Differential Association of Plasma Angiopoietin-Like Proteins 3 and 4 With Lipid and Metabolic Traits

Nidhi Mehta, Arman Qamar, Liming Qu, Atif N. Qasim, Nehal N. Mehta, Muredach P. Reilly, Daniel J. Rader

Objective—Angiopoietin-like protein 3 (ANGPTL3) and 4 (ANGPTL4) are secreted proteins that inhibit lipoprotein lipase in vitro. Genetic variants at the ANGPTL3 and ANGPTL4 gene loci are significantly associated with plasma lipid traits. The aim of this study was to evaluate the association of plasma ANGPTL3 and ANGPTL4 concentrations with lipid and metabolic traits in a large community-based sample.

Approach and Results—Plasma ANGPTL3 and ANGPTL4 levels were measured in 1770 subjects using a validated ELISA assay. A Pearson unadjusted correlation analysis and a linear regression analysis adjusting for age, sex, and race were performed. ANGPTL3 levels were significantly positively associated with low-density lipoprotein cholesterol and high-density lipoprotein cholesterol levels (both \( P<2 \times 10^{-5} \)) but not triglycerides. In contrast, ANGPTL4 levels were significantly negatively associated with low-density lipoprotein cholesterol and high-density lipoprotein cholesterol (both \( P<2 \times 10^{-5} \)) and positively associated with triglycerides (\( P=0.003 \)). In addition, ANGPTL4, but not ANGPTL3, levels were significantly positively associated with fasting blood glucose and metabolic syndrome.

Conclusions—Despite having similar biochemical effects in vitro, plasma ANGPTL3 and ANGPTL4 concentrations have nearly opposite relationships with plasma lipids. ANGPTL4 is strongly negatively associated with low-density lipoprotein cholesterol and high-density lipoprotein cholesterol and positively with multiple features of the metabolic syndrome including triglycerides, whereas ANGPTL3 is positively associated with low-density lipoprotein cholesterol and high-density lipoprotein cholesterol and not with metabolic syndrome traits including triglycerides. Although ANGPTL3 and ANGPTL4 both inhibit lipoprotein lipase in vitro and influence lipoprotein metabolism in vivo, the physiology of these related proteins and their effects on lipoproteins is clearly divergent and complex.

Key Words: angiopoietins ■ cholesterol, HDL ■ cholesterol, LDL ■ diabetes mellitus ■ epidemiology ■ lipid metabolism ■ lipids ■ lipoproteins ■ triglycerides

The family of angiopoietin-like proteins (ANGPTLs) are secreted proteins characterized by key structural motifs including an N-terminal signal peptide directing secretion, a N-terminal coiled-coiled domain, a linker region, and a C-terminal fibrinogen-like domain. Two of the members that have been extensively studied are ANGPTL3 and ANGPTL4. Functional studies in vitro have shown that ANGPTL3 reversibly inhibits and ANGPTL4 irreversibly inhibits lipoprotein lipase (LPL), suggesting a role in lipoprotein metabolism. Transgenic mouse models have shown that ANGPTL3 and ANGPTL4 overexpression leads to increased triglyceride levels. ANGPTL3 knockout mice have at least a 50% reduction in both plasma triglyceride and high-density lipoprotein cholesterol (LDL-C). ANGPTL4 knockout mice have a 65% to 90% decrease in triglyceride levels, lower circulating very-low-density lipoprotein cholesterol levels, and increased LPL activity.

Consistent with these data, genome-wide association studies identified that common variants at the ANGPTL3 locus are associated with triglycerides and low-density lipoprotein cholesterol (LDL-C) and common variants at the ANGPTL4 locus are associated with HDL-C levels. Population-based resequencing in the Dallas Heart Study found that nonsynonymous loss-of-function variants in both proteins were associated with lower triglyceride levels. Exome sequencing in a family with familial combined hypolipidemia identified loss-of-function mutations in ANGPTL3 that were causally associated with decreased triglycerides, LDL-C, and HDL-C.

Despite the biological interest in these proteins, the fact that they are secreted proteins detectable in the plasma, and their potential for therapeutic targeting, there are limited data on plasma levels of ANGPTL3 and ANGPTL4 and their relationship with plasma lipids and metabolic parameters. We therefore undertook the first large-scale study to assay plasma...
ANGPTL3 and ANGPTL4 levels and address their association with lipids and metabolic traits.

**Materials and Methods**

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

**Results**

**Distribution and Demographic Associations With Plasma ANGPTL3 and ANGPTL4 Levels**

The characteristics of the study population (n=1770) are described in Table 1. The sample was predominantly white, majority male, and baseline median age was 53 years. Approximately half of the sample had metabolic syndrome or type 2 diabetes mellitus.

The distribution of plasma ANGPTL3 levels is shown in Figure 1A (mean, 236±87 ng/mL and median, 224 ng/mL) and of plasma ANGPTL4 levels in Figure 1B (median, 128 [interquartile range, 85–198] ng/mL).

Higher levels of ANGPTL3 and ANGPTL4 levels were observed with increasing age quartiles as shown in Figure 2 (r=0.17 and r=0.31, respectively; P<1×10−13 for both) based on Pearson correlation.

ANGPTL3 levels were significantly lower in men (mean, 227±82 ng/mL) than in women (mean, 253±94 ng/mL; P=2.38×10−9) in a logistic regression analysis. There was no significant difference in ANGPTL4 levels between men (mean, 160±108 ng/mL and median, 130 ng/mL) and women (mean, 153±96 ng/mL and median, 126 ng/mL). Although the black population was small (6%), there were significant racial differences in ANGPTL3 and ANGPTL4, with black subjects having lower plasma levels for both proteins (P<0.01 for both) in a logistic regression analysis.

Plasma levels of ANGPTL3 and ANGPTL4 were weakly but significantly correlated with each other. Figure 3 shows a scatter plot of the measured ANGPTL3 and ANGPTL4 concentrations. Based on Pearson correlation, ANGPTL3 and ANGPTL4 were correlated (r=0.18; P<0.0001). When adjusted for age, sex, and race, the same findings held true (P<0.0001).

**ANGPTL3 and ANGPTL4 Are Associated With LDL-C and HDL-C in Opposing Directions**

Plasma ANGPTL3 levels were significantly positively associated with LDL-C (P<0.002), HDL-C (P<10⁻⁵), and total cholesterol (P<10⁻⁷), but were not associated with triglycerides or very-low-density lipoprotein cholesterol (Table 2). The age-, sex-, and race-adjusted linear regression analysis supported these findings (Table 1 in the online-only Data Supplement).

In a second model, a linear regression analysis adjusting for demographics and statin use showed no significant change in the associations observed (Table II in the online-only Data Supplement). Lastly, the same associations were observed between ANGPTL3 and lipid phenotypes among patients with and without diabetes mellitus. Apolipoprotein A-I was positively associated with plasma ANGPTL3 levels based on Pearson correlation and linear regression analysis. Apolipoproteins A-II, B, and C-III were not associated with ANGPTL3 levels in a demographic-adjusted linear regression model (Table I in the online-only Data Supplement).

In contrast, plasma ANGPTL4 levels were significantly negatively associated with LDL-C, HDL-C, and total cholesterol (all P<10⁻⁴) and positively associated with triglycerides.
and very-low-density lipoprotein cholesterol ($P<0.001$; Table 2). The age-, sex-, and race-adjusted linear regression analysis supported these findings (Table I in the online-only Data Supplement). A second model adjusting for age, sex, race, and statin use showed no significant change from the association observed for the demographic-adjusted models (Table II in the online-only Data Supplement).

An interaction analysis was performed between ANGPTL4 and diabetes mellitus status to determine whether diabetes mellitus was affecting the association of ANGPTL4 with lipid parameters. For HDL-C, there was a significant interaction with ANGPTL4 and diabetes mellitus status ($P=0.02$). Interaction analysis of ANGPTL4 and diabetes mellitus was not significant when performing analysis for triglycerides and LDL-C. Therefore, a stratified analysis was performed which showed that among those without diabetes mellitus, there was no significant association between ANGPTL4 and HDL-C, LDL-C, total cholesterol, and triglycerides. Among those with diabetes mellitus, there was a significant negative association between ANGPTL4 and HDL-C ($P<10^{-3}$) and a positive association with triglycerides ($P=0.02$).

In addition, a substratified analysis was performed with metabolic syndrome in those with and without diabetes mellitus. First, there was no association observed between lipid parameters and ANGPTL4 in those without diabetes mellitus with or without metabolic syndrome. All the patients with diabetes mellitus had metabolic syndrome; therefore, a substratified analysis could not be performed.

In support of the lipid findings, apolipoproteins A-I and B were also negatively associated with ANGPTL4.

Figure 1. A, Distribution of angiopoietin-like protein 3 (ANGPTL3) plasma concentrations. B, Distribution of ANGPTL4 plasma concentrations.
levels ($P<0.01$), whereas apolipoprotein C-III, a marker of triglyceride-rich lipoproteins, was positively associated with ANGPTL4 levels ($P<0.02$; Table 2). In a demographic-adjusted linear regression analysis, apolipoproteins A-I, B, and C-III showed similar findings as the Pearson correlation analysis (Table I in the online-only Data Supplement).

**Angiopoietin-Like Protein 3**

**ANGPTL4 Has a Much Stronger Relationship With Metabolic Parameters Than ANGPTL3**

In an unadjusted model, ANGPTL4 had a more significant positive association with body mass index (BMI) and waist circumference ($P<2\times10^{-16}$ for both) compared with ANGPTL3 ($P<10^{-4}$; Table 3). In addition, ANGPTL4, but not ANGPTL3, was significantly and positively associated with fasting plasma glucose ($P<2\times10^{-16}$). ANGPTL4 levels in those with diabetes mellitus were 2 times higher than those without diabetes mellitus (181 [interquartile range, 136–237] versus 93 [interquartile range, 69–134] ng/mL; $P<2.0\times10^{-16}$). ANGPTL4 was also significantly and positively associated with insulin, free fatty acids, and leptin, whereas plasma adiponectin levels were significantly and negatively associated with ANGPTL4 levels. In an age-, sex-, and race-adjusted model, linear regression analysis supported the above findings (Table III in the online-only Data Supplement).
In contrast, although modest positive associations were observed with ANGPTL3 and waist circumference, BMI, hemoglobin A1C, insulin, free fatty acids, and leptin on unadjusted analyses, after adjustment for demographic parameters, ANGPTL3 was only modestly significantly associated with BMI, waist circumference, adiponectin, and leptin (Table III in the online-only Data Supplement).

Given the significant associations observed with all factors of metabolic syndrome—waist circumference, systolic blood pressure, blood glucose, triglycerides, and HDL-C, there was a clear relationship between plasma ANGPTL4 levels and the number of metabolic syndrome parameters, as observed in Figure 4B ($P < 2 \times 10^{-16}$). In contrast, no association was observed between plasma ANGPTL3 levels and metabolic syndrome (Figure 4A).

### Discussion

In the largest study of plasma concentrations of ANGPTL3 and ANGPTL4 to date, we identified highly significant correlations of each protein with lipid and metabolic traits. Remarkably, despite their known similar biochemical properties (specifically inhibition of LPL), plasma concentrations of these 2 proteins have relatively weak associations with plasma triglyceride levels and opposite relationships with LDL-C and HDL-C levels. ANGPTL3 concentrations are significantly positively correlated with LDL-C and HDL-C levels; conversely, ANGPTL4 concentrations are significantly negatively correlated with LDL-C and HDL-C levels. Although ANGPTL4 has an expected (albeit modest) positive correlation with triglyceride levels, ANGPTL3 has no correlation with triglycerides. Strikingly, ANGPTL4 is strongly associated with multiple features of the metabolic syndrome, whereas ANGPTL3 has much less association. Previous studies had small sample sizes or less accurate assays, which resulted in inconsistent associations between these proteins and phenotypic parameters.3,5,13–18 Thus, these results provide new information about these 2 related proteins that are clearly important in lipoprotein biology in humans.

In humans, ANGPTL3 expression occurs predominantly in the liver. ANGPTL3 expression is markedly induced by activation of liver X receptor, which binds to its response element in the ANGPTL3 promoter. ANGPTL3 is proteolytically cleaved by proprotein convertases to yield the biologically active N-terminal fragment and an inactive C-terminal fragment. The N-terminal fragments form multisubunit complexes to protect against degradation. N-terminal ANGPTL3 modestly suppresses LPL catalytic activity in vitro.1 Studies of gain of function of ANGPTL3 in mouse models have shown elevation of fasting triglyceride levels because of suppression of very-low-density lipoprotein clearance via inhibition of LPL, which is more pronounced in the fed state.19 Loss of function in mice results in reduction of both triglyceride and HDL-C levels.5

Common variants at the ANGPTL3 gene locus are significantly associated with LDL-C and triglycerides. Sequencing studies of the coding region of ANGPTL3 revealed that the variant M259T among blacks (mean allele frequency, 5%) was significantly associated with lower triglyceride levels.10 The M259T variant was not associated with other metabolic parameters such as glucose and BMI. Most compellingly, exome sequencing of a large family with pan-hypolipidemia revealed 2 rare ANGPTL3 nonsense mutations that cosegregated with reduced plasma lipid levels.12 Although increased LPL activity attributable to lack of ANGPTL3 might explain the reduced triglyceride levels in this family, it does not explain the reduced LDL-C or HDL-C levels. ANGPTL3 also inhibits endothelial lipase, which hydrolyzes HDL-C phospholipids, and the effect on HDL-C levels could be related to ANGPTL3 inhibition of endothelial lipase activity.5 However, the effect on LDL-C levels remains unexplained.

Studies of plasma ANGPTL3 are sparse and provide conflicting results. One study with 250 Finnish subjects showed that ANGPTL3 was positively associated with HDL-C and negatively associated with triglycerides.14 In a second population of predominantly end-stage kidney disease and healthy subjects (n=394), ANGPTL3 was positively associated with HDL-C and LDL-C on univariate analysis.15 Our finding in
a significantly larger community-based sample that plasma levels of ANGPTL3 are strongly and positively associated with LDL-C and HDL-C levels is novel and largely consistent with the human genetic data. However, we did not observe the expected positive association with triglycerides. This suggests that there are other factors involved in modulating the relationship between ANGPTL3 levels and triglyceride metabolism.

In humans, ANGPTL4 expression occurs in the liver, adipose tissue, small intestine, and heart. ANGPTL4 expression is stimulated by peroxisome proliferator-activated receptor α, which binds to response elements in the ANGPTL4 promoter. Oligomerization occurs with intermolecular disulfide bonds followed by secretion of the protein. ANGPTL4 is proteolytically cleaved via proprotein convertases into an active N-terminal fragment and inactive C-terminal fragment containing the fibrinogen-like domain. N-terminal ANGPTL4 interacts with the extracellular matrix through heparin sulfate proteoglycans and has been shown to irreversibly inhibit LPL. Studies of gain and loss of function of ANGPTL4 in mouse models have shown both elevation and reduction of triglycerides, respectively.

Common variants at the ANGPTL4 gene locus are significantly associated with HDL-C. Population-based resequencing of the coding regions in 3551 subjects from the Dallas Heart Study found 1 nonsynonymous sequence variant, E40K, in European Americans (mean allele frequency, 1.3%) associated with lower plasma triglyceride and LDL-C levels and higher HDL-C levels (P=0.004). These findings were reproduced in 2 larger patient populations. This variant was not genotyped in our study population. ANGPTL4 also inhibits hepatic lipase, which is involved in HDL-C and LDL-C metabolism. Unlike ANGPTL3, no patients with genetic deficiency of ANGPTL4 have been reported.

Thus far, the largest scale study of plasma ANGPTL4 levels was in 666 healthy men and showed that plasma ANGPTL4 levels were inversely associated with HDL-C, but not associated with LDL-C or triglycerides. Smaller scale studies in a Finnish population showed no correlation of plasma ANGPTL4 levels with triglycerides, LDL-C, or HDL-C. Metabolic and inflammatory parameters such as BMI, free fatty acids, and C-reactive protein were positively associated with the protein. Our current study showed that plasma ANGPTL4 concentrations are significantly inversely associated with LDL-C and HDL-C and positively associated with triglycerides. Although the directional associations with triglycerides and HDL-C are consistent with predictions from the human genetics, the inverse association with LDL-C is not. This suggests that there are other factors involved in modulating the relationship between ANGPTL4 levels and LDL metabolism.

The biological explanation for the difference between the associations of ANGPTL3 and ANGPTL4 levels with lipid traits is not obvious. Their mechanisms of inhibition of LPL are different. ANGPTL3 inhibits endothelial lipase, and ANGPTL4 inhibits hepatic lipase; and although it is not clear that these are distinct qualitative differences between the 2 proteins, this could provide some explanation. Of note, there is remarkable variation in their amino acid sequence, and only 30% similarity between the 2 proteins exists. They differ in tissue expression, with ANGPTL4 being more broadly expressed including in intestine and adipose, whereas ANGPTL3 is more specific to liver. Finally, regulation of expression differs, with ANGPTL3 more responsive to liver X receptor and ANGPTL4 to peroxisome proliferator-activated receptor α.

Furthermore, we found that ANGPTL4 levels were highly significantly associated with all aspects of the metabolic syndrome (increased BMI and waist circumference, increased systolic blood pressure, elevated triglycerides, low HDL-C, elevated glucose, and hemoglobin A1C). There are limited data on the effects of ANGPTL4 and glucose metabolism. ANGPTL4 is directly stimulated by free fatty acids via activation of peroxisome proliferator-activated receptor α. ANGPTL4 inhibits extracellular LPL-mediated lipolysis to prevent excess free fatty acid uptake into the cell. ANGPTL4 knockout mice studies show that ANGPTL4 deficiency has a significant inhibitory effect on macrophage foam cell production. However, in transgenic mice, ANGPTL4 overexpression decreased macrophage content by 41% (P<0.05). Human recombinant ANGPTL4 significantly decreased macrophage uptake of oxidized LDL-C.

By an unknown mechanism, ANGPTL4 stimulates intracellular lipolysis in adipocytes and myocytes, particularly in the fasting state. At hyperinsulinemic–euglycemic clamp in 24-hour fasting mice, transgenic ANGPTL4 mice showed reduced glucose utilization compared with controls (125% versus 59%; P<0.05). These data suggest that there is peripheral insulin resistance seen with ANGPTL4 overexpression.
Further mechanistic studies are needed to better elucidate the metabolic functions of ANGPTL4.

One of the limitations of this study was the predominantly white population; therefore, the observed associations may not be generalizable across races. The study population was enriched for diabetes mellitus and metabolic syndrome. In addition, the assays that were used for measuring plasma ANGPTL3 and ANGPTL4 concentrations may have included truncated portions of the protein, which are not involved in lipid metabolism. Because this is a cross-sectional analysis, causal relationships of ANGPTL3 or ANGPTL4 cannot be inferred with lipid and metabolic parameters; only associations can be drawn.

The goal of this study was to determine relationships between ANGPTL3 and ANGPTL4 with various lipid and metabolic parameters. These circulating proteins clearly influence human physiology and may be potential targets for lipid-lowering therapies.

Sources of Funding
This work was supported by grants R37 HL055323 and RC2 HL101834 from the National Heart, Lung, and Blood Institute.

Disclosures
None.

References

Significance
Angiopoietin-like protein 3 (ANGPTL3) and 4 (ANGPTL4) are secreted proteins that inhibit lipoprotein lipase in vitro. Genetic variants at the ANGPTL3 and ANGPTL4 gene loci are significantly associated with plasma lipid traits. However, few data exist regarding the relationship of plasma levels of ANGPTL3 and ANGPTL4 with lipid and metabolic traits. The current study is the largest scale study that highlights the associations between these biomarkers and lipid and energy metabolism. Despite having similar biochemical effects in vitro, plasma ANGPTL3 and ANGPTL4 concentrations have nearly opposite relationships with plasma lipids. ANGPTL4, but not ANGPTL3, is strongly negatively associated with low-density lipoprotein cholesterol and positively with multiple features of the metabolic syndrome, whereas ANGPTL3 is positively associated with low-density lipoprotein cholesterol and high-density lipoprotein cholesterol. These novel biomarkers may be potential targets for lipid-lowering therapies.
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Arterioscler Thromb Vasc Biol. published online March 13, 2014;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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SUPPLEMENTAL MATERIAL

ANGPTL3 ELISA Validation

The Biovendor assay (Biovendor Laboratory Medicine, Prague, Czech Republic) was previously validated in published literature (Robciuc, M et al. JLR 2010, Stejskal, M, et al. Gen Physiol. Biophys 2007). Our internal validation consisted of running different positive controls. We obtained an inter-assay variability of 5.7%. Pre and post-heparinized plasma samples were compared to determine if heparin affects the ANGPTL3 concentrations. A pre and post-heparin analysis was done and the average net change between pre- and post-heparin was 4.6% (Figure I) \(R^2 = 0.93\).

Figure I. Comparison of ANGPTL3 Concentration in Pre-heparin and Post-heparin Samples

ANGPTL4 ELISA Validation

The ANGPTL4 ELISA validation involved testing different commercialized assay kits, which included: R & D DuoSet (R&D Systems, Minneapolis, MN), RayBio Human ANGPTL4 (RayBiotech, Inc., Norcross, GA) and Biovendor (Biovendor Laboratory, Prague, Czech Republic). For the R & D assay, the linearity in dilution (1x to 32x) was inconsistent with diluted samples having significantly lower ANGPTL4 concentrations.
The same finding was observed with the spike and recovery. Next, for the RayBio assay, the linearity of dilution was more consistent than the R & D assay. However, the spike and recovery for this assay was reduced (4%-50% recovery of spiked sample). Lastly, validation was performed with the Biovendor Assay. First, 30 healthy volunteers were tested twice for reproducibility and coefficient of variation ranged from 2% to 12% (outliers, 18% and 28%). Next, average recovery for spiked standard samples was 107%. Lastly, linearity of dilution (1x to 16x) resulted in a 1.5% - 6% coefficient of variation among the subjects. The final concentrations of the diluted samples were on average 82% of the expected value (based on the recombinant ANGPTL4 standard).
Table I. ANGPTL3 and ANGPTL4 Association with Lipid Parameters Adjusted for Demographics*

<table>
<thead>
<tr>
<th>Lipid Parameter</th>
<th>ANGPTL3</th>
<th>ANGPTL4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β coefficient</td>
<td>P</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
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<tr>
<td>VLDL-C, mg/dl</td>
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<td>0.82</td>
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<tr>
<td>LDL-C, mg/dl</td>
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<td>HDL-C, mg/dl</td>
<td>1.60</td>
<td>1.98 x 10^{-6}</td>
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<tr>
<td>Total cholesterol, mg/dl</td>
<td>5.74</td>
<td>1.04 x 10^{-8}</td>
</tr>
<tr>
<td>Apolipoprotein A-I, mg/dl</td>
<td>1.21</td>
<td>0.03</td>
</tr>
<tr>
<td>Apolipoprotein A-II, mg/dl</td>
<td>0.17</td>
<td>0.27</td>
</tr>
<tr>
<td>Apolipoprotein B, mg/dl</td>
<td>0.75</td>
<td>0.19</td>
</tr>
<tr>
<td>Apolipoprotein C-III, mg/dl</td>
<td>0.004</td>
<td>0.84</td>
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*Linear regression model adjusted for age, gender, and race. Beta coefficient indicates increment (positive or negative) in lipid parameter per 1-standard deviation increase in ANGPTL3 or log transformed ANGPTL4 concentrations. LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol. Triglycerides, VLDL-C, and Apolipoprotein C-III were log transformed.
Table II. ANGPTL3 and ANGPTL4 Association with Lipid Parameters Adjusted for Demographics and Statin Use*

<table>
<thead>
<tr>
<th></th>
<th>ANGPTL3</th>
<th></th>
<th>ANGPTL4</th>
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<tr>
<td></td>
<td>β coefficient</td>
<td>P</td>
<td>β coefficient</td>
<td>P</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>0.01</td>
<td>0.445</td>
<td>0.04</td>
<td>0.008</td>
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<tr>
<td>LDL-C, mg/dl</td>
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<td>-2.85</td>
<td>5.15 x 10^{-4}</td>
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<tr>
<td>HDL-C, mg/dl</td>
<td>1.52</td>
<td>6.78 x 10^{-6}</td>
<td>-2.02</td>
<td>4.1 x 10^{-9}</td>
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<tr>
<td>Total Cholesterol</td>
<td>4.07</td>
<td>1.77 x 10^{-5}</td>
<td>-3.44</td>
<td>4.18 x 10^{-4}</td>
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</tbody>
</table>

*Linear regression model adjusted for age, gender, race, and statin use. Beta coefficient indicates increment (positive or negative) in lipid parameter per 1-standard deviation increase in ANGPTL3 or log transformed ANGPTL4 concentrations. LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol. Triglycerides were log transformed.
### Table III. ANGPTL3 and ANGPTL4 Association with Metabolic Parameters*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ANGPTL3 β coefficient</th>
<th>ANGPTL3 P</th>
<th>ANGPTL4 β coefficient</th>
<th>ANGPTL4 P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass index, kg/m²</td>
<td>0.65</td>
<td>1.3 x 10⁻⁵</td>
<td>1.54</td>
<td>2.0 x 10⁻¹⁶</td>
</tr>
<tr>
<td>Waist Circumference, inches</td>
<td>0.66</td>
<td>8.1 x 10⁻⁶</td>
<td>1.67</td>
<td>2.0 x 10⁻¹⁶</td>
</tr>
<tr>
<td>Blood Pressure, mmHG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>-0.18</td>
<td>0.63</td>
<td>0.73</td>
<td>0.0205</td>
</tr>
<tr>
<td>Diastolic</td>
<td>-0.24</td>
<td>0.32</td>
<td>-0.67</td>
<td>0.014</td>
</tr>
<tr>
<td>Fasting Blood Glucose, mg/dl</td>
<td>-0.02</td>
<td>0.014</td>
<td>0.09</td>
<td>2.0 x 10⁻¹⁶</td>
</tr>
<tr>
<td>Hemoglobin A1C, %</td>
<td>0.04</td>
<td>0.33</td>
<td>0.48</td>
<td>2.0 x 10⁻¹⁶</td>
</tr>
<tr>
<td>Insulin, IU/ml</td>
<td>0.03</td>
<td>0.11</td>
<td>0.24</td>
<td>2.0 x 10⁻¹⁶</td>
</tr>
<tr>
<td>Free Fatty Acids, mEq/ml</td>
<td>0.004</td>
<td>0.81</td>
<td>0.15</td>
<td>6.4 x 10⁻¹⁴</td>
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<tr>
<td>Leptin, ng/ml</td>
<td>0.056</td>
<td>0.016</td>
<td>0.15</td>
<td>3.91 x 10⁻⁸</td>
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<tr>
<td>Adiponectin, mg/ml</td>
<td>0.064</td>
<td>0.003</td>
<td>-0.12</td>
<td>3.8 x 10⁻⁸</td>
</tr>
</tbody>
</table>

*Linear regression model adjusted for age, gender, and race. Beta coefficient indicates increment (positive or negative) in cardiometabolic outcome per 1-standard deviation increase in ANGPTL3 or log transformed ANGPTL4 concentrations. Fasting blood glucose, insulin, free fatty acids, leptin, and adiponectin were log transformed.
Materials and Methods

The Penn Coronary Artery Calcification sample included predominantly European Caucasian ancestry subjects recruited from three separate parallel studies: the Study of Inherited Risk of Coronary Atherosclerosis (N=617), the Penn Diabetes Heart Study (N=711), and the Philadelphia Area Metabolic Syndrome Network (N=442). Details of each of these studies have been discussed previously.\(^1,2\)

The Study of Inherited Risk of Coronary Atherosclerosis is a cross-sectional study of factors associated with coronary calcium in a community-based sample of asymptomatic subjects and their families. Subjects were healthy adults aged 30-75 who had a family history of premature coronary artery disease (CAD). Subjects were excluded if they reported evidence of CAD on screening questionnaire, total cholesterol higher than 300 g/dl, cigarette smoking of pack or more per day, blood pressure higher than 160/100 mmHg, had reported a history of diabetes mellitus, or had a serum creatinine >3.0 mg/dl.

The Penn Diabetes Heart Study is a cross-sectional community-based study of type 2 diabetic subjects without clinical evidence of CAD or overt chronic kidney disease. Subjects were aged 35 to 75 years; had a clinical diagnosis of type 2 diabetes (defined as fasting blood glucose >126 mg/dl, 2-h postprandial glucose >200 mg/dl, or use of oral hypoglycemic agents/insulin in a subject greater than age 40 years); and had negative pregnancy test if female. Subjects were excluded if they had evidence for clinical CAD, a clinical diagnosis of type 1 diabetes (insulin use prior to age 35), a serum creatinine >2.5 mg/dl, or weight >300 pounds.

The Philadelphia Area Metabolic Syndrome Network is a cross-sectional study of patients with one or more metabolic syndrome risk factors, as defined by the National Cholesterol Education Program Adult Treatment Panel III. Participants were recruited between 2004-2009 via the University of Pennsylvania Health System primary care providers, word of mouth in the community, and Penn health fairs for cardiovascular disease risk factors. Subjects were aged 18-75 years and had one or more metabolic syndrome risk factors. Subjects with known type 1 diabetes or clinical atherosclerotic coronary vascular disease were excluded. All Penn study protocols were approved by the Penn IRB, and all subjects provided written informed consent.

After a12-hour overnight fast, clinical parameters such as blood pressures, laboratory values, and body mass index were measured as described previously. Plasma lipids were measured enzymatically (Hitachi 912 AutoAnalyzer; Roche Diagnostics GmbH, Basel, Switzerland) in lipoprotein fractions after ultracentrifugation (B-quantification technique) at the University of Pennsylvania Center for Disease Control and Prevention-certified lipid laboratory. Subjects were classified as having metabolic syndrome using the definition of the National Cholesterol Education Program. All subjects were classified as having National Cholesterol Education program metabolic syndrome glucose criteria.

Plasma levels of ANGPTL3 and ANGPTL4 were measured according to the manufacturer’s instructions (Biovendor Laboratory Medicine, Prague, Czech Republic). All samples were assayed and pooled human plasma samples were included to assess variability. Intra- and interassay coefficients of variation were 3.2% and 18.7% for ANGPTL3 and 3.8% and 17.5% for ANGPTL4. The manufacturer performed cross-reactivity analysis and found no cross-reactivity between ANGPTL3, ANGPTL4, and angiopoietin-like protein 2 assays. Testing with other commercialized ELISA kits are described in the Supplemental material. Radioimmunoassay was performed to measure insulin levels.
The statistical analysis was performed with R statistics software. Descriptive characteristics were performed for key clinical risk factors through the use of mean, median, and interquartile ranges for continuous variables and percentages for categorical variables. Plasma ANGPTL4, TG, VLDL-C, apolipoprotein C-III, fasting blood glucose, insulin, free fatty acids, leptin, and adiponectin concentrations did not follow a normal distribution and a natural logarithmic (log) transformation was performed. An unadjusted Pearson’s correlation was performed to summarize associations between continuous risk factors and plasma ANGPTL3 and ANGPTL4 levels. A linear and logistic regression analysis was performed for the key clinical risk factors adjusting for age, gender, and race.

References