Rare loss-of-function mutations in *ANGPTL* family members contribute to plasma triglyceride levels in humans

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The relative activity of lipoprotein lipase (LPL) in different tissues controls the partitioning of lipoprotein-derived fatty acids between sites of fat storage (adipose tissue) and oxidation (heart and skeletal muscle). Here we used a reverse genetic strategy to test the hypothesis that 4 angiopoietin-like proteins (ANGPTL3, -4, -5, and -6) play key roles in triglyceride (TG) metabolism in humans. We re-sequenced the coding regions of the genes encoding these proteins and identified multiple rare nonsynonymous (NS) sequence variations that were associated with low plasma TG levels but not with other metabolic phenotypes. Functional studies revealed that all mutant alleles of *ANGPTL3* and *ANGPTL4* that were associated with low plasma TG levels interfered either with the synthesis or secretion of the protein or with the ability of the ANGPTL protein to inhibit LPL. A total of 1% of the Dallas Heart Study population and 4% of those participants with a plasma TG in the lowest quartile had a rare loss-of-function mutation in *ANGPTL3*, *ANGPTL4*, or *ANGPTL5*. Thus, ANGPTL3, ANGPTL4, and ANGPTL5, but not ANGPTL6, play nonredundant roles in TG metabolism, and multiple alleles at these loci cumulatively contribute to variability in plasma TG levels in humans.

Introduction

In individuals consuming Western diets, more than 100 grams of triglycerides (TGs) are transported each day from the liver and small intestines to peripheral tissues. The partitioning of TG between sites of storage (adipose tissue) and oxidation (primarily heart and skeletal muscle) is determined by the relative activity of lipoprotein lipase (LPL), an enzyme located on the lumenal surfaces of capillaries. LPL catalyzes the hydrolysis of the lipoprotein TG, releasing FFAs, which are taken up by adjacent tissues.

LPL activity is regulated at the transcriptional and posttranslational levels. The hormonal and nutritional milieu of tissues modulates transcription of the *LPL* gene (1). LPL is regulated at the posttranscriptional level by 2 angiopoietin-like proteins (ANGPTLs), ANGPTL3 and ANGPTL4 (2), which belong to a family of 7 structurally similar secreted proteins (ANGPTL1–ANGPTL7). The ANGPTL proteins contain a signal sequence followed by a helical domain predicted to form a coiled coil and a globular, fibrinogen-like domain at the C terminus (3). Both ANGPTL3 and ANGPTL4 inhibit LPL activity in vitro and in vivo (4–6), and mice lacking *Angptl3* or *Angptl4* have increased LPL activity and reduced levels of plasma TG (7, 8).

Although ANGPTL3 and ANGPTL4 both inhibit LPL activity, the 2 proteins have different patterns of expression. ANGPTL3 is expressed almost exclusively in liver (9), an organ that expresses little or no LPL in adults (1), and is presumed to function as a circulating inhibitor of LPL. In contrast to ANGPTL3, ANGPTL4 is expressed in multiple tissues, with the highest level of expression in mice being in adipose tissue (10). Originally, this member of the ANGPTL family was referred to as "fasting-induced adipocyte factor," since its expression is highly induced by fasting (10). It has been proposed that ANGPTL4 inhibits LPL activity in adipose tissue to reroute fatty acids away from fat to muscle and other tissues during fasting (2).

A third member of the ANGPTL family, ANGPTL6 (also referred to as "angiopoietin-related growth factor") has also been implicated in energy metabolism and lipid partitioning. Genetic deletion of *Angptl6* in mice is associated with significant (>80%) embryonic lethality (11). Surviving *Angptl6*$^{-/-}$ mice are markedly obese and hyperinsulinemic and accumulate significant amounts of TG in liver and skeletal muscle. Circulating TG levels are not altered in these mice, but the levels of cholesterol and FFA in serum are increased. An *ANGPTL6* transgene under the control of a β-actin promoter was expressed at high levels in multiple tissues (brown fat, heart, and skeletal muscle) and resulted in reduced white adipose tissue mass and resistance to diet-induced obesity (11).

The physiological roles of these ANGPTL proteins in humans have been inferred largely from studies in mice. Recently, we used a population-based resequencing strategy to examine the metabolic role of ANGPTL4 in humans (12). By resequencing the coding region and proximal intronic regions of *ANGPTL4* in a multiethnic sample of 3,551 individuals, we showed that sequence variations

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Nonstandard abbreviations used: ANGPTL, angiopoietin-like protein; ARIC, Atherosclerosis Risk in Communities (study); DHS, Dallas Heart Study; Fs, frameshift; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; LPL, lipoprotein lipase; MAF, minor allele frequency; NS, nonsynonymous; TG, triglyceride.

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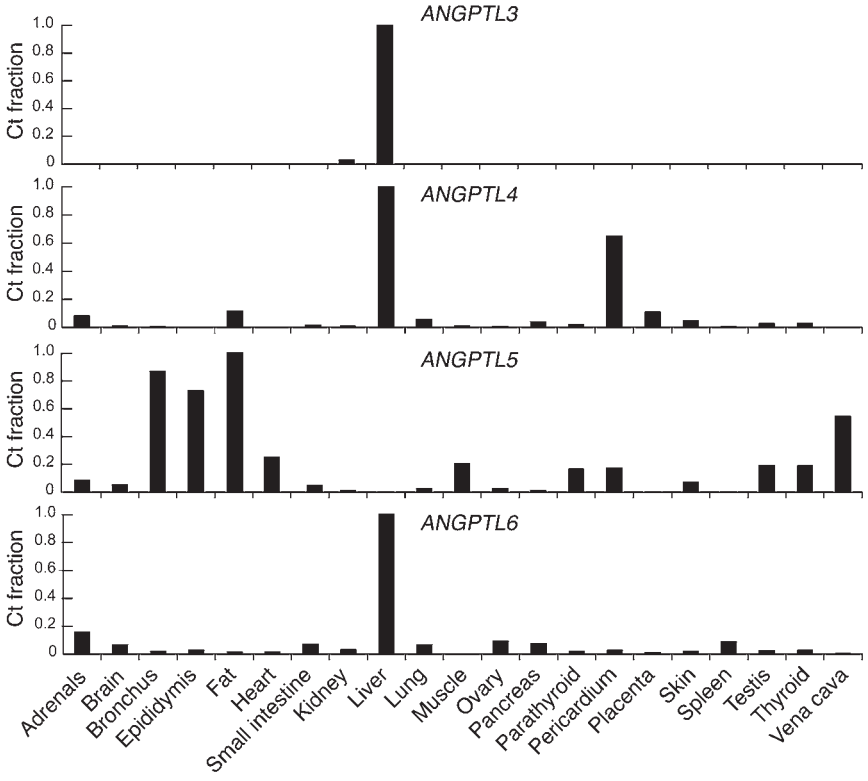

Figure 1
Expression of *ANGPTL3*, *ANGPTL4*, *ANGPTL5*, and *ANGPTL6* in human tissues. Quantitative real-time PCR was used to determine mRNA levels of the 4 *ANGPTL* family members in commercial cDNA arrays of 48 tissues prepared from normal humans (Origene). The 21 tissues in which the signal was detected are shown. Each bar represents the average of triplicate measurements expressed as a fraction of the Ct value obtained from the tissue expressing the highest levels of mRNA for that gene.

that alter an amino acid (nonsynonymous [NS] sequence variations) in ANGPTL4 were more prevalent in individuals with TG levels in the lowest quartile than in the highest quartile ($P = 0.016$). One variant (E40K), which was present in approximately 3% of Americans of mixed European descent, was associated with significantly lower plasma levels of TG and LDL cholesterol (LDL-C) and higher levels of HDL cholesterol (HDL-C) in the Atherosclerosis Risk in Communities (ARIC) study and the Copenhagen City Heart Study (12). These findings confirmed that ANGPTL4 is involved in TG metabolism in humans and revealed that the protein also plays roles in the metabolism of HDL and LDL, which was not apparent from studies in genetically modified mice.

To glean insights into the physiological roles of the other ANGPTL family members in humans, we resequenced *ANGPTL3, ANGPTL5*, and *ANGPTL6* in a large multiethnic population. We complemented these studies with cell-based and in vitro assay studies to examine the effects of the mutations identified on protein synthesis, secretion, and function. We found that 1% of participants in the Dallas Heart Study (DHS) and 4% of those with plasma TG in the lowest quartile had a rare loss-of-function mutation in *ANGPTL3*, *-4*, or *-5*. Functional studies showed that the mutant alleles of *ANGPTL3* and *ANGPTL4* had major detrimental effects on the synthesis, secretion, or function of the protein.

Results

ANGPTL mRNA levels in human tissues. As a first test of the hypothesis that multiple members of the ANGPTL family regulate TG metabolism in different tissues, we examined the levels of mRNA from each gene in 48 human tissues (Figure 1). Expression of ANGPTL3 was largely restricted to liver, consistent with the pattern seen previously in mice (9). The levels of *ANGPTL4* mRNA were also highest in liver, with the next highest level being in the pericardium. The level of the ANGPTL4 transcript in adipose tissue was only 10% that found in liver, despite fat having the highest expression level of ANGPTL4 in mice (10). Low levels of *ANGPTL4* transcript (<10% of liver) were also present in the adrenal glands, lung, pancreas, and placenta, with only trace amounts detected in other tissues.

ANGPTL5 was most highly expressed in adipose tissue, with the bronchus, epididymis, and vena cava having the next highest levels of expression. The transcript was identified at very low levels in many other tissues. A previous study reported that *ANGPTL5* was expressed most strongly in heart (13), but those authors did not examine mRNA from adipose tissue. Although we detected ANGPTL5 mRNA in the heart, it was present at only 20% of the level found in adipose tissue.

Finally, *ANGPTL6* was expressed at the highest level in the liver and at much lower levels in other tissues.

Thus, 3 of the 4 ANGPTLs analyzed in this study (*ANGPTL3*, *ANGPTL4*, and *ANGPTL6*) were most highly expressed in the liver, and 2 of the 4 family members were expressed in adipose tissue (*ANGPTL4* and *ANGPTL5*). This finding is consistent with the hypothesis that these genes coordinate fuel trafficking and homeostasis in response to changes in energy demands.

Multiple sequence variants in the coding regions of ANGPTL3, ANGPTL5, and ANGPTL6. To develop a comprehensive inventory of sequence variations in the coding regions of *ANGPTL3*, *ANGPTL5*, and *ANGPTL6*, we sequenced the exons of the 3 genes in the DHS, a multiethnic, probability-based population (including 1,870 African Americans, 1,045 individuals of mixed European descent, and 601 Hispanics) as previously described (12). A total of 255 variants were identified, most of which were rare: more than half (155/255) were found in only a single individual, and 86% (220/255) had a minor allele frequency (MAF) below 1% (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/

Table 1
Numbers of individuals with NS sequence variations identified in *ANGPTL3*, *ANGPTL4*, *ANGPTL5*, and *ANGPTL6* in the upper or lower quartile of the distribution of selected traits in the DHS

Parameter	ANGPTL3			ANGPTL4			ANGPTL5			ANGPTL6		
	≤25th	≥75th	*P*	≤25th	≥75th	*P*	≤25th	≥75th	*P*	≤25th	≥75th	*P*
BMI	6	12	0.235	4	9	0.26	5	12	0.141	4	12	0.075
Glucose	16	3	**0.002**	6	8	0.593	12	5	0.085	6	8	0.593
Insulin	11	6	0.330	2	7	0.180	4	9	0.264	7	12	0.355
HOMA-IR	11	4	0.110	2	9	0.064	3	9	0.144	6	8	0.789
Systolic BP	11	7	0.479	6	3	0.507	9	4	0.265	7	11	0.353
Diastolic BP	6	8	0.597	6	4	0.754	9	5	0.425	6	6	1
Hepatic TG[A]	7	5	0.773	4	6	0.753	8	6	0.789	5	9	0.421
Cholesterol	11	7	0.495	11	6	0.332	8	9	1	3	14	**0.007**
HDL-C	8	3	0.232	6	8	0.79	5	9	0.286	10	3	0.097
LDL-C	13	10	0.676	14	5	0.063	4	3	1	8	11	0.496
TGs	14	5	0.064	13	2	**0.016**	9	1	**0.022**	6	13	0.107

[A]Hepatic TG content was determined using magnetic resonance imaging (37). NS sequence variations found in both extremes were excluded from the analysis. For each trait, the number of individuals with variants unique to one extreme is provided. To determine whether the distribution of sequence variations was significantly different between the 2 extremes, a contingency table was generated and evaluated using Fisher's exact test. Statistically significant *P* values are shown in bold. HOMA-IR, homeostatic model assessment — insulin resistance.

JCI37118DS1). NS variants were more common than synonymous variants in all 3 genes (97 vs. 27 variants). The density of coding sequence variants was similar in the 3 genes: 1 sequence substitution per 33 nucleotides in *ANGPTL3* and *ANGPTL5* and 1 substitution per 30 nucleotides in *ANGPTL6*.

To determine the phenotypic effects of sequence variations in the 3 genes, we stratified the DHS population by race, sex, and trait level for metabolic phenotypes associated with TG removal from the circulation (plasma TG level), TG accumulation (BMI and hepatic TG content), and indices of energy homeostasis (fasting blood glucose, fasting insulin, homeostatic model assessment — insulin resistance [HOMA-IR]). In addition, we tested for association with systolic blood pressure, diastolic blood pressure, and plasma levels of cholesterol, TG, HDL-C, and LDL-C. We used a strategy similar to that employed to assess the effect of rare sequence variations in other genes on quantitative traits (14, 15). For each phenotype we compared the number of individuals with NS sequence variants in the top and bottom quartiles (Table 1; ANGPTL4 was included for comparison). Any sequence variation present in both the top and the bottom quartiles was eliminated from the analysis.

Rare and common sequence variations in ANGPTL3 are associated with reduced plasma TG levels. A total of 35 NS sequence variations were identified in *ANGPTL3* (Supplemental Table 2). An excess of sequence variants in the lower quartile for plasma TG levels (14 vs. 5 variants) approached the nominal significance threshold ($P = 0.064$; Figure 2A). All sequences likely to be loss-of-function alleles (frameshift 122 [Fs122], FsQ192, and FsK455) were in the lowest quartile of TG levels, suggesting that decreased levels of circulating ANGPTL3 are associated with reduced plasma levels of TG. One of the missense mutations associated with a low plasma TG level, K63T, is predicted to disrupt a heparin binding motif (^{61}VHKTKG^{66}) that is required for LPL inhibition in mice (4).

To further examine the relationship between sequence variation in *ANGPTL3* and plasma TG levels, we tested for association between plasma TG and the common SNPs at the locus (MAF >1%). None of these SNPs was associated with plasma TG levels (data not shown), except for M259T. M259T was present at appreciable frequency among African Americans (MAF = 5%) and was significantly associated with plasma TG levels ($P = 0.006$) in this ethnic group (Supplemental Table 3 and Figure 2B). The allele was rare among individuals of mixed European descent (MAF, 0.1%). To validate this association, we assayed the M259T SNP in African Americans in the ARIC study (16) (Supplemental Table 3). This analysis confirmed that the M259T SNP was significantly associated with plasma levels of TG ($P = 0.014$). Taken together, these data indicate that a spectrum of sequence variants in *ANGPTL3* contributes to variation in plasma TG levels.

The number of NS sequence variants in *ANGPTL3* was similar in the upper and lower quartiles of the distribution for BMI ($P = 0.235$) and hepatic TG content ($P = 0.773$). Similarly, the common allele associated with plasma TG levels (M259T) was not associated with either parameter. A significant excess of NS sequence variants was found among individuals in the lowest quartile for blood glucose levels (16 vs. 3 variants; $P = 0.002$) in the DHS, but in both the DHS and in ARIC, the M259T variant was not associated with blood glucose, insulin, or with any of the other metabolic parameters examined (Supplemental Table 3). Taken together, these findings indicate that sequence variation in *ANGPTL3* was associated with plasma levels of TG, but not with other indices of fat accumulation or metabolism, although we cannot exclude the possibility that some rare variants in ANGPTL3 affect plasma glucose levels.

Excess of rare sequence variations in ANGPTL5 in individuals with low plasma levels of TG. A similar strategy was used to analyze the metabolic effects of sequence variations in *ANGPTL5* (Figure 3A), a gene of unknown function that is not expressed in mice (13). A significant excess of NS variants was found in the lowest quartile of TG levels ($n = 9$) compared with the highest quartile ($n = 1$) ($P = 0.022$). No frameshift or nonsense mutations in this gene were identified in the lowest quartile, but a single mutation in a consensus splice donor site (IVS8+1) was found. Only 1 NS variant (T268M) in *ANGPTL5* had a MAF of greater than 1%, and this variant was not consistently associated with plasma TG levels in the DHS or in ARIC (data not shown). Sequence variations in *ANGPTL5* were not associated with any of the other metabolic phenotypes examined.

Sequence variations in ANGPTL6 are not associated with plasma TG levels. In contrast to *ANGPTL3, ANGPTL4,* and *ANGPTL5*, in which NS variants were significantly associated with plasma TG levels, we

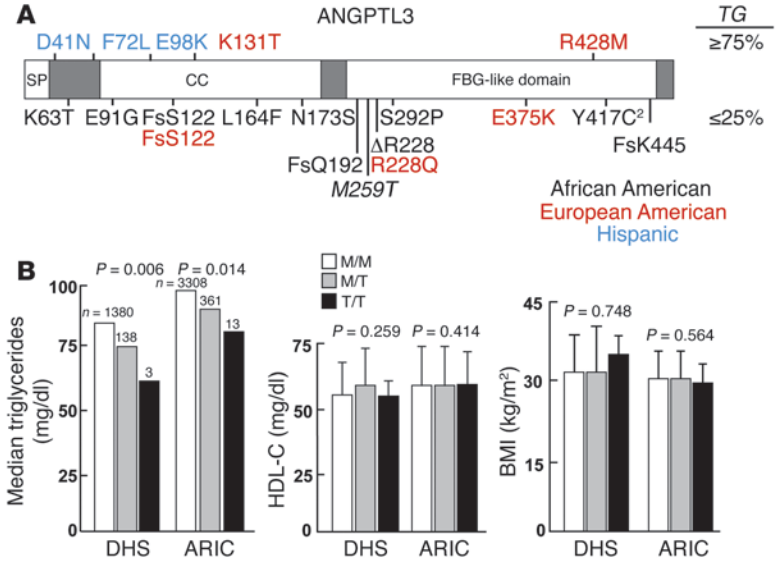

Figure 2
Schematic representation of ANGPTL3 with positions of NS sequence variations identified in the upper and lower quartiles of TG distribution in the DHS. (**A**) The deduced 460–amino acid ANGPTL3 protein has the characteristic features of angiopoietins: a signal peptide (SP), an extended helical domain predicted to form coiled coils (CC), and a globular fibrinogen homology domain (FBG-like domain) at the C terminus. An excess of NS sequence variations was found in lower quartile of TG distribution compared with the upper quartile (14 vs. 5 variations; $P = 0.064$). Coiled coil domains were predicted using COILS (http://www.ch.embnet.org/software/COILS_form.html), and the fibrinogen-like domain sequence was obtained from NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Protein&itool=toolbar). Δ, deletion of an amino acid. (**B**) A common allele of *ANGPTL3* (M259T) present in 10% of African Americans was associated with lower plasma levels of TG in 2 the DHS and the ARIC study. The variant was not associated with HDL-C or BMI. M/M, M/T, and T/T refer to predicted amino acids at position 259 of ANGPTL3.

found no evidence of association between sequence variants in *ANGPTL6* and plasma TG levels. The number of individuals with NS variants in *ANGPTL6* did not differ significantly in the upper and lower quartiles of the TG distribution (Figure 3B). Two NS variants in *ANGPTL6* had a MAF of greater than 1% (R96P and R358C). Neither of these variants was associated with TG levels in the DHS (data not shown). A statistically significant excess of NS variants in *ANGPTL6* was found in upper quartile of plasma cholesterol levels when compared with the lowest quartile of cholesterol levels, but not in the upper quartile of LDL-C or HDL-C levels (Table 1).

Figure 3C summarizes the relationship between plasma TG levels and sequence variations in 3 of the ANGPTL family members analyzed in this paper (ANGPTL3, ANGPTL5, and ANGPTL6), together with data from our prior analysis of ANGPTL4. The numbers of individuals with NS variants in the top and bottom quartiles for plasma TG are given for each gene. As was observed for ANGPTL3 and ANGPTL5, sequence variations in ANGPTL4 were more common among individuals with plasma levels of TG in the lowest quartile compared with the highest quartile.

The majority of NS variants in ANGPTL3, ANGPTL4, and ANGPTL5 associated with low plasma TG levels interfere with protein secretion. Several of the sequence variants in ANGPTL3, ANGPTL4, and ANGPTL5 that were found in the lowest quartile of plasma TG levels were nonsense, frameshift, or splice-site mutations (Figures 2 and 3). This finding suggested that loss-of-function alleles of these genes are associated with low plasma TG levels. The remainder of the mutations found in the lowest quartile of TG levels were missense mutations. To determine whether these mutations also interfere with protein function, we generated cDNA expression constructs for each mutant allele and compared the expression and secretion of the mutant proteins with wild-type ANGPTL in cultured human embryonic kidney (HEK293A) cells. Immunoblot analysis of the cell lysates and the medium are shown in Figure 4A. Whereas wild-type ANGPTL3 was readily detected in the medium, 5 of the 9 missense mutations present in the lowest TG quartile, but none of the 5 missense variants in the high-TG group abolished secretion of ANGPTL3 from cells.

Similarly, when the same experiment was performed to examine the NS sequence variants that we previously identified in *ANGPTL4* (12), 5 of the 7 missense alleles had either a partial or complete deficit in ANGPTL4 secretion (Figure 4B).

Finally, in contrast to wild-type ANGPTL5 or the allele containing the missense mutation found in the high TG group (I233V) 3 of the 7 missense mutations in *ANGPTL5* associated with a low plasma TG level failed to be secreted from the cells (Figure 4C).

Thus, of the 23 missense mutations in the 3 ANGPTL family members that were found in individuals in the lower quartile of plasma TGs, more than half ($n = 13$) impaired protein secretion, presumably by interfering with the proper folding of the protein. These mutations were located almost exclusively in the highly structured fibrinogen-like domains located at the C terminus of the proteins (Figure 2).

Effects of ANGPTL3 and ANGPTL4 mutations on LPL-mediated hydrolysis of TG in vitro. Previously, ANGPTL3 and ANGPTL4 were shown to inhibit LPL activity in vitro (6, 17). ANGPTL4 appears to disrupt catalytically active LPL dimers (17). To determine whether the *ANGPTL* variants identified in the lowest quartile of plasma TG levels that were secreted normally had a reduced ability to suppress LPL activity, we tested the effect of the mutant proteins on LPL activity in vitro. Addition of conditioned medium from cells expressing ANGPTL3 reproducibly suppressed LPL activity by more than 50% (Figure 5A). In contrast, conditioned media from cells expressing *ANGPTL3-259T* (Figure 5A) or the rare *ANGPTL3* alleles associated with low plasma levels of TG (Figure 5B) failed to suppress LPL activity.

Conditioned medium from cells expressing wild-type ANGPTL4 consistently suppressed LPL activity by more than 90% (Figure 5C). These data are consistent with prior data showing that ANGPTL4 has more potent inhibitory effect on LPL activity than does ANGPTL3 (5, 18). Conversely, when conditioned medium containing equivalent quantities of mutant ANGPTL4 proteins that were found in the low TG group was added to the lipase assay, no suppression of LPL activity was observed. Furthermore, the mutant ANGPTL4 proteins failed to suppress LPL even when added at concentrations 10-fold higher than those at which the wild-type protein completely inhibited the enzyme (data not shown). The inhibitory effects of both ANGPTL3 and ANGPTL4 were specific for the salt-sensitive component of post-heparin plasma lipase activity (data not shown). Both proteins had negligible effects on the salt-resistant lipase (hepatic lipase) activity.

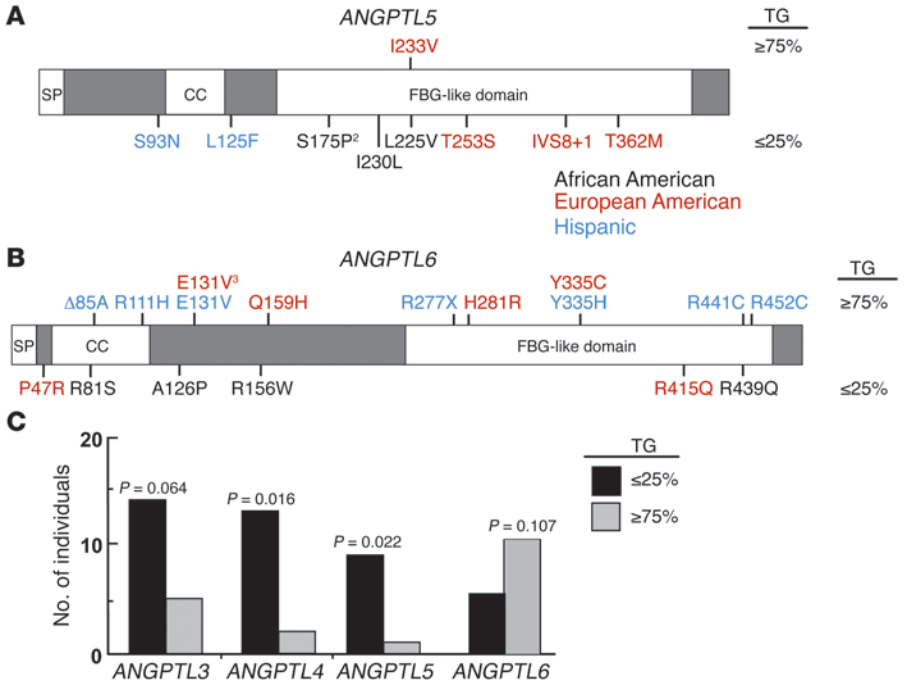

Figure 3
Schematic representation of ANGPTL5 and ANGPTL6 with positions of NS variants identified in the upper and lower quartiles of the TG distribution in the DHS. (**A**) Individuals carrying NS sequence variations in *ANGPTL5* were more prevalent in the bottom quartile than in the top quartile (9 vs. 1 individuals, respectively; $P = 0.012$). (**B**) A corresponding analysis of *ANGPTL6* revealed no significant difference in allele frequencies (6 vs. 13 individuals; $P = 0.107$). (**C**) Number of subjects with plasma TG levels in the upper quartile ($n = 878$) and the lower quartile ($n = 897$) of the DHS who had nonsense, missense, and splicing mutations in *ANGPTL3*, *ANGPTL4*, *ANGPTL5*, and *ANGPTL6*. The difference in numbers of subjects in the upper and lower quartiles is due to differences in the number of individuals with plasma TG levels at the thresholds for the quartiles. IVS, intron.

ANGPTL5 was expressed at much lower levels than was ANGPTL4 and ANGPTL3, and we were unable to obtain comparable levels of this protein in the media. Therefore, we were unable to examine the effects of this protein on LPL activity. ANGPTL6 was efficiently secreted from cells but did not inhibit LPL activity at any of the concentrations tested (Figure 5D).

The mutations in ANGPTL3 and ANGPTL4 that interfered with their ability to inhibit LPL activity were in the N-terminal region of the protein, which is consistent with the observation of Sukonina et al. (17) that the N-terminal portion of ANGPTL4 interacts with the enzyme.

Comparison of in vitro and in silico analysis of ANGPTL mutations. A total of 31 NS variants were identified in the low-TG group and 8 in the high-TG group (Table 2). Of the 31 variants in the low-TG group, 8 (26%) introduced a premature termination codon or altered a consensus splice site, 13 interfered with secretion of the protein from cells (Figure 4), and 6 resulted in proteins that were secreted but failed to inhibit LPL activity. Thus, for ANGPTL3 and ANGPTL4, all variants found only in the low-TG group severely compromised the function of the protein. Three of the 7 mutations in ANGPTL5 prevented expression or secretion; we were not able to assess the effects of the remaining 4 mutations on LPL activity.

Multiple in silico programs have been developed to predict the functional effects of sequence variations, although these perform with variable success (19). Our analysis allowed us to assess the utility of 2 computer programs, PolyPhen (20) and SIFT (21), which predict the functional consequences of amino acid substitutions. PolyPhen correctly predicted 10 of the 19 mutations (53%), whereas SIFT predicted 12 of the variations (63%). Of the 7 variants that showed no functional defect, 2 were predicted to be deleterious by PolyPhen and 3 were predicted to be deleterious by SIFT. Thus, the false positive rates were 29% and 43% for PolyPhen and SIFT, respectively.

Cumulative analysis of rare NS sequence variations in ANGPTL3, ANGPTL4, ANGPTL5, and plasma TG levels. To assess the cumulative effects of rare variants in the 3 genes in the DHS, we pooled the data for *ANGPTL3*, *ANGPTL4*, and *ANGPTL5* and found a greater than 4-fold excess of individuals with NS sequence variations in the low-TG group ($n = 36$) compared with the high-TG group ($n = 8$) (Figure 6). The total number of synonymous sequence variations ($n = 8$) was identical in the high-TG and low-TG groups. Although each variant was rare (MAF, <1%), taken together, 1 of every 24 participants (4%) in DHS with TG levels below the 25th percentile had a NS variant in 1 of these 3 genes.

Discussion

In this study we used resequencing in a well-phenotyped population of unselected individuals to define the physiological roles of ANGPTL proteins in humans. The major finding was that multiple loss-of-function alleles in *ANGPTL3* and *ANGPTL5*, together with those previously reported in *ANGPTL4* (12), are associated with lower plasma levels of TG, suggesting a common role for these proteins in the metabolism of TG-rich lipoproteins. One percent of participants in the DHS (and 4% of those with a TG in the lowest quartile) had a rare sequence variant in 1 of these 3 genes that was associated with low plasma levels of TG. Most of the sequence variants identified in this study prevented (or markedly reduced) secretion of the mutant proteins from cells. Resequencing also revealed an allele of *ANGPTL3* that was common in African Americans and was significantly associated with plasma TG levels in 2 large populations. The sequence variants associated with low plasma TG levels were not associated with the amount of fat in the body or in the liver or with indices of glucose metabolism. Taken together, these findings indicate that *ANGPTL3*, *ANGPTL4*, and *ANGPTL5* regulate the uptake of TG-derived fatty acids from the circulation but do not have a major impact on the overall accumulation of TG in the body, on the partitioning of TG between the liver and peripheral tissues, or on the efficiency of glucose utilization.

Genetic manipulation of mice is a powerful and widely used strategy to assess the physiological roles of genes. The finding that a reduction in plasma TG levels is the major consequence of loss-of-function

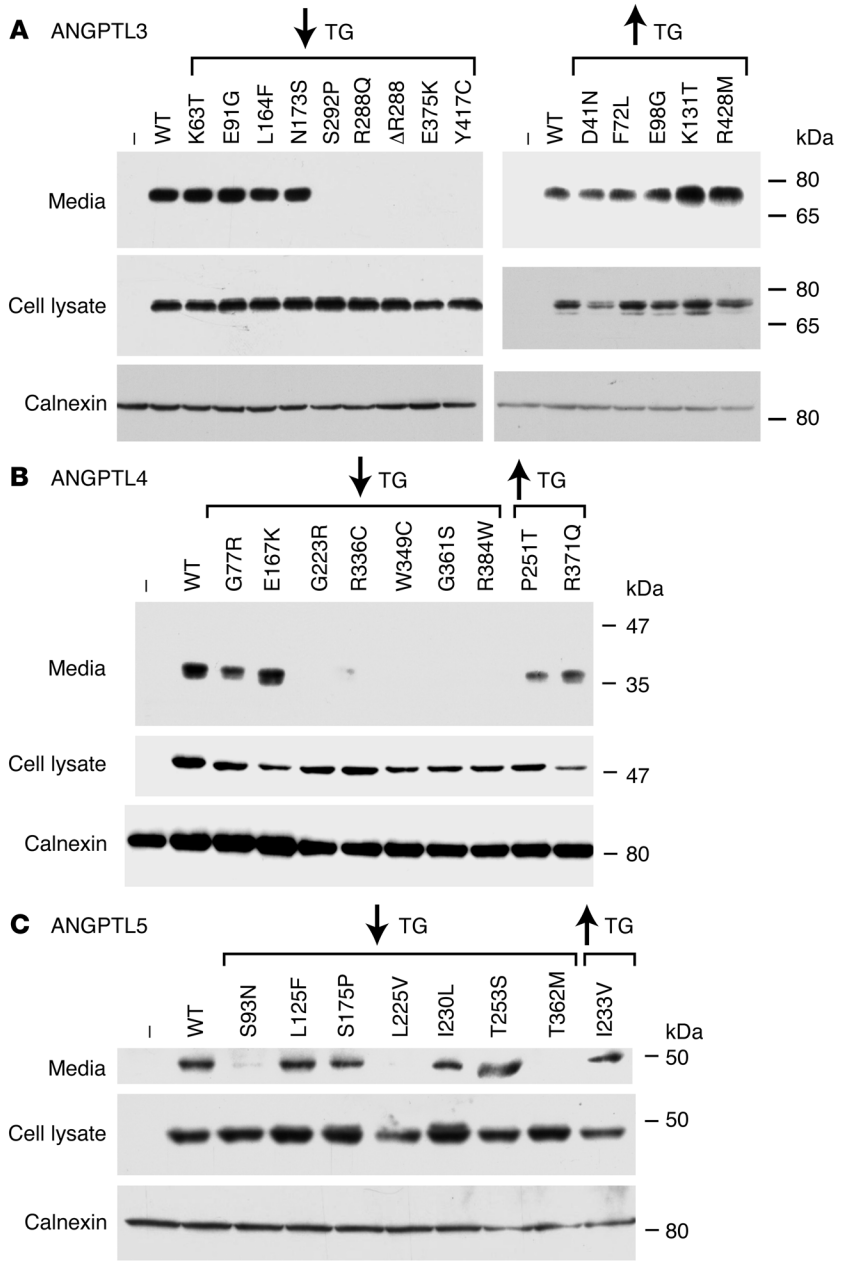

Figure 4
Effects of sequence variations on the synthesis and secretion of ANGPTL3, ANGPTL4, and ANGPTL5. (**A**) ANGPTL3, (**B**) ANGPTL4, and (**C**) ANGPTL5. Wild-type and mutant forms of ANGPTL3, ANGPTL4, and ANGPTL5 were expressed in HEK293A cells, and immunoblotting was performed on the cell lysates and medium using an anti-V5 mAb as described in Methods. Calnexin was used as a loading control. This experiment was repeated 3 times with similar results. ↓TG, sequence variations found in individuals with TG in the ≤ 25th percentile; ↑TG, sequence variations found in individuals with TG in the ≥ 75th percentile; —, vector only.

mutations in *ANGPTL3* and *ANGPTL4* both in humans and in mice affirms the value of mouse models for metabolic studies. However, other phenotypes observed in *Angptl*-knockout mice were not recapitulated in humans with loss-of-function alleles in these genes. In mice, genetic deletion of *Angptl3* is associated with an approximately 50% reduction in plasma levels of HDL-C, perhaps due to increased activity of endothelial lipase, an enzyme that hydrolyzes HDL phospholipids (22, 23). Since ANGPTL3 inhibits endothelial lipase in vitro, it has been proposed that ANGPTL3 normally suppresses endothelial lipase–mediated catabolism of HDL, resulting in higher plasma levels of HDL (22, 24). In support of this model, inactivation of *Angptl3* in vivo, either genetically or enzymatically, is associated with a substantial reduction in HDL phospholipid and HDL-C levels in mice (22, 24). In the present study, loss-of-function mutations in *ANGPTL3* were not associated with a decrease in plasma levels of HDL-C. Thus, our data are not consistent with a significant role for ANGPTL3 in determining plasma levels of HDL-C in humans.

Angptl6$^{-/-}$ mice are obese and have both hepatic steatosis and hyperinsulinemia (11). We found no evidence that genetic variability in *ANGPTL6* contributes to BMI, hepatic TG content or fasting glucose or insulin levels in humans. The apparent differences in the phenotypic effects of *Angptl6* mutations in mice and humans may be due to loss-of-function alleles at this locus being recessive in humans. We did not identify any individuals in the DHS with mutations in both *ANGPTL6* alleles. Alternatively, only a single line of *Angptl6* knockout mice has been described (11), and some of the phenotypic effects reported in that mouse line may be due to factors unrelated to the inactivation of *Angptl6*. These findings underscore the essential role for human studies in validating observations from model organisms.

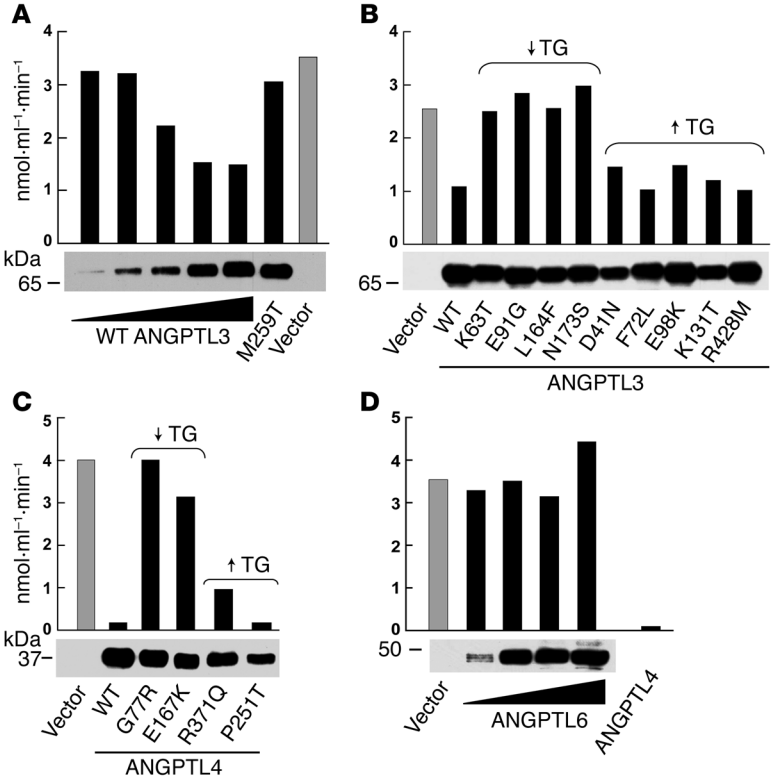

Figure 5
Effects of sequence variations in ANGPTL3 and ANGPTL4 on LPL activity. Wild-type or mutant forms of ANGPTL3 (**A** and **B**), ANGPTL4 (**C**), and ANGPTL6 (**D**) were expressed in HEK293A cells, and conditioned medium was collected and concentrated. The effect of increasing concentrations (range of 20-fold) of protein was evaluated for wild-type ANGPTL3 (**A**) and ANGPTL6 (**D**) as described in Methods.

The association between sequence variations in *ANGPTL5* and plasma TG levels demonstrates that the reverse genetic strategy employed in this study can provide insights into the physiological roles of genes even without a priori information from physiological studies or observations in model systems. *ANGPTL5* was initially identified during a large-scale sequencing study of a human fetal brain library (25). No ortholog of *ANGPTL5* has been identified in mice, and functional analysis of the protein is limited to a single report in which recombinant ANGPTL5 was shown to support ex vivo expansion of human cord blood hematopoietic stem cells (26). The observation that loss-of-function mutations in *ANGPTL5* are associated with low plasma levels of TG suggests that ANGPTL5 also plays a role in the metabolism of TG-rich lipoproteins. We believe that this finding provides the first insight into the physiological function of ANGPTL5 in humans.

Recently, 2 large genome-wide association studies reported that plasma levels of TG were associated with multiple sequence variants spanning a 250-kb interval on chromosome 7 that contains *ANGPTL3*, *DOK7*, and *USP1* (27, 28). The specific variants responsible for the associations were not identified. The *ANGPTL3* alleles identified in the present study are unlikely to explain these associations, since all the sequence variations found in individuals of mixed European descent, the population represented in the genome-wide association studies, were rare. The only common *ANGPTL3* allele (*ANGPTL3-259T*) associated with plasma TG levels was largely restricted to African Americans and had a frequency of 0.1% in individuals of mixed European descent. Taken together, these findings indicate that a spectrum of rare and common sequence variants in *ANGPTL3* contributes to variation in plasma TG levels in the general population. No sequence variants in *ANGPTL4* or *ANGPTL5* reached genome-wide significance in either study.

The observation that sequence variations in *ANGPTL3*, *ANGPTL4*, and *ANGPTL5* contribute independently to plasma TG levels indicates that the 3 proteins are not functionally redundant. Since *ANGPTL3* and *ANGPTL4* are both expressed primarily in the liver in humans, and loss-of-function mutations in the 2 genes are associated with very similar lipoprotein phenotypes, it is possible that the 2 proteins function as a heterodimer. Both proteins contain coiled coil domains as well as a fibrinogen domain at the C terminus, and prior studies have shown that ANGPTL4 forms higher-order oligomers (29) and that oligomerization is required for activity (30). Coexpression of the 2 proteins in cultured cells (including cultured hepatoctyes) failed to reveal any evidence that they form a stable complex (data not shown), but additional studies in different cell types will be required to rule out this possibility.

An alternative possibility is that *ANGPTL3*, *ANGPTL4*, and *ANGPTL5* perform similar roles but in different tissues or different metabolic states. This hypothesis is consistent with our finding that *ANGPTL3*, *ANGPTL4*, and *ANGPTL5* have different patterns of expression (Figure 1) and with previous reports that *ANGPTL4* is strongly upregulated by fasting and is suppressed by refeeding (10), whereas *ANGPTL3* mRNA levels do not change significantly in response to changes in food intake (18). Further studies will be required to determine the specific effects of ANGPTL3, ANGPTL4, and ANGPTL5 on the uptake of TG-derived fatty acids in different tissues and under different nutritional states.

Interestingly, the mutations we identified in these 4 members of the ANGPTL family were not evenly distributed among the 3 major ethnic groups in the DHS. The majority of rare mutations in *ANGPTL4* (8 of 13), as well as a common sequence variation in this gene (E40K) that was associated with lower plasma TG levels (E40K) were found in individuals of mixed European descent (12).

Table 2
Observed and predicted functional effects of the NS variants in ANGPTLs in the top and bottom quartiles of the DHS

ANGPTL	TG quartile	NS variants[A]	In vitro assays[B]			In silico predictions[C]	
			Expression	Secretion	LPL inhibition	PolyPhen	SIFT
ANGPTL3	25th	K63T	–	–	X	+	++
		E91G	–	–	X	+	++
		FsS122	X				
		L164F	–	–	X	N	+
		N173S	–	–	X	N	N
		FsQ192	X				
		ΔR288	–	X			
		R288Q	–	X		N	++
		S292P	–	X		+	N
		E375K	–	X		N	N
		Y417C	–	X		++	+
		FsK445	X				
	75th	D41N	–	–	–	N	++
		F72L	–	–	–	+	N
		E98K	–	–	–	N	N
		K131T	–	–	–	N	++
		R428M	–	–	–	+	N
ANGPTL4	25th	G77R	–	–	X	+	++
		IVS3+1	X				
		E167K	–	–	X	N	N
		K217X	X				
		G223R	–	X		++	++
		FsK245	X				
		FsS302	X				
		R336C	–	X		N	++
		W349C	–	X		++	++
		G361S	–	X		+	++
		R384W	–	X		++	++
	75th	P251T	–	–	–	++	N
		R371Q	–	–	–	N	++
ANGPTL5	25th	S93N	–	X		N	N
		L125F	–	–	?	N	N
		S175P	–	–	?	N	N
		L225V	–	X		N	+
		I230L	–	–	?	N	N
		T253S	–	–	?	N	N
		IVS8+1	X				
		T362M	–	X		+	+
	75th	I233V	–	–	?	N	N

[A]NS sequence variations found only in individuals with TG levels in the lowest quartile (25th) or in the highest quartile (75th) in the DHS. [B]Nonsense, frameshift, and splicing mutations were assumed to interfere with protein synthesis and were not included in the analysis. Recombinant proteins were expressed in HEK293A cells and assayed for secretion and inhibition of LPL activity (see Methods). X, variant showed defective function; —, no defect observed; ?, experiment not performed due to insufficient expression levels. Empty cells indicate that no data were available for the sequence variant either in vitro or in silico. [C]SIFT (21) and PolyPhen (20) were used to predict the effects of the amino acid substitutions on protein function. N, neutral; +, possibly damaging to protein function; ++, probably damaging to protein function, as predicted using the PolyPhen and SIFT algorithms.

Conversely, 10 of the 13 alleles in *ANGPTL3* identified in the low-TG group were present in African Americans, and a common allele associated with low plasma TG levels (M259T) was also largely restricted to this population. In contrast to these 2 genes, in which the mutations clustered in a single ethnic group, the NS sequence variations identified in *ANGPTL5* were found in all 3 ethnic groups in approximately equal proportion (Figure 3). Hispanics were overrepresented among those individuals in the highest quartile of TG who had mutations in *ANGPTL6* (Figure 3). These ethnic differences in allele frequencies may reflect historic differences in selective pressures among the 3 ethnic groups.

The finding that approximately 1% of the individuals in this study had a sequence variant that profoundly altered protein function is consistent with our findings in other genes (14, 31, 32) and with in silico predictions from Sunyaev and colleagues that about 1% of the alleles of a typical 500–amino acid protein will contain a deleterious mutation (33). Thus severe loss-of-function mutations, though individually rare, are collectively common in the population. This observation provides a strategy for reverse genetics in humans. Historically, the major advances in human genetics have been made almost exclusively by using forward genetic approaches in which individuals are selected based on a phenotypic classifi-

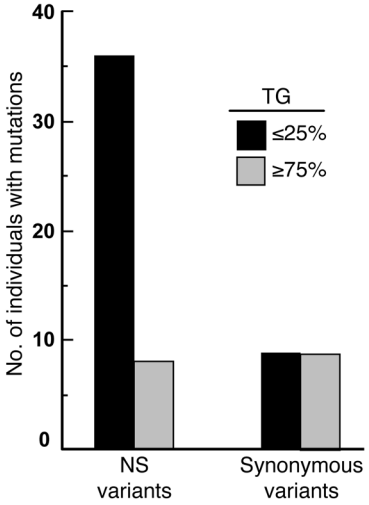

Figure 6
Cumulative frequency of NS and synonymous sequence variations in *ANGPTL3*, *ANGPTL4*, and *ANGPTL5* in the upper and lower quartiles of plasma TG distribution in the DHS. The number of individuals in each quartile was $n = 878$ (upper quartile) and $n = 897$ (lower quartile).

cation and then screened for causal sequence variations. Reverse genetic approaches have been highly informative in model organisms, where genetic manipulations can be performed to assess the phenotypic consequences of inactivation, overexpression, or alteration of the sequence of a gene, but these approaches have played little role in human genetics. Since it is not feasible to manipulate genes in humans, human geneticists rely on naturally occurring variations that are almost invariably ascertained indirectly as a consequence of their associated phenotypes. Advances in sequencing technology are poised to shift the focus of resequencing from selected candidate genes to the entire exome, and possibly to the genome. By resequencing large cohorts of well-characterized individuals, the sequence variants identified can be tested against multiple traits to determine the roles of genes to human physiology.

Methods

Study populations. The coding regions of the *ANGPTL3*, *ANGPTL5*, and *ANGPTL6* gene were sequenced in all DHS participants ($n = 3,551$) who underwent phlebotomy (34). The DHS is a population-based probability sample of Dallas County (52% African American, self-identified as "black;" 29% individuals of mixed European descent, self-identified as "white;" 17% self-identified as "Hispanic;" and 2% of other ethnicities), in which ethnicity was self-assigned according to US census categories. Only the individuals of mixed European descent, African Americans, and Hispanics were included in our analysis. The DHS was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center, and all subjects provided written informed consent before participating in the study.

A subset of the genetic associations observed in the DHS were validated in the ARIC study (16). The ARIC study is a prospective study of atherosclerosis in 4 communities in the USA (Jackson, Mississippi; Minneapolis, Minnesota; Forsyth County, North Carolina; and Washington County, Maryland). A randomly selected cohort of approximately 4,000 individuals ages 45–64 years was selected from each community. The protocol for the study was approved by the institutional review boards of the Johns Hopkins University, the University of North Carolina, the University of Minnesota, and the University of Mississippi, and all participants provided written informed consent that included consent for genetic studies.

Assay of plasma lipids and lipoproteins. Plasma and lipoprotein cholesterol and TG concentrations were determined colorimetrically by using commercial enzymatic reagents.

DNA sequencing and genotyping. The exons and flanking introns of *ANGPTL3*, *ANGPTL5*, and *ANGPTL6* were sequenced in both directions in the 3,551 DHS participants, as previously described (12). All sequence variants identified were verified by manual inspection of the chromatograms, and missense changes were confirmed by an independent resequencing reaction.

Fluorogenic 5′-nucleotidase assays to detect the M259T polymorphism in *ANGPTL3* were developed using the TaqMan assay system (Applied Biosystems). The assays were performed on a 7900HT Fast Real-Time PCR instrument with probes and reagents purchased from Applied Biosystems.

Expression of ANGPTL in cultured cells. Expression constructs for human ANGPTL3, ANGPTL4, ANGPTL5, and ANGPTL6 were made in pcDNA3.1 (Supplemental Table 4) under the control of the cytomegalovirus promoter-enhancer (pCMV-ANGPTL3-V5, pCMV-ANGPTL4-V5, pCMV-ANGPTL5-V5). A V5 epitope tag (GKPIPNPLLGLDST) was placed at the C-terminus of each construct. Single base pair changes were introduced into these constructs using QuikChange (Stratagene). The presence of the desired mutation and the fidelity of each construct were confirmed by DNA sequencing.

HEK293A cells were seeded (1×10^5 cells/well) in 6-well plates and grown in DMEM with 10% fetal calf serum (Cellgro). Expression plasmids (4 μg/well) were used to transfect HEK293A cells using Lipofectamine 2000 (2.5 μl/μg of plasmid DNA) in DMEM according to the manufacturer's protocol (Invitrogen). After 48 h, the medium was collected and centrifuged for 5 min (5,000 *g* at 4°C) and the supernatants were collected. The cells were washed twice in PBS and then incubated in 0.3 ml of 1× RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% [vol/vol] NP-40, 0.5% [vol/vol] sodium deoxycholate, 0.1 % SDS, and complete mini EDTA-free protease inhibitor cocktail [Roche]) for 15 min at 4°C. Cells were harvested by scraping with a rubber policeman and transferred to 1.5-ml tubes prior to centrifugation for 15 min (15,000 *g* at 4°C). Aliquots from the medium and cells were subjected to SDS-PAGE and immunoblot analysis.

SDS-PAGE and immunoblot analysis. Protein concentrations in the cell lysates were determined using the Bio-Rad bicinchoninic acid assay, according to the manufacturer's protocol. Equivalent amounts of protein from the cell lysate and medium were added to the sample loading buffer (final concentration of 1×). The samples were heated to 95°C for 5 min, size-fractionated on 12% SDS-polyacrylamide gels at 150 V, and then transferred to nitrocellulose membranes at 100 V for 1 h. Membranes were incubated in PBST buffer (1× PBS plus 0.1% Tween-20) with 5% dry milk for 60 min at room temperature before addition of the primary antibodies. Monoclonal anti-V5 antibody (Invitrogen) and a polyclonal antibody to calnexin (Stressgen) were diluted to 1:5,000 in PBST buffer with 5% dry milk and incubated with the membranes for 60 min. Membranes were washed 3 times for 10 min each time in PBST buffer. Horseradish peroxidase–conjugated donkey anti-rabbit IgG or goat anti-mouse IgG (Pierce Biotechnology) was diluted (1:10,000) in PBST buffer with 5% dry milk and incubated with membranes for 60 min. Membranes were washed 3 times for 10 min each time in PBST and were visualized using SuperSignal-enhanced chemiluminescence (Pierce Biotechnology).

In vitro assay of LPL. LPL activity assay was measured using a modification of the procedure described by Nilsson-Ehle and Schotz (35). Media harvested from HEK293A cells transfected with ANGPTL expression constructs were concentrated 8-fold and buffer-exchanged into 1× PBS (pH 7.4). The concentrated media were mixed with 7.5 μl mouse post-heparin plasma, brought up to a volume of 50 μl with 0.2 M Tris HCl buffer (pH 8.0), and incubated at 20°C or 25°C. Radiolabeled substrate (16.7 μl) composed of 9,10-^{3}H(N) triolein (American Radiolabeled Chem-

icals), triolein and phosphatidylcholine (Sigma-Aldrich), 16.7 μl heat-inactivated fetal calf serum, and bovine serum albumin (3% in 66.6 μl of 0.2 M Tris HCl buffer [pH 8.0]) were added and the mixtures were incubated for 15 min at 37°C in the presence or absence of 1 M NaCl, which inhibits LPL activity (36). The reactions were terminated by adding heptane/chloroform/methanol (1:1.25:1.41), mixed, and centrifuged at 3,000 g for 15 min. A 1-ml aliquot of upper (aqueous) phase was taken into scintillation tubes and counted. The amount of [^{3}H]-fatty acid released was calculated as previously described (35).

Real-time PCR. The expression of ANGPTL3, -4, -5, and -6 in human tissues was examined using cDNA prepared from 48 tissues (Human Normal cDNA Panel). The cDNA was standardized to 2 ng using GAPDH as a calibrator. Oligonucleotides specific to each gene were used to amplify by PCR in 2× SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 66 μl according to the manufacturer's instructions. For each gene, the tissue expressing the highest activity was used as a reference and assigned an arbitrary expression level of 1. The mean of the 3 CT measurements in each tissue was expressed as a fraction of the level observed in the highest-expressing tissue.

Statistics. The prevalence of NS variants in the upper and lower quartiles of the DHS was compared using Fisher's exact test. Individuals with diabetes, as defined previously (12), and subjects on lipid-lowering medications were not included in the analysis. Men who consumed more than 30 g (and women who consumed more than 20 g) of alcohol per day were also excluded from the analyses. Risk factor levels between carriers of each *ANGPTL* variant with a MAF of greater than 1% and noncarriers were compared by ANOVA. For the comparisons of plasma lipid levels between the genotype groups, we included age, sex, and diabetes mellitus as covariates in the model. Plasma levels of TG and insulin were log-transformed before analysis.

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1. Merkel, M., Eckel, R.H., and Goldberg, I.J. 2002. Lipoprotein lipase: genetics, lipid uptake, and regulation. *J. Lipid Res.* **43**:1997–2006.
2. Li, C. 2006. Genetics and regulation of angiopoietin-like proteins 3 and 4. *Curr. Opin. Lipidol.* **17**:152–156.
3. Yoon, J.C., et al. 2000. Peroxisome proliferator-activated receptor gamma target gene encoding a novel angiopoietin-related protein associated with adipose differentiation. *Mol. Cell. Biol.* **20**:5343–5349.
4. Ono, M., et al. 2003. Protein region important for regulation of lipid metabolism in angiopoietin-like 3 (ANGPTL3): ANGPTL3 is cleaved and activated in vivo. *J. Biol. Chem.* **278**:41804–41809.
5. Yoshida, K., Shimizugawa, T., Ono, M., and Furukawa, H. 2002. Angiopoietin-like protein 4 is a potent hyperlipidemia-inducing factor in mice and inhibitor of lipoprotein lipase. *J. Lipid Res.* **43**:1770–1772.
6. Shimizugawa, T., et al. 2002. ANGPTL3 decreases very low density lipoprotein triglyceride clearance by inhibition of lipoprotein lipase. *J. Biol. Chem.* **277**:33742–33748.
7. Koishi, R., et al. 2002. Angptl3 regulates lipid metabolism in mice. *Nat. Genet.* **30**:151–157.
8. Koster, A., et al. 2005. Transgenic angiopoietin-like (angptl)4 overexpression and targeted disruption of angptl4 and angptl3: regulation of triglyceride metabolism. *Endocrinology.* **146**:4943–4950.
9. Conklin, D., et al. 1999. Identification of a mammalian angiopoietin-related protein expressed specifically in liver. *Genomics.* **62**:477–482.
10. Kersten, S., et al. 2000. Characterization of the fasting-induced adipose factor FIAF, a novel peroxisome proliferator-activated receptor target gene. *J. Biol. Chem.* **275**:28488–28493.
11. Oike, Y., et al. 2005. Angiopoietin-related growth factor antagonizes obesity and insulin resistance. *Nat. Med.* **11**:400–408.
12. Romeo, S., et al. 2007. Population-based resequencing of ANGPTL4 uncovers variations that reduce triglycerides and increase HDL. *Nat. Genet.* **39**:513–516.
13. Zeng, L., et al. 2003. Identification of a novel human angiopoietin-like gene expressed mainly in heart. *J. Hum. Genet.* **48**:159–162.
14. Cohen, J.C., et al. 2004. Multiple rare alleles contribute to low plasma levels of HDL cholesterol. *Science.* **305**:869–872.
15. Cohen, J., et al. 2006. Multiple rare variants in NPC1L1 associated with reduced sterol absorption and plasma low-density lipoprotein levels. *Proc. Natl. Acad. Sci. U. S. A.* **103**:1810–1815.
16. Atherosclerosis Risk in Communities Study. 1987. *ARIC manual of operations: No 2, cohort component procedures.* ARIC Coordinating Center, School of Public Health, University of North Carolina. Chapel Hill, North Carolina, USA.
17. Sukonina, V., Lookene, A., Olivecrona, T., and Olivecrona, G. 2006. Angiopoietin-like protein 4 converts lipoprotein lipase to inactive monomers and modulates lipase activity in adipose tissue. *Proc. Natl. Acad. Sci. U. S. A.* **103**:17450–17455.
18. Ge, H., et al. 2005. Differential regulation and properties of angiopoietin-like proteins 3 and 4. *J. Lipid Res.* **46**:1484–1490.
19. Kotowski, I.K., et al. 2006. A spectrum of PCSK9 alleles contributes to plasma levels of low-density lipoprotein cholesterol. *Am. J. Hum. Genet.* **78**:410–422.
20. Sunyaev, S., et al. 2001. Prediction of deleterious human alleles. *Hum. Mol. Genet.* **10**:591–597.
21. Ng, P.C., and Henikoff, S. 2003. SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Res.* **31**:3812–3814.
22. Shimamura, M., et al. 2007. Angiopoietin-like protein3 regulates plasma HDL cholesterol through suppression of endothelial lipase. *Arterioscler. Thromb. Vasc. Biol.* **27**:366–372.
23. McCoy, M.G., et al. 2002. Characterization of the lipolytic activity of endothelial lipase. *J. Lipid Res.* **43**:921–929.
24. Jin, W., et al. 2007. Hepatic proprotein convertases modulate HDL metabolism. *Cell Metab.* **6**:129–136.
25. Xu, J., et al. 2001. Cloning, expression and characterization of a novel human REPS1 gene. *Biochim. Biophys. Acta.* **1522**:118–121.
26. Zhang, C.C., Kaba, M., Iizuka, S., Huynh, H., and Lodish, H.F. 2008. Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. *Blood.* **111**:3415–3423.
27. Willer, C.J., et al. 2008. Newly identified loci that influence lipid concentrations and risk of coronary artery disease. *Nat. Genet.* **40**:161–169.
28. Kathiresan, S., et al. 2008. Six new loci associated with blood low-density lipoprotein cholesterol, high-density lipoprotein cholesterol or triglycerides in humans. *Nat. Genet.* **40**:189–197.
29. Ge, H., et al. 2004. Oligomerization and regulated proteolytic processing of angiopoietin-like protein 4. *J. Biol. Chem.* **279**:2038–2045.
30. Ge, H., Yang, G., Yu, X., Pourbahrami, T., and Li, C. 2004. Oligomerization state-dependent hyperlipidemic effect of angiopoietin-like protein 4. *J. Lipid Res.* **45**:2071–2079.
31. Cohen, J., et al. 2005. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nat. Genet.* **37**:161–165.
32. Fahmi, S., Yang, C., Esmail, S., Hobbs, H.H., and Cohen, J.C. 2008. Functional characterization of genetic variants in NPC1L1 supports the sequencing extremes strategy to identify complex trait genes. *Hum. Mol. Genet.* **17**:2101–2107.
33. Kryukov, G.V., Pennacchio, L.A., and Sunyaev, S.R. 2007. Most rare missense alleles are deleterious in humans: implications for complex disease and association studies. *Am. J. Hum. Genet.* **80**:727–739.
34. Victor, R.G., et al. 2004. The Dallas Heart Study: a population-based probability sample for the multidisciplinary study of ethnic differences in cardiovascular health. *Am. J. Cardiol.* **93**:1473–1480.
35. Nilsson-Ehle, P., and Schotz, M.C. 1976. A stable, radioactive substrate emulsion for assay of lipoprotein lipase. *J. Lipid Res.* **17**:536–541.
36. Berbee, J.F., van der Hoogt, C.C., Sundararaman, D., Havekes, L.M., and Rensen, P.C. 2005. Severe hypertriglyceridemia in human APOC1 transgenic mice is caused by apoC-I-induced inhibition of LPL. *J. Lipid Res.* **46**:297–306.
37. Szczepaniak, L.S., et al. 2004. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *Am. J. Physiol. Endocrinol. Metab.* **288**:E462–E468.