Angptl3 Deficiency Is Associated With Increased Insulin Sensitivity, Lipoprotein Lipase Activity, and Decreased Serum Free Fatty Acids

Marius R. Robciuc,* Marianna Maranghi,* Anna Lahikainen, Daniel Rader, Andre Bensadoun, Katarina Öörni, Jari Metso, Ilenia Minicocci, Ester Ciociola, Fabrizio Ceci, Anna Montali, Marcello Arca, Christian Ehnholm, Matti Jauhiainen

Objective—Angiopoietin-like 3 (Angptl3) is a regulator of lipoprotein metabolism at least by inhibiting lipoprotein lipase activity. Loss-of-function mutations in ANGPTL3 cause familial combined hypolipidemia through an unknown mechanism.

Approach and Results—We compared lipolytic activities, lipoprotein composition, and other lipid-related enzyme/lipid transfer proteins in carriers of the S17X loss-of-function mutation in ANGPTL3 and in age- and sex-matched noncarrier controls. Gel filtration analysis revealed a severely disturbed lipoprotein profile and a reduction in size and triglyceride content of very low density lipoprotein in homozygotes as compared with heterozygotes and noncarriers. S17X homozygotes had significantly higher lipoprotein lipase activity and mass in postheparin plasma, whereas heterozygotes showed no difference in these parameters when compared with noncarriers. No changes in hepatic lipase, endothelial lipase, paraoxonase 1, phospholipid transfer protein, and cholesterol ester transfer protein activities were associated with the S17X mutation. Plasma free fatty acid, insulin, glucose, and homeostatic model assessment of insulin resistance were significantly lower in homozygous subjects compared with heterozygotes and noncarriers subjects.

Conclusions—These results indicate that, although partial Angptl3 deficiency did not affect the activities of lipolytic enzymes, the complete absence of Angptl3 results in an increased lipoprotein lipase activity and mass and low circulating free fatty acid levels. This latter effect is probably because of decreased mobilization of free fatty acid from fat stores in human adipose tissue and may result in reduced hepatic very low density lipoprotein synthesis and secretion via attenuated hepatic free fatty acid supply. Altogether, Angptl3 may affect insulin sensitivity and play a role in modulating both lipid and glucose metabolism. (Arterioscler Thromb Vasc Biol. 2013;33:1706-1713.)

Key Words: ANGPTL3 protein, human ■ ANGPTL4 protein, human ■ endothelial lipase, human ■ familial combined hypolipidemia ■ fatty acids, nonesterified ■ hepatic lipase, human ■ lipoprotein lipase

It was recently reported that individuals affected by familial combined hypolipidemia (FHBL2, OMIM #605019) are either homozygotes or compound heterozygotes for loss-of-function (LOF) mutations in the ANGPTL3 gene.1 FHBL2 was originally described in a kindred identified because of low plasma total and LDL-cholesterol,2 but a careful evaluation of affected individuals demonstrated that also very low density lipoprotein (VLDL) and high density lipoprotein (HDL) fractions were comprehensively reduced, suggesting the definition of familial combined hypolipidemia. Subsequently, other studies have reported additional probands with similar phenotype, further demonstrating that FHBL2 is associated with LOF mutations in the ANGPTL3 gene that causes absence or marked reduction of the Angiopoietin-like 3 (Angptl3) protein in plasma.3-5 To date, FHBL2 is considered to segregate as a recessive trait, so that apolipoprotein B (apoB) and apolipoprotein A-I (apoA-I) containing lipoproteins are comprehensively affected by the total deficiency of Angptl3.

Detailed characterization of FHBL2 subjects reveals that this condition does not perturb whole-body cholesterol...
homeostasis and is not associated with adverse clinical sequelae. Moreover, among the low cholesterol syndromes, FHBL2 was found to be quite common and, accordingly in a cohort of subjects with primary hypobetalipoproteinemia, the prevalence of ANGPTL3 gene mutations responsible for this phenotype was reported to be ≈10%. Insights into the function of Angptl3 came after the identification of a mutation in ANGPTL3 gene in KK mice that have low plasma triglyceride (TG) levels (KK/San). Angptl3 deficiency in these animals accelerates the clearance of VLDL particles from plasma because of increase in lipoprotein lipase (LPL) activity, whereas overexpression or intravenous injection of recombinant Angptl3 has opposite effects. As suggested mechanism, Angptl3 enhances the cleavage of LPL by proprotein convertases causing dissociation of LPL from the cell surface. Angptl3 has also been reported to inhibit in vitro endothelial lipase (EL), an enzyme involved in HDL catabolism, and to be positively correlated to plasma HDL cholesterol and phospholipid levels. Angptl3 protein is expressed almost exclusively in the liver and is released into the circulation where it undergoes cleavage by hepatic proprotein convertases. This cleavage has been shown to be essential for activation of Angptl3.

A role for Angptl3 in regulating VLDL and HDL metabolism in humans has also been observed in genome wide association studies, and analysis of ANGPTL3 exons by resequencing revealed rare and common alleles associated with plasma TG and HDL cholesterol levels. Based on this knowledge, it was suggested that the FHBL2 phenotype attributable to deficiency of Angptl3, at least in part, might be caused by increased activity of LPL and EL causing accelerated catabolism of VLDL and HDL. Previously, we reported that FHBL2 subjects showed reduced TG levels mainly in VLDL and HDL, and to a lesser extent in LDL particles. These observations support a possible role of the LPL enzyme. However, a direct demonstration of this mechanism has not been provided.

In vivo kinetic studies indicated that FHBL2 individuals have decreased production rates of VLDL by the liver, but the reason for this has not been elucidated yet. It could be that the reduced synthesis of VLDL is linked to a reduced supply of free fatty acid (FFA) to the liver. This hypothesis seems to be supported by the observation in experimental animals that deficiency of Angptl3 causes reduced availability of circulating FFAs, a well-known regulator of hepatic VLDL synthesis and secretion. However, this possibility has never been tested in the human context.

We identified in the small town of Campodimele (Latina, Italy) a large cohort of individuals affected by FHBL2 because of LOF ANGPTL3 mutations, of which the S17X was the most prevalent. Homozygous S17X carriers showed complete absence of Angptl3 in their plasma, whereas heterozygous carriers had only partial Angptl3 deficiency. The aim of the present study was to investigate the effect of Angptl3 deficiency on the activities and mass of LPL, hepatic lipase (HL), and EL activities as well as on plasma lipoprotein composition, distribution and size, and plasma FFA levels in subjects carrying the S17X mutation in the ANGPTL3 gene. Furthermore, we evaluated plasma levels of glucose, insulin, and calculated homeostatic model assessment of insulin resistance (HOMA-IR) as index of insulin resistance according to the ANGPTL3 LOF mutation status in a larger Campodimele cohort. This approach should provide novel insights into the mechanism(s) by which Angptl3 deficiency results in the FHBL2 phenotype.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

We reported that Angptl3-deficient subjects have reduced cholesterol levels in all major (VLDL, LDL, HDL) plasma lipoprotein fractions and a marked reduction of TGs especially in VLDL and HDL. Also, in the present study, these parameters were comprehensively reduced in homozygotes compared with noncarriers. Significant reduction of the major apolipoproteins of VLDL, LDL, and HDL, apoB-100 and apoA-I in homozygotes was also evident compared with noncarriers and heterozygotes. Heterozygous individuals showed partial 42% reduction in Angptl3, and only total cholesterol was significantly reduced (Table 1). Angptl4 levels were significantly reduced (P<0.05) in S17X homozygotes as compared with heterozygotes and noncarriers.

**Lipoprotein Composition in Angptl3 S17X Carriers and Noncarriers**

Analysis of lipoprotein profiles in noncarriers, heterozygotes, and homozygotes demonstrated that noncarriers and heterozygotes have similar lipoprotein profiles with only a small reduction in VLDL-associated lipid levels, whereas homozygotes have reduced levels of lipids in all major plasma lipoprotein classes (Figure 1A through 1E). It is noteworthy that only in homozygous subjects all the measured lipid components, that is, TGs, total cholesterol, free cholesterol, cholesterol esters (CEs), and phospholipids, were significantly reduced. Also the major

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**Table 1. Clinical Characteristics of the Study Subjects**

<table>
<thead>
<tr>
<th></th>
<th>Homozygotes (n=5)</th>
<th>Heterozygotes (n=17)</th>
<th>Noncarrier (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (W/M)</td>
<td>2/3</td>
<td>8/9</td>
<td>11/11</td>
</tr>
<tr>
<td>Age, y</td>
<td>63.6±10</td>
<td>50.1±20</td>
<td>51.8±19</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>31.5±7</td>
<td>28.3±2</td>
<td>28.3±5</td>
</tr>
<tr>
<td>CHOL, mmol/l</td>
<td>2.3±0.4*</td>
<td>4.6±0.8</td>
<td>5.2±1.1</td>
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<tr>
<td>LDL-C, mmol/l</td>
<td>1.4±0.2*</td>
<td>2.6±0.6</td>
<td>3.1±0.8</td>
</tr>
<tr>
<td>HDL-C, mmol/l</td>
<td>0.7±0.21*</td>
<td>1.5±0.3</td>
<td>1.5±0.4</td>
</tr>
<tr>
<td>TG, mmol/l</td>
<td>0.5±0.10*</td>
<td>1.1±0.6</td>
<td>1.5±0.7</td>
</tr>
<tr>
<td>Angptl3, ng/mL</td>
<td>0 *</td>
<td>97±96 *</td>
<td>233±145</td>
</tr>
<tr>
<td>ApoB, g/l</td>
<td>1.0±0.08*</td>
<td>1.5±0.3</td>
<td>1.5±0.4</td>
</tr>
<tr>
<td>ApoA-I, g/l</td>
<td>0.5±0.2*</td>
<td>1.3±0.3</td>
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</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>4.8±0.7</td>
<td>5.1±0.67</td>
<td>5.1±1</td>
</tr>
<tr>
<td>Insulin, µU/mL</td>
<td>5.2±5.6</td>
<td>4.9±5.2</td>
<td>6±3.4</td>
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<tr>
<td>HOMA-IR</td>
<td>0.8±0.9</td>
<td>1.1±0.8</td>
<td>1.4±1</td>
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</tbody>
</table>

*Data are reported as mean±SD. *P<0.05 compared with noncarriers. 1-way ANOVA, Kruskal–Wallis test with Dunn Multiple Comparison Post Test. Angptl3 indicates angioptin-like 3; ApoA-I, apolipoprotein A-I; ApoB, apolipoprotein B; BMI, body mass index; CHOL, cholesterol; HDL-C, high-density lipoprotein-C; HOMA-IR, homeostatic model assessment of insulin resistance; LDL-C, low-density lipoprotein-C; and TG, triglyceride.
apolipoprotein components of VLDL, LDL, and HDL, that is, apoB and apoA-I when analyzed from the gel filtration fractions were significantly reduced in homozygous S17X carriers as compared with heterozygotes and noncarriers (Figure 1F and 1G). Analysis of VLDL and LDL particle size using dynamic light scattering demonstrated that homozygotes have a dramatic reduction in VLDL particle size with an average diameter of 44 nm compared with 82 nm measured in VLDL derived from noncarriers. A small reduction in LDL particle size was also seen in homozygotes (20 nm) compared with noncarriers (21 nm). Once we observed a dramatic reduction of VLDL, LDL, and HDL levels in homozygote S17X carriers, we next analyzed mass composition of lipoprotein particles especially the distribution of neutral core lipids (Table 2). VLDL particles of homozygote subjects displayed a significant reduction in TG/CE ratio (mol/mol) as compared with VLDL of noncarriers (0.28 versus 1.17, homozygotes versus noncarriers). The change in this ratio was mainly attributable to reduction of TGs in VLDL particles of S17X homozygotes. The TG/CE ratio increased in LDL of homozygotes because of reduction in particle CE content (0.39 versus 0.17, homozygotes versus noncarriers).

We also calculated neutral lipid ratio to apoB in VLDL and LDL fractions. Homozygotes had a 4.3-fold reduction in the TG/ApoB ratio in VLDL, and this ratio increased 1.6-fold in LDL when compared with noncarriers. Furthermore, there was a tendency for a reduction of CE/apoB ratio in VLDL and LDL of homozygotes compared with noncarriers (all P<0.07). This is in accordance with the smaller size of VLDL and LDL particles derived from S17X homozygotes. Plasma level of the major HDL protein, apoA-I, in homozygotes was ≈60% of that observed in heterozygotes and noncarriers (P<0.01, Table 1). HDL composition in homozygous subjects was also drastically changed (Table 2) with major reduction in all measured lipid classes (Table 2 and Figure 1A through 1D).

**Lipase Activities in Angptl3 S17X Carriers and Noncarriers**

To determine whether Angptl3 deficiency affects LPL, HL, and EL, we measured these lipases in postheparin plasma samples derived from the 3 study groups. Compared with noncarriers, heterozygous carriers did not show any significant differences neither in postheparin LPL mass nor activity, whereas...
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homozygotes demonstrated almost 3.5-fold higher levels of both LPL activity and mass (all \( P < 0.05 \); Figure 2A and 2B). No differences in plasma levels of HL mass or activity (neither in TG or phospholipid lipolytic activity) were observed between the 3 genotypes (Figure 2C through 2E). Neither did EL activity differ between noncarriers and homozygotes (Figure 2F).

In addition, we calculated the specific activity for LPL and HL (Figure 2G and 2H, respectively). The results showed no differences in LPL- or HL-specific activities between homozygotes and noncarriers, whereas heterozygotes had slightly elevated LPL specific activity when compared with noncarriers but did not differ from that of homozygotes.

Table 2. Lipoprotein Particles Composition in Homozygotes Compared With Noncarriers

<table>
<thead>
<tr>
<th></th>
<th>Homozygotes (n=6–8)</th>
<th>Noncarrier (n=5–8)</th>
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<tbody>
<tr>
<td></td>
<td>VLDL</td>
<td>LDL</td>
</tr>
<tr>
<td>TG, mmol/l</td>
<td>0.013±0.01*</td>
<td>0.092±0.03</td>
</tr>
<tr>
<td>CE, mmol/l</td>
<td>0.046±0.03*</td>
<td>0.239±0.10*</td>
</tr>
<tr>
<td>TG/CE</td>
<td>0.283±0.10*</td>
<td>0.389±0.05*</td>
</tr>
<tr>
<td>ApoB, µmol/l</td>
<td>0.030±0.03</td>
<td>0.269±0.13*</td>
</tr>
<tr>
<td>TG/ApoB (mol/mol)</td>
<td>747±635*</td>
<td>418±145*</td>
</tr>
<tr>
<td>CE/ApoB (mol/mol)</td>
<td>1472±580</td>
<td>1076±338</td>
</tr>
</tbody>
</table>

Gel filtration fractions corresponding to elution positions for VLDL, LDL, and HDL were pooled (VLDL, fractions 17; LDL, fractions 24–25; HDL, fractions 33–34) and analyzed for triglyceride and cholesterol esters as described in Materials and Methods. Data are reported as mean±SD. *\( P < 0.05 \) compared with noncarriers, 2-sided \( t \) test. ApoB indicates apolipoprotein B; CE, cholesterol ester; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride; and VLDL, very low-density lipoprotein.

Figure 2. Levels of Lipoprotein lipase (LPL), hepatic lipase (HL), and endothelial lipase (EL) in postheparin plasma. Postheparin plasma collected from noncarriers, S17X heterozygotes and homozygotes, was analyzed for the following: LPL activity (A) and mass (B); HL triglyceride (C) and phospholipase (E) activity, and mass (D); and EL triglyceride hydrolysis activity (F). Specific LPL (G) and HL (H) activity was calculated from LPL/HL activity and mass. Individual values are scattered in dot plots, and median line is shown. *\( P < 0.05 \), 1-way ANOVA. HL-PL indicates phospholipase activity of hepatic lipase.
To test whether postheparin plasma-LPL activity is related to variation in circulating Angptl3 protein levels, we performed linear regression analysis combining heterozygotes and noncarrier subjects. No correlation was observed between LPL activity and Angptl3 plasma levels in noncarriers and heterozygotes (Figure 3A). In addition although there was a 7.5-fold difference in median Angptl3 levels between the lowest and highest Angptl3 quintiles (Figure 3B), LPL activities were similar (Figure 3C).

To analyze whether the reduced Angptl3 levels might result in changes in enzymes or proteins associated with VLDL or HDL metabolism, we measured CE transfer protein, phospholipid transfer protein, and paraoxonase 1 activities, and apoE levels. As depicted in Figure I in the online-only Data Supplement, there were no differences in these parameters between mutation carriers and noncarriers.

Because Angptl3 has been shown to regulate the release of FFAs from adipose tissue in mice, we determined plasma FFA levels in fasting plasma samples of our study subjects. Among the 3 study groups, plasma FFA levels were decreased in heterozygotes and homozygotes as compared with noncarriers (Figure 4A), suggesting that Angptl3 deficiency is associated with reduced mobilization of FFA from fat stores in human adipose tissue. We also analyzed glycerol levels in fasting plasma samples, and homozygotes displayed slightly lower levels when compared with heterozygotes and noncarriers (8.76+/−2.02 μmol/L versus 10.23+/−3.32 μmol/L versus 11.72+/−4.33 μmol/L, mean+/−SD in homozygotes, heterozygotes, and noncarriers, respectively; P>0.2 1-way ANOVA). These data strengthen the link between Angptl3 and adipose tissue lipolysis. Encouraged by these results, we performed a more thorough study and obtained new samples to confirm the above data (Figure 4A) and extended the measurements of FFA, insulin, glucose, and calculated HOMA-IR in a larger Campodimele cohort comprising ANGPTL3 LOF carriers and noncarriers (Table 3). Unadjusted comparison did not reveal significant differences among the groups. After correction for age, sex, and body mass index, homozygous subjects showed significantly lower plasma concentration of FFA, glucose, and insulin as compared with heterozygotes and noncarriers. They also showed significantly lower values of HOMA-IR (adjusted data), suggesting higher insulin sensitivity in homozygous Angptl3 LOF carriers. In correlation analyses of the entire study cohort, plasma concentrations of FFAs associated with plasma levels of Angptl3 with borderline significance (r=0.179; P=0.080), but significantly and positively with HOMA-IR (r=0.223; P=0.018). Conversely, no correlation between plasma concentration of Angptl3 and HOMA-IR values was observed (r=0.076; P=0.448). In addition, Angptl4 plasma levels were reduced in carriers of S17X mutation, reaching statistical significance in homozygotes as compared with noncarriers (P<0.05; Figure 4B). Furthermore, Angptl4 levels were strongly correlated with FFA levels (Figure 4C) but not with LPL (Figure 4D), HL, or EL activities (data not shown). No direct correlations between Angptl3 and FFAs and Angptl4 plasma levels were observed (all P>0.05).

Discussion

It has been reported that Angptl3 can, at least in vitro, act as an inhibitor of LPL, HL, and EL. 3 enzymes that are involved in the lipolytic degradation of VLDL and HDL particles. It has, therefore, been proposed that the FHBL2 phenotype caused by the deficiency of Angptl3 could result from dysfunction of 1 or several of these lipolytic enzymes. Taking advantage of cohorts of homozygote and heterozygote carriers of the LOF mutation S17X in the ANGPTL3 gene who display either complete or partial absence of Angptl3 protein in the circulation, we tested whether the FHBL2 phenotype could be caused by changes in lipase activities of LPL, HL, or EL.

A major finding in the study was that homozygote carriers of the S17X mutation demonstrated a marked increase in LPL activity in postheparin plasma. This is in full agreement with previous work by Koster et al demonstrating that postheparin plasma (PHP)-LPL activity was 9-fold higher in male

Figure 3. Relationship between plasma angiopoietin-like 3 (Angptl3) levels and lipoprotein lipase (LPL) activity. A, Linear regression analysis for plasma-LPL activity and Angptl3 levels in noncarriers and heterozygotes. B, plasma Angptl3 levels and C) postheparin plasma-LPL activity are shown in the highest (Q5) and lowest (Q1) quintile of Angptl3 levels. Individual values are shown as dot plots, and median line is shown (B, C). *P<0.05, Mann-Whitney test.
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Angptl3-deficient mice, compared with male wild-type mice. However, as shown in the present study, postheparin plasma-LPL activity was not different in Angptl3 S17X heterozygotes as compared with noncarriers, although a significant reduction in plasma Angptl3 levels was observed. These data suggest that Angptl3 is a very potent LPL inhibitor but does not act in a concentration-dependent manner in vivo, which is in contrast to in vitro data. It can be speculated that Angptl3 proteolysis by proprotein convertases rather than plasma Angptl3 levels are important in regulating LPL activity and plasma triacylglycerol levels. The large increase in LPL mass in ANGPTL3 deficiency could support the notion that the lack of Angptl3 may influence synthesis or degradation of LPL enzyme.

It has been reported that Angptl3 can act as inhibitor of HL and thereby regulate HDL metabolism. Our data here do not support this notion and suggest that Angptl3 does not regulate HDL metabolism via HL function. Of note is the recent observation by Christensen et al that in mice inhibition of apoB synthesis via antisense-apoB treatment caused a concomitant reduction of both VLDL and HDL secretion from liver cells. One explanation for the low level of HDL seen in our study subjects is that the ANGPTL3 S17X mutation could directly target hepatocyte lipoprotein secretion and clearance as suggested recently. We and others reported previously that Angptl3 plasma levels are highly correlated with both HDL cholesterol and apoA-I levels in a normal population.

Shimamura and coworkers showed that Angptl3 inhibits EL in vitro and is positively correlated with HDL cholesterol and phospholipid levels in human plasma, suggesting that Angptl3 may regulate HDL levels by inhibiting EL. In the present study, we show that Angptl3 deficiency, although associated with decreased HDL levels, does not affect heparin releasable EL, phospholipase/TG lipase activity. This suggests that the mechanism by which Angptl3 regulates LPL activity is independent of EL activity and does not explain the low HDL levels found in S17X homozygote subjects.

Angptl3 deficiency in mice was shown to decrease plasma FFAs, whereas injection of recombinant or adenoviral over-expression of Angptl3 robustly increased plasma FFAs. This effect is explained by increased intracellular adipose tissue lipolysis followed by FFA release into circulation. Because adipose tissue is the main contributor to the plasma FFA pool, our observation that plasma FFA levels are reduced in carriers of ANGPTL3 null alleles might suggest that these subjects have an attenuated rate of adipose tissue lipolysis. Angptl3 deficiency could modulate the flow of FFAs to the liver, providing lesser substrate for the assembly of apoB and TGs into nascent VLDL particles.

Table 3. Metabolic Parameters of the Campodimele Cohort According to LOF Mutation in ANGPTL3 Gene

<table>
<thead>
<tr>
<th></th>
<th>Homozygous Carriers (n=8)</th>
<th>Heterozygous Carriers (n=61)</th>
<th>Noncarriers (n=205)</th>
<th>Adjusted P Value‡</th>
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<tbody>
<tr>
<td>FFA (μmol/L)</td>
<td>344.5±292.2</td>
<td>486.2±239.9</td>
<td>563.9±249.9</td>
<td>0.028</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.4 (3.7–5.1)</td>
<td>4.9 (4.5–5.5)</td>
<td>4.9 (4.4–5.4)</td>
<td>0.004</td>
</tr>
<tr>
<td>Insulin (µU/mL)</td>
<td>4.6 (1.5–9.9) * †</td>
<td>5.8 (3.8–8.8)</td>
<td>5.9 (3.6–8.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.77 (0.26–2.1)</td>
<td>1.35 (0.8–1.8)</td>
<td>1.34 (0.76–1.9)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Data are reported as mean±SD or as median (IQ). Plasma FFA was measured in 7 homozygotes, 47 heterozygotes, and 58 noncarriers. Plasma insulin was measured in 8 homozygotes, 59 heterozygotes, and 204 noncarriers; glucose values were measured in 8 homozygotes, 61 heterozygotes, and 205 noncarriers; HOMA-IR index was calculated in 8 homozygotes, in 59 heterozygotes, and 202 controls; *comparison between homozygotes and noncarriers; †comparison between heterozygotes and noncarriers; P<0.05; ‡Statistical comparison adjusted for age, sex, and body mass index (BMI) under a recessive model: homozygous vs. heterozygous and noncarriers. FFA indicates free fatty acid; and HOMA-IR, homeostatic model assessment of insulin resistance.
the TGs to CE ratio in VLDL particles together with reduced plasma apoB level in S17X homozygotes suggests defects in hepatic VLDL assembly and secretion. Indeed, a reduced production rate of VLDL particles has been previously reported in FHBL2 subjects. Additional explanation for the observed low TG/apoB and CE/apoB ratios in VLDL would be that these lipoproteins undergo extensive delipidation by LPL because postheparin plasma-LPL activity is significantly elevated in S17X homozygotes.

A novel observation was that homozygous subjects showed significantly lower plasma insulin levels and a tendency toward lower glucose values. As a consequence, the HOMA-IR index, which is an indirect measure of insulin resistance, was significantly lower in homozygotes. This seems in agreement with data of a population study reporting higher prevalence of nonsynonymous (probably inactivating) variations in the ANGPTL3 genes among subjects in the lowest quartile of blood glucose levels. The mechanisms by which Angptl3 influences insulin sensitivity are unknown. Because chronically elevated FFAs have been reported to impact on insulin action, it can be speculated that the reduced FFAs levels associated with complete Angptl3 deficiency may improve tissue insulin action. It is noteworthy that Angptl3 does not influence both FFAs and insulin sensitivity in a concentration-dependent manner, because only its complete deficiency seems to determine detectable effects on these pathways. Collectively, these data support the notion that Angptl3 might play an important role in the crosstalk between liver and adipose tissue and can affect insulin sensitivity and FFA transport and thereby acting like a hepatokine.

It has been reported that another angiopoietin-like protein, Angptl4, is also strongly correlated with serum FFA levels. This is of interest because Angptl4 was recently reported to induce adipose tissue lipolysis and was inversely correlated with obesity parameters in humans. Of note we found that serum Angptl4 levels were also decreased in S17X homozygote subjects. It is well established that FFAs induce cellular expression of Angptl4 via peroxisome proliferator–activated receptor-δ activation. Therefore, one could interpret that the reduced serum FFAs levels seen in S17X heterozygotes and homozygotes could cause reduced peroxisome proliferator–activated receptor-δ-dependent expression and secretion of Angptl4. These findings might suggest a coordinate regulation of both Angptl3 and Angptl4 by FFA, but additional data are required to clarify this point.

Although the reduction of plasma levels of apoB-containing lipoproteins can be explained by increased LPL activity and reduced apoB secretion from the liver, the reduction in HDL remains unexplained. The function of Angptl3 in regulating lipoprotein metabolism was recently reported to be more complicated because of identification of another ANGPTL family member, ANGPTL8 or RIFL, suggested to play a role in Angptl3 activation. This observation needs further studies to verify the interaction between Angptl3 and Angptl8 in humans and how it affects the circulating lipoprotein phenotype. Our results further provide the first evidence that complete lack of Angptl3 in humans is associated with low levels of FFAs and improved insulin sensitivity. When confirmed in larger studies these preliminary evidences could pave the way for the existence of a comprehensive role for Angptl3 in regulating not only lipid but also glucose metabolism.

Acknowledgments

We are grateful to Dr John Brunzell, University of Washington, for providing us with the 2 monoclonal antibodies, SP9 and SD2, used in the ELISA of human LPL and to Ana Curinha and Sari Nuutinen for excellent assistance during the work.

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Disclosures

None.

References

Angiopoietin-like 3 (Angptl3) is expressed and secreted by liver and circulates in plasma associated with lipoproteins. The best characterized function of Angptl3 is to inhibit lipoprotein lipase activity and thereby decrease plasma triglyceride hydrolysis. In humans, Angptl3 deficiency causes reduction of all major lipoproteins, a phenotype called familiar combined hyperlipidemia. We present here a detailed biochemical characterization of Angptl3-deficient subjects, which reveals that Angptl3 deficiency does not only reduce plasma lipids but also alters lipoprotein particle size and composition. Several key enzymes enrolled in lipoprotein metabolism were studied, but postheparin plasma lipoprotein lipase was the only significantly changed parameter. Plasma free fatty acids, insulin, glucose, and homeostatic model assessment of insulin resistance were significantly lower in Angptl3-deficient subjects. Our results provide the first evidence that complete lack of Angptl3 in humans is associated with improved insulin sensitivity and, thus, for a role of Angptl3 in regulating not only lipid but also glucose metabolism.

**Significance**

Angiopoietin-like 3 (Angptl3) is expressed and secreted by liver and circulates in plasma associated with lipoproteins. The best characterized function of Angptl3 is to inhibit lipoprotein lipase activity and thereby decrease plasma triglyceride hydrolysis. In humans, Angptl3 deficiency causes reduction of all major lipoproteins, a phenotype called familial combined hyperlipidemia. We present here a detailed biochemical characterization of Angptl3-deficient subjects, which reveals that Angptl3 deficiency does not only reduce plasma lipids but also alters lipoprotein particle size and composition. Several key enzymes enrolled in lipoprotein metabolism were studied, but postheparin plasma lipoprotein lipase was the only significantly changed parameter. Plasma free fatty acids, insulin, glucose, and homeostatic model assessment of insulin resistance were significantly lower in Angptl3-deficient subjects. Our results provide the first evidence that complete lack of Angptl3 in humans is associated with improved insulin sensitivity and, thus, for a role of Angptl3 in regulating not only lipid but also glucose metabolism.
Angptl3 Deficiency Is Associated With Increased Insulin Sensitivity, Lipoprotein Lipase Activity, and Decreased Serum Free Fatty Acids
Marius R. Robciuc, Marianna Maranghi, Anna Lahikainen, Daniel Rader, Andre Bensadoun, Katariina Öörni, Jari Metso, Ilenia Minicocci, Ester Ciociola, Fabrizio Ceci, Anna Montali, Marcello Arca, Christian Ehnholm and Matti Jauhiainen

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Supplemental figure I. A, Angptl3, B, CETP, C, PLTP, D, apoE and E, PON1 levels were measured in non-carriers and carriers of S17X mutation. Individual values are shown as dot plots and the median line is presented for each group. * p < 0.05, one way ANOVA.
Materials and methods

Study population and blood samples. Individuals previously identified in the population of Campodimele as carriers of the S17X mutation were considered for the present study (1). Twenty two age, gender and BMI-matched non-carriers selected among individuals living in the same community and not carrying any known functional variants in the ANGPTL3 gene were included as controls. The characteristics of the study population and the screening procedures have been described in detail (1). Briefly, subjects were examined in the morning after overnight fast according to a standardized protocol. Personal and familial medical history, presence of cardiovascular risk factors (smoking, hypertension, diabetes) and use of medication were surveyed by questionnaire. Alcohol consumption and physical activity was assessed by a standardized questionnaire. Blood pressure, height, and weight were measured. Hypertension was defined as a blood pressure (BP) ≥140/90 mm Hg and/or use of antihypertensive medication. Diabetes mellitus was defined as a fasting blood glucose ≥126 mg/dl and/or use of hypoglycemic drugs. Fasting blood samples for laboratory determinations were obtained. In addition, postheparin plasma (PHP) samples were collected from 5 homozygotes, 17 heterozygotes and 22 non-carriers by injecting (i.v.) 100 IU heparin/kg of body weight and collecting blood samples 15 min post-injection. Furthermore, we evaluated plasma levels of glucose, insulin and calculated HOMA-IR as index of insulin-resistance according to the ANGPTL3 LOF mutation status in a larger Campodimele cohort to confirm the results obtained from our discovery sample. Informed consent was obtained from all subjects under a protocol approved by the Ethical Committee of University of Rome La Sapienza.

Measurements of lipoprotein lipase (LPL) and hepatic lipase (HL) activity. Lipase activities were measured as previously described (2). Briefly, [Carboxyl-14C]-Triolein (S.A. 2.2 GBq/mmol, PerkinElmer) and glyceryl trioleate (Sigma) emulsified in the presence of gum arabic was used as substrate. PHP was incubated with the substrate and human serum (as a source for apoC-II, LPL cofactor) for 1 hour at 37°C. Reaction was stopped by addition of 3.25 ml of methanol-chloroform-heptane (1.4:1.25:1.00, vol/vol/vol) and 0.75 ml of potassium carbonate/borate buffer (pH 10.5). Hydrophilic phase (BSA-FAs) and hydrophobic phase (triol) were separated by centrifugation and radioactivity was measured from both fractions by liquid scintillation counting (Wallac LS Counter, Turku, Finland). To distinguish between HL and LPL activities, HL lipolytic activity was measured in the presence of high salt (1 M NaCl) as this is known to inhibit lipoprotein lipase activity but not hepatic lipase activity. Values obtained using high salt were attributed to HL activity and LPL activity was calculated by subtracting the HL activity from the total PHP lipolytic activity. Lipolytic activity was expressed as µmol of free FAs released per hour per ml of PHP. Excessive hemolysis impaired accurate measurements of the lipase activities in two non-carriers, three heterozygotes and one homozygote, and these subjects were excluded from the final data analyses.

Measurements of endothelial lipase activity (EL). Endothelial lipase activity was measured as described (3). Results are reported as µmol of FAs released per min per ml of PHP.

Measurements of lipoprotein lipase and hepatic lipase mass. Plasma lipoprotein lipase mass was measured by a sandwich ELISA using monoclonal antibodies 5F9 for capture and 5D2 for detection (4). Plasma hepatic lipase mass was also
determined by sandwich ELISA using monoclonal antibodies 3-6a and 1-1c for capture and detection, respectively (5).

**Lipoprotein profile analysis by fast-performance liquid chromatography (FPLC).** FPLC lipoprotein profiles were obtained as previously described (1). Serum aliquots of 500 µl were applied on a Superose 6HR gel filtration column (Pharmacia Biotech, Uppsala, Sweden) at a flow rate of 0.5 ml/min in PBS (pH 7.4) containing 1 mM EDTA. Chromatography fractions (0.5 ml) were collected and analyzed for total cholesterol, unesterified cholesterol, cholesterol esters, phospholipids, TGs, apoA-I and apoB concentrations.

**Lipoprotein particle size determination.** The size-exclusion chromatography fractions 16-18 were combined for VLDL particle size analysis and fractions 22-27 for LDL particle size analysis. Fractions were chosen based on the highest concentration of apoB and TGs. Particle size analysis was done using dynamic light scattering (DLS) by Zetasizer Nano (Malvern Instruments, Malvern Works, UK). Briefly, a 50-µl aliquot of the combined lipoprotein samples was placed in a micro cuvette, the temperature of the sample was allowed to stabilize in the instrument for 5 min prior to the measurements at 37 °C. Each sample was measured in triplicate. The instrument uses a 633 nm laser and the scattered light is collected at an angle of 173 °.

**Other laboratory determinations.** Blood samples were collected in EDTA-containing tubes and plasma was immediately obtained by centrifugation at 4°C. Aliquots were added with EDTA (0.04%), NaN₃ (0.05%) and PMSF (0.015%) to prevent lipid and lipoprotein modifications. Some were used within 2-4 hours for lipids and blood glucose determination, while others were stored at -80°C for additional measurements. Plasma lipids, were determined as reported (3). Fasting blood glucose and liver enzymes were assayed using standard methods. Angiopoietin-like 3 and 4 serum levels were determined using an ELISA assay (6). Cholesteryl ester transfer protein (CETP) (7), phospholipid transfer protein (PLTP) (8) and serum paraoxonase/arylesterase 1 (PON1) (9) activity, apoA-I and apoE (10) protein levels were measured as reported. ApoB was measured with the ELISA method (Mabtech). Plasma concentration of free fatty acids (FFA) and glycerol were determined using a commercial methods (Wako and BioVision, respectively). Furthermore, we measured, in a larger Campodimele cohort (205 non-carriers and 69 carriers of ANGPLT3 LOF mutations, mostly the S17X) plasma FFA, insulin and glucose and calculated the HOMA-IR index. Values were compared according to the mutation status. Plasma insulin concentrations were measured by using a electrochemiluminescence immunoassay (ECLIA) (Elecys Insulin–Roche Diagnostic GmbH, D-68298 Mannheim) on the Roche/Hitachi COBAS CE 6000 analyzer. The inter-assay variation coefficient was 2.7%. Insulin resistance was estimated by the homeostasis model of insulin-resistance (HOMA-IR) calculated as follows: serum insulin (µU/I) x fasting plasma glucose (mmol/L)/22.5. Plasma concentration of free fatty acids (FFA) was measured by enzymatic colorimetric assay following the protocol provided by manufacturer (Roche Diagnostics, Mannheim, Germany). All determinations were run in duplicates. The mean intra and inter-assay coefficient of variation were 5.5% and 9.2% respectively

**Statistical Analysis.** All statistical analyses were performed with GraphPad Prism software (GraphPad Software, Inc.) and IBM SPSS Statistics 20 for Windows. Values above or below three standard deviations were considered outliers and excluded from the calculation. Differences between genotypes were tested using one-way ANOVA and Kruskal-Wallis test with Dunn’s Multiple Comparison post test if not otherwise specified. Bivariate correlations were performed after values were
logarithmically transformed and Pearson correlation coefficient and significance level (2-tailed) are reported. P value < 0.05 was considered significant. Normal distribution assumption was checked with Kolmogorov-Smirnov test. Normally distributed data were expressed as mean (± SD), whereas variables with a skewed distribution were reported as median and interquartile (IQ) ranges. Non parametric variables were log transformed before comparison. Statistical tests included Student's t test, Mann-Whitney, \( \chi^2 \) or Fisher's exact test when appropriate. Adjustment for age, sex and BMI was performed by using the generalized linear model (GLM) under a recessive model. Correlations among the study variables were tested by the Pearson's coefficient.

References


