB mutations. The following apoB mutations were identified in the FHBL group: 2534delA apoB-18, Q1309X apoB-29, R2507X apoB-55, 11712delC apo-B8617 (Table 2). Subjects did not use lipid-lowering drugs. There was no significant difference in blood pressure, smoking, body mass index or alcohol consumption between FHBL subjects and controls. In line with their diagnosis, apoB-, LDL-C and total cholesterol levels were significantly lower in the FHBL group. The type of apoB truncation, but not plasma LDL-C or apoB levels, was modestly correlated with the degree of liver steatosis using Spearman’s rho method (r = 0.336, p=0.002). Mean levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyltransferase (GGT) levels were significantly higher in the FHBL group as compared to the controls (Table 3).
Table 2  Apolipoprotein B mutations

<table>
<thead>
<tr>
<th>exon</th>
<th>mutation</th>
<th>WT</th>
<th>MT</th>
<th>Bp position</th>
<th>predicted size</th>
<th># of carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>2534delA</td>
<td>A</td>
<td>delA</td>
<td>2534</td>
<td>ApoB-18</td>
<td>14</td>
</tr>
<tr>
<td>25</td>
<td>Q1309X</td>
<td>CAA</td>
<td>TAA</td>
<td>4006</td>
<td>ApoB-29</td>
<td>4</td>
</tr>
<tr>
<td>26</td>
<td>R2507X</td>
<td>CGA</td>
<td>TGA</td>
<td>7600</td>
<td>ApoB-55</td>
<td>1</td>
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<td>26</td>
<td>11712delC</td>
<td>C</td>
<td>delC</td>
<td>11712</td>
<td>ApoB-86</td>
<td>14</td>
</tr>
</tbody>
</table>

The reference sequence used was NM_000384, with the A of the ATG translation initiation codon numbered nucleotide +1 and the methionine numbered as amino acid -27. (Adapted from Fouchier et al. J Med Genet. 2005 Apr;42(4):e23)

Table 3  Laboratory characteristics for FHBL subjects and controls

<table>
<thead>
<tr>
<th></th>
<th>FHBL</th>
<th>Controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mmol/L)</td>
<td>2.95 ± 0.88</td>
<td>2.96 ± 0.88</td>
<td>5.26 ± 0.95</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.73 ± 0.59</td>
<td>1.76 ± 0.60</td>
<td>1.55 ± 0.33</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>1.04 ± 0.50</td>
<td>1.02 ± 0.50</td>
<td>3.07 ± 0.76</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>0.40 (0.18-0.55)</td>
<td>0.39 (0.17-0.53)</td>
<td>1.06 (0.77-1.81)</td>
</tr>
<tr>
<td>ApoB (g/L)</td>
<td>0.36 ± 0.13</td>
<td>0.35 ± 0.12</td>
<td>0.92 ± 0.22</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.0 (4.8-5.1)</td>
<td>4.9 (4.8-5.1)</td>
<td>4.9 (4.6-5.2)</td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td>1.7 (0.8-3.0)</td>
<td>1.8 (1.0-3.0)</td>
<td>1.6 (0.7-3.5)</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>30 (23-35)</td>
<td>30 (23-35)</td>
<td>25 (22-30)</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>32 (22-54)</td>
<td>32 (22-54)</td>
<td>23 (17-32)</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>25 (18-43)</td>
<td>25 (18-43)</td>
<td>19 (15-35)</td>
</tr>
<tr>
<td>Alk. Phos. (U/L)</td>
<td>62 (51-73)</td>
<td>62 (53-73)</td>
<td>67 (54-80)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD, except for glucose, TG, hs-CRP, ALT, GGT and Alk. Phos. which are given as median (interquartile range). * P-value indicates difference between FHBL group (n=41) and controls (n=41). † P-value indicates difference between FHBL-DM group (n=37) and controls (n=41). FHBL indicates familial hypobetalipoproteinemia; DM, diabetes mellitus; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; TG, triglycerides; ApoB, apolipoprotein B; hs-CRP, high-sensitivity C-reactive protein; Alk. Phos., alkaline phosphatase.

However, this was mainly caused by the 7 subjects with severe steatosis who had the highest levels of transaminases. Three of these 7 subjects had ALT levels more than twice the upper limit of normal (ULN) but still < 3x ULN. The highest value of ALT, observed in a diabetic patient, was 102. None of these 7 subjects had AST values > 2x ULN. AST and ALT were both significantly associated with hepatic steatosis (p=0.04 and p= 0.0002, respectively). Levels of hs-CRP and glucose were comparable between the two groups. HDL-C levels were significantly higher in the FHBL minus DM group compared to the controls (1.76 ± 0.59 mmol/L vs. 1.55 ± 0.33 mmol/L, p=0.01).

Liver Ultrasound
We observed a significantly higher prevalence of liver steatosis in the FHBL group compared to the control group (54% vs. 29%; p=0.01). In addition, FHBL subjects were also characterized by a more severe degree of hepatic steatosis. Seven (17%) of the subjects in the FHBL group were classified as severe steatosis compared to none in the control group (Figure 1). The distribution
of steatosis severity was significantly different between groups (p=0.004). This was even more apparent when comparing the controls with the subgroup of FHBL with mutations (p=0.001). The 4 diabetic subjects were equally distributed over the 4 steatosis categories. Separate analyses with exclusion of these 4 subjects lead to an equal statistical difference for steatosis severity between groups.

Figure 1. Grade of Liver Steatosis

Hepatic steatosis was not more severe in those subjects carrying truncated apoB100s not secreted into the plasma (apoB-18 and ApoB-29) compared to the carriers of longer truncations (p=0.68). Plasma LDL-C and apoB levels were also not different between these subgroups (p=0.77 and p=0.81, respectively). Nine of the 14 carriers of Apo-86 had liver steatosis compared to 3 of the 8 FHBL subjects without mutations. FHBL was positively associated with liver steatosis on univariate analysis (p=0.02). When adjusted for gender and smoking on multivariate analysis, FHBL, age, and BMI were independent predictors for the development of liver steatosis (p=0.001, p<0.0001 and p=0.0014, respectively). Similar significant results were found when running the analyses with the subgroup of FHBL subjects with mutations (n=33) compared to the healthy controls. Results were not significant when this was done for the subgroup of FHBL without mutations (n=8). However, the latter is likely to be caused by a lack of power due to the limited number of subjects in this group.

Vascular measurements
Mean carotid IMT (± SD) was 0.63 ± 0.14 mm in the FHBL group compared to 0.65 ± 0.15 mm in the control group. The latter difference on univariate analysis (p=0.049) lost significance when adjusting for age, gender, smoking and BMI on multivariate analysis. Arterial stiffness was significantly lower in the FHBL minus DM group compared to the controls on univariate analysis (p=0.04) whereas a similar trend was seen for the whole FHBL group (p=0.06). When comparing
the 2 subgroups of FHBL, (with and without mutations), FHBL was still significantly associated with arterial stiffness in the first group (p=0.04) but this was not significant for the subgroup without mutations. Again, this could reflect a lack of power, since the subgroup without mutations contained only 8 subjects. To evaluate a potential interaction between risk factors and apoB-containing lipoproteins, we attributed a cumulative risk score to each subject. The cumulative risk score comprised age, systolic blood pressure and smoking. These variables were chosen because their individual relationship with cardiovascular risk has been proven beyond any doubt as attested to by their incorporation in both the PROCAM-\cite{27} and Framingham Risk Score\cite{28}, the 2 most widely used risk calculators for predicting cardiovascular disease. Moreover, these risk factors have an independent, strong association with arterial stiffness.\cite{29,35} In our study these parameters were also strongly correlated with arterial stiffness with the exception of smoking ($r = 0.732$, $p<0.001$, $r = 0.550$, $p<0.001$ and $r = 0.267$, $p=0.018$, respectively). In line, both FHBL and control subjects showed a gradual increase in arterial stiffness with increasing cumulative risk scores. However, using linear regression analysis, the slope for the FHBL group was markedly decreased compared to the controls, indicating decreased stiffening in the presence of non-lipid risk factors in the FHBL group ($p=0.03$) (Figure 2). This difference remained significant ($p=0.04$) when comparing the FHBL minus DM group with the control subjects.

Figure 2. Arterial Stiffness versus Cumulative Risk Score in FHBL

The Cumulative Risk Score is based on 3 variables: age, smoking and systolic blood pressure (SBP). Results of each variable, except for smoking, were divided into tertiles. For age and SBP, patients received scores of 1, 2 or 3 with each increasing tertile. Smoking was scored as either 0 for non-smoking or 3 for smoking. Minimal and maximal attainable scores were 2 and 9, respectively.

The $p$-value indicates the difference in slope between the two regression lines.
Discussion

Subjects with FHBL are characterized by an increased prevalence of fat accumulation in the liver as well as by a more severe degree of such hepatic steatosis. Whereas these findings are not novel, we show that FHBL subjects exhibit decreased arterial stiffness. Notably, the increase in arterial stiffness under the influence of ‘traditional’ non-lipid risk factors was markedly attenuated in FHBL subjects.

Biochemical analyses

Subjects with FHBL are characterized by significantly decreased levels of apoB-containing lipoproteins, including low LDL-C as well as low triglyceride-rich particles. Also, slightly higher levels of HDL-C were found in these FHBL subjects. Presumably, the latter is the consequence of low levels of triglycerides, thus minimizing exchange of cholesterol esters from HDL-C to apoB-containing particles through the action of cholesterol ester transfer protein. Another explanation could be that the truncated apoBs are only present in the density range of HDL. However, we excluded the latter option by performing agarose gel electrophoresis on HDL-fractions, from patients with apoB-55 and apoB-86, which were isolated after ultracentrifugation. No LDL-bands were present in the HDL-density range. Additionally, apoB could also not be detected in the HDL fraction using immunonephelometry (data not shown).

Hepatosteatosis

The prevalence of fatty liver disease in healthy controls (29%) is in the same order of magnitude as reported by others. Hypothesis The increased prevalence of hepatosteatosis in subjects with FHBL is in agreement with previous results from Schonfeld and Tanoli who showed that these subjects had a ~3-fold increase in mean liver fat content, as assessed by MRS. The most likely cause for this increase in hepatic steatosis is the impaired secretion of VLDL-TG from the liver, leading to accumulation of VLDL-TG in the liver. There is a large body of evidence suggesting that accumulation of liver triglycerides may give rise to increased oxidative stress in the hepatocytes. However, distinct proof, in the human setting, that this process invariably translates into progression of liver steatosis to NASH is lacking. Recent work by Youssef and colleagues revealed that up to 25% of patients with FLD may progress to NASH, of whom 20% may eventually progress into cirrhosis. Notably, in our FHBL group, transaminase levels were only modestly elevated with none of the subjects exceeding a three-fold increase in ULN. Most studies reporting long term outcome of fatty liver disease, use the AST/ALT ratio as a marker for the risk of disease progression. A ratio of less than 1 indicates a ‘low risk’ for steatosis and in our FHBL subjects, AST/ALT ratios were all below 1. It should be kept in mind, however, that the absence of liver enzyme elevations does not completely preclude advanced fibrosis or cirrhosis in these subjects. To date, long-term follow-up data with regard to liver outcome in FHBL are lacking. Nevertheless, risk factors for FLD such as hypertriglyceridemia, obesity, alco-
hol, diabetes mellitus and certain drugs are likely to aggravate hepatic steatosis in FHBL. Hence, it is prudent to avoid these risk factors, and recommend a diet with low to moderate amounts of fat and energy, limited use of alcohol as well as avoiding obesity in these individuals.

**Cardiovascular Risk**

Numerous studies have established a strong correlation between levels of LDL-C and progression of IMT. In the present study, however, we could not show an independent statistical difference in terms of carotid IMT values between FHBL subjects and controls. Nevertheless, data have accumulated recently, that show the predictive value of the assessment of vascular function, such as arterial stiffness, for future cardiovascular events. Arterial stiffness is closely correlated with increasing age, smoking and hypertension. The impact of these risk factors is augmented in the presence of hypercholesterolemia and can be reverted by statin therapy. In our FHBL group, we observed a significant decrease in arterial stiffness. Of note, this difference was observed in spite of the fact that traditional risk factors such as smoking and diabetes occurred more frequently in the FHBL group compared to controls. In earlier studies, apoB-containing lipoproteins have been put forward as a pivotal ‘permissive’ factor for the development of atherogenic changes of the vessel wall. To evaluate a potential interaction between apoB-containing lipoproteins and other traditional risk factors, we constructed a cumulative risk index including age, smoking, and systolic blood pressure in FHBL subjects as well as controls. In both groups, there was a linear relationship between increased risk score and arterial stiffness. Interestingly, the increase in arterial stiffness, also in presence of these risk factors was decreased significantly in the FHBL group compared to controls. These data suggest that apoB-containing lipoproteins indeed have the ability to potentiate the impact of traditional risk factors on vascular function. Tentatively, these observations might suggest that lowering of apoB-containing lipoproteins should have a beneficial impact also in subjects with ‘non-cholesterol’ risk factors. Indeed, recent studies have validated the beneficial effects of statin therapy in normcholesterolemic subjects with non-lipid risk factors, such as hypertension.

This study has some limitations. We used the less sensitive ultrasonography method to evaluate fatty liver disease rather than magnetic resonance spectroscopy. However, in view of the carefully standardized methodology and the fact that both patients and controls were evaluated using the same methodology, it is unlikely that the latter has affected our outcomes. With regard to the IMT measurement, we could not find a clear relationship between LDL-C levels and carotid IMT. Several reasons may have attributed to the absence of a relation. First, we studied a relatively young cohort with an inherently low risk for cardiovascular disease and hence low IMT values. Second, we studied IMT in a case control design to show thinner IMTs compared to healthy controls. A priori, it is very difficult to demonstrate decreased IMT thickness in ‘low-risk’ groups compared to healthy controls. We have estimated that inclusion of more than 1000 subjects per group would have been necessary to be able to detect signifi-
Hepatic and cardiovascular consequences of FHBL

Significantly thinner IMTs compared to healthy controls with a “normal” risk factor distribution, as seen in western populations.

In summary, our study shows that subjects with FHBL are at increased risk of developing FLD. Whereas long-term sequelae of FLD in FHBL subjects remain to be established, it is prudent to give lifestyle advice in affected individuals. As is illustrated by decreased vascular wall stiffness, our findings suggest that the vessel wall in FHBL subjects is relatively protected by the (life-long) reduced levels of exposure to apoB-containing lipoproteins. The attenuated gradual increase in vascular stiffness in the presence of classical, non-lipid cardiovascular risk factors in FHBL subjects is of interest and suggests that apoB-containing particles constitute a central factor in atherogenesis, amplifying any risk mediated by non-lipid risk factors. Further confirmation of this finding is needed in larger cohorts to ascertain its impact on cardiovascular risk.

Acknowledgements

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References


