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ORIGINAL RESEARCH

ANGPTL3 (Angiopoietin-Like 3) Preferentially Resides on High-Density Lipoprotein in the Human Circulation, Affecting Its Activity

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BACKGROUND: ANGPTL3 (angiopoietin-like protein 3) is an acknowledged crucial regulator of lipid metabolism by virtue of its inhibitory effect on lipoprotein lipase and endothelial lipase. It is currently unknown whether and to which lipoproteins ANGPTL3 is bound and whether the ability of ANGPTL3 to inhibit lipase activity is affected by binding to lipoproteins.

METHODS AND RESULTS: Incubation of ultracentrifugation-isolated low-density lipoprotein (LDL) and high-density lipoprotein (HDL) fractions from healthy volunteers with recombinant ANGPTL3 revealed that ANGPTL3 associates with both HDL and LDL particles ex vivo. Plasma from healthy volunteers and a patient deficient in HDL was fractionated by fast protein liquid chromatography, and ANGPTL3 distribution among lipoprotein fractions was measured. In healthy volunteers, ≈75% of lipoprotein-associated ANGPTL3 resides in HDL fractions, whereas ANGPTL3 was largely bound to LDL in the patient deficient in HDL. ANGPTL3 activity was studied by measuring lipolysis and uptake of ^{3}H-trioleate by brown adipocyte T37i cells. Unbound ANGPTL3 did not suppress lipase activity, but when given with HDL or LDL, ANGPTL3 suppressed lipase activity by 21.4±16.4% (P=0.03) and 25.4±8.2% (P=0.006), respectively. Finally, in a subset of the EPIC (European Prospective Investigation into Cancer) Norfolk study, plasma HDL cholesterol and amount of large HDL particles were both positively associated with plasma ANGPTL3 concentrations. Moreover, plasma ANGPTL3 concentrations showed a positive association with incident coronary artery disease (odds ratio, 1.25 [95% CI, 1.01–1.55], P=0.04).

CONCLUSIONS: Although ANGPTL3 preferentially resides on HDL, its activity was highest once bound to LDL particles.

Key Words: adipocytes ■ atherosclerosis ■ cholesterol ■ drug therapy/hypolipidemic drugs ■ lipase/lipoprotein ■ triglycerides

ANGPTL3 (angiopoietin-like protein 3) is a member of a family of 8 angiopoietin-like proteins[1,2] and is primarily expressed and secreted by the liver.[3] ANGPTL3 recently emerged as a key player in lipid metabolism following the observation that loss-of-function mutations in *ANGPTL3* result in a distinct phenotype called familial combined hypolipidemia, which is characterized by low plasma triglyceride, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol concentrations.[1,4–9] Accordingly, inhibition of ANGPTL3 by a monoclonal antibody reduced plasma LDL cholesterol concentrations up to 50% in healthy volunteers[10–12] as well as in patients with homozygous familial hypercholesterolemia.[13] The latter

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RESEARCH PERSPECTIVE

What New Question Does This Study Raise?

- ANGPTL3 (angiopoietin-like protein 3) resides on low-density and high-density lipoprotein particles with a preference for large high-density lipoprotein.
- ANGPTL3 has the highest lipase inhibitory activity when residing on low-density lipoprotein.

What Question Should Be Addressed Next?

- The directionality of the association between large high-density lipoprotein and ANGPTL3 should be explored by investigating whether the presence of large high-density lipoprotein particles results in more plasma ANGPTL3 due to a buffering effect.

Nonstandard Abbreviations and Acronyms

ANGPTL	angiopoietin-like protein
EPIC	European Prospective Investigation Into Cancer
FFA	free fatty acid
FPLC	fast protein liquid chromatography
LPL	lipoprotein lipase
TRL	triglyceride-rich lipoprotein

finding suggests that the lipid-lowering property of ANGPTL3 inhibition is independent of the functioning of the LDL receptor,[14] providing a therapeutic option in those patients with severely reduced or even absent LDL receptor functioning. ANGPTL3 inhibition reduced plasma triglyceride concentrations in patients with elevated plasma triglycerides but only in patients with residual lipoprotein lipase (LPL) activity,[15] highlighting the importance of the interaction between this lipase and ANGPTL3. Indeed, various other studies also show that ANGPTL3 inhibits LPL activity,[16,17] especially in conjunction with ANGPTL8.[18]

The causality of the relationship between plasma ANGPTL3 and lipoprotein concentrations remains ambiguous. Although ANGPTL3 presumably controls plasma lipoprotein concentrations via its inhibitory actions on LPL and endothelial lipase,[19] a reciprocal regulatory effect of lipoproteins on plasma ANGPTL3 concentrations cannot be ruled out. It might be that lipoproteins control plasma ANGPTL3 concentrations by binding this protein, a feature that might affect the ability of ANGPTL3 to suppress LPL activity. Here, we explored the distribution of ANGPTL3 over the main lipoprotein classes and determined the differential effect on LPL activity of ANGPTL3 bound to HDL and LDL particles. In addition, we determined the plasma ANGPTL3 concentrations and its association with lipoproteins in a subset of the EPIC (European Prospective Investigation into Cancer) Norfolk study.

METHODS

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Human Lipoprotein Profiling, Isolations, and Incubations

Blood was obtained from 3 healthy subjects and 1 subject with Tangier disease in EDTA-containing vacutainers. Plasma was isolated as described[20] and concentrations of total cholesterol, HDL-cholesterol, and triglyceride were measured using commercially available assays (Wako Chemicals, Neusss, Germany; DiaSys Diagnostic Systems, Holzheim, Germany; Roche Diagnostics, Almere, the Netherlands) on a Vitalab Selectra E analyzer (Vital Scientific, Dieren, the Netherlands). Plasma LDL cholesterol concentrations were calculated by the Friedewald formula.[21] Plasma was fractionated into 250-μL fractions using fast protein liquid chromatography (FPLC) with a superpose-6 increase column at a flow rate of 0.5 mL/min using an ÄKTA Explorer 10XS (GE Healthcare, Chicago, IL). The cholesterol and ANGPTL3 concentrations of these fractions were measured using a commercially available assay (DiaSys Diagnostic Systems) and a human ANGPTL3 DuoSet ELISA (R&D Systems, Minneapolis, MN), respectively. Immunoblotting was performed on a subset of the isolated fractions.

The plasma was also used to isolate LDL and HDL by gradient ultracentrifugation as described before[22] and dialyzed overnight against PBS with 12- to 14-kD molecular weight cutoff membranes (Medicell Membranes Ltd., London, UK). Part of the isolated HDL and LDL fractions were incubated 30 minutes at 37°C with recombinant human ANGPTL3 (3829-AN, R&D Systems) or PBS as control. Following incubation, samples were fractionated by FPLC into volumes of 250 μL as described earlier, and the cholesterol and ANGPTL3 concentrations were measured in these fractions.

Lipoprotein Preparations for in Vitro Assays

Triglyceride-rich lipoproteins (TRLs), LDL, and HDL were isolated by gradient ultracentrifugation and

dialyzed as described from the plasma obtained from a healthy male donor 2 hours after ingestion of a fat-rich meal (ie, 2 ham and cheese croissants that contained 20.6 grams of fat, of which 11 grams are saturated[23]).

Cell Culture

The brown preadipocyte cell line T37i[24] (provided by Dr M. Lombès, Inserm U1185, France), was cultured with DMEM/F12 with L-glutamine (21 041 025, Gibco-Invitrogen, Breda, the Netherlands) supplemented with 10% fetal bovine serum (Serena, Brandenburg, Germany), penicillin–streptomycin (Gibco-Invitrogen), and 20 mmol/L HEPES (Gibco-Invitrogen). Two days after reaching full confluence (differentiation day 0), the cells were differentiated by adding 2 nmol/L triiodothyronine and 20 nmol/L insulin (both from Sigma-Aldrich, Zwijndrecht, the Netherlands) to the medium. This medium was refreshed every 2 to 3 days until full differentiation on day 9 when cells were used to measure the effect on (1) TRL lipolysis, (2) radioactive LPL activity, (3) LPL protein concentration, and (4) *Lpl*, *Ucp1*, and *Angptl4* mRNA expression.

TRL Lipolysis Experiment

The medium of the fully differentiated T37i cells was removed and the cells were washed twice with PBS. For the overnight incubation experiments, the cells were incubated with lipid-depleted medium (DMEM/F12 containing 10% lipoprotein-depleted serum and penicillin–streptomycin) supplemented with or without 500 ng/mL recombinant human ANGPTL3 (3829-AN, R&D Systems) in combination with 167 ng/mL recombinant human ANGPTL8 (10159-AN, R&D Systems) in the presence or absence of 10% ultracentrifugation-isolated HDL or LDL from the healthy male donor. For the short incubation experiments, the cells were incubated overnight with lipid-depleted medium without additional supplementations.

In both experiments the medium was substituted after the overnight incubation by lipid-depleted medium supplemented with or without 500 ng/mL recombinant human ANGPTL3 in combination with 167 ng/mL recombinant human ANGPTL8 in the presence or absence of 10% ultracentrifugation-isolated HDL or LDL from the healthy male donor. Thirty minutes later, ultracentrifugation-isolated TRLs from the same healthy male subject were added to the medium to a final concentration of 10%. The free fatty acid (FFA) concentrations were measured from the samples medium using a commercially available assay (Fujifilm NEFA-HR[2]; Wako Chemicals, Neuss, Germany) before and 30 and 90 minutes after the addition of TRLs.

Radioactive LPL Activity Experiment

The medium of the fully differentiated T37i cells was removed and the cells were washed twice with PBS after which the cells were incubated overnight with lipid-depleted medium. After this incubation, the medium was removed, and lipid depleted medium was added, but now supplemented with or without recombinant human ANGPTL3 in combination with recombinant human ANGPTL8 in the presence or absence of 10% ultracentrifugation-isolated HDL or LDL from the healthy male donor. Thirty minutes after start of incubation, a mixture of [9,10-^{3}H(N)]-trioleylglycerol (Perkin-Elmer, Waltham, MA), unlabeled trioleylglycerol and lecithin (both from Sigma-Aldrich) emulsified in glycerol (Sigma-Aldrich) was added to the medium. After 30 minutes, the medium was collected, and the reaction was stopped by adding a mixture of chloroform–methanol–heptane (33:40:27) and a buffer containing 0.1 M K_2CO_3 and 0.1 M H_3BO_3, pH 10.5, to the medium. These mixtures were centrifuged at 3000*g* for 5 minutes, after which the upper layer was collected. The cells were washed 3 times with PBS and collected with RIPA buffer (Pierce, Rockford, IL). Radioactivity was measured in the collected upper layer and in the cell lysates. The sum of radioactivity determined in the upper phase, and the lysate is a readout for LPL activity.

LPL Protein Content Experiment

The medium of the fully differentiated T37i cells was removed, and the cells were washed twice with PBS after which they were incubated overnight with lipid-depleted medium supplemented with or without recombinant human ANGPTL3 in combination with recombinant human ANGPTL8 in the presence or absence of 10% ultracentrifugation-isolated HDL or LDL from a healthy subject. After incubation, the medium was removed and replaced by the same medium but now also supplemented with 10 IE/mL heparin (LEO Pharma, Ballerup, Denmark). This medium was collected 30 minutes later and used for LPL immunoblotting, as described in a later section.

Gene Expression Experiment

The medium of the fully differentiated T37i cells was removed and the cells were washed twice with PBS. The cells were incubated overnight with lipid-depleted medium (DMEM/F12 or DMEM containing 10% lipoprotein depleted serum and penicillin–streptomycin) supplemented with or without 500 ng/mL recombinant human ANGPTL3 in combination with 167 ng/mL recombinant human ANGPTL8 in the presence or absence of 10% ultracentrifugation-isolated HDL or LDL from the healthy male subject. After the overnight

incubation, the cells were immediately stored at −80 °C until RNA isolation.

Immunoblotting

Selected fractions and media were diluted in reducing sample buffer and denatured at 95 °C for 10 minutes after which they were electrophoresed on an 4–12% Bis-Tris gel (Invitrogen, Breda, the Netherlands) and blotted onto a PDVF membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in 3% skim milk powder in tris-buffered saline (TBS) containing 0.1% Tween–20 for 1 hour at room temperature and incubated overnight at 4°C with antibodies against human ANGPTL3 (BAF3829, R&D Systems), apolipoprotein A1 (ab7613; Abcam, Cambridge, UK), apolipoprotein E (18–272-197 662; GenWay Biotech, San Diego, CA), or LPL (AF7197; R&D Systems) in 3% skim milk powder in TBS containing 0.1% Tween-20. Next, membranes were washed and incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated goat antirabbit or rabbit antigoat secondary antibody (Dako, Santa Clara, CA) in 3% skim milk powder in TBS containing 0.1% Tween-20. After washing with TBS containing 0.1% Tween-20, bands were visualized with the SuperSignal West Femto Substrate (Thermo Fisher) on a ChemiDoc MP (Bio-Rad).

Gene Expression Analysis

Total RNA from T37i cells was isolated using Tripure Isolation Reagent (Roche Applied Sciences, Almere, the Netherlands) according to the instructions of the manufacturer. Reverse transcription was performed using a cDNA synthesis kit (SensiFAST cDNA synthesis kit, Bioline, London, UK) according to the instructions of the manufacturer. Quantitative real-time polymerase chain reaction was performed using SensiFAST SYBRgreen (Bioline) with a CFX384 Real-Time PCR System (Bio-Rad). Primer sequences are listed in Table S1. The expression of each gene was reported in arbitrary units after normalization to the average expression level of the reference genes *Actb* and *Rplp0* using the $2^{-\Delta\Delta Ct}$ method.[25]

EPIC-Norfolk Prospective Population Study

We performed analyses in a previously described subpopulation of the EPIC (European Prospective Investigation Into Cancer) cohort,[26] a collaboration of multiple cohort studies in 10 European countries, set up to investigate multiple diseases. The EPIC-Norfolk cohort, which is part of the EPIC consortium, recruited 25 663 apparently healthy men and women between 40 and 79 years old, all residents of Norfolk, United Kingdom between 1993 and 1997. A baseline survey and clinical and laboratory measurements were performed. In the follow-up, participants were adjudicated as having coronary artery disease (CAD) if they had a hospital admission or died with CAD as underlying cause. CAD was defined as code 410–414 according to the *International Classification of Diseases, Ninth Revision (ICD-9)*. In 2004, a prospective nested case–control study among participants of the EPIC-Norfolk cohort was initiated.[27] Cases were defined as participants who developed CAD during follow-up through 2003. Controls were study participants who remained free of any cardiovascular disease during 7.4 years of follow-up. Controls were matched in a 2-to-1 ratio to cases by sex, age (within 5 years), and date of visit (within 3 months). The study was approved by the Norwich District Health Authority Ethics Committee, and all participants gave signed informed consent.

Nonfasting samples were obtained at baseline and plasma concentrations of total cholesterol, HDL cholesterol, and triglyceride were measured using an RA 1000 auto-analyzer (Bayer Diagnostics, Basingstoke, UK). LDL cholesterol concentrations were calculated with the Friedewald formula.[21] Automated nuclear magnetic resonance spectroscopic assay was used to measure the lipoprotein size and subclass particle concentrations.[28] ANGPTL3 plasma concentrations were measured with a human ANGPTL3 DuoSet ELISA (R&D Systems).

Statistical Analysis

Results were compared using Mann–Whitney *U* tests for nonpaired analyses with GraphPad Prism (Version 9, GraphPad Software, Inc.). Plasma ANGPTL3 concentrations in the EPIC-Norfolk case–control cohort were analyzed using RStudio version 3.6.1 (R foundation, Vienna, Austria). The correlation between plasma ANGPTL3 plasma concentrations and lipid fractions was investigated by calculating Spearman correlation coefficients. For the association between ANGPTL3 and CAD, the plasma values were divided into quintiles. Logistic regression models were used to analyze the odds ratio of the highest ANGPTL3 quintile versus the other ANGPTL3 quintiles on incident CAD. The first model was adjusted for age and sex and the second model was adjusted for the known atherosclerotic cardiovascular disease risk factors age, sex, systolic blood pressure, LDL cholesterol, log-transformed triglyceride, diabetes, and current smoking status. *P* values <0.05 were considered statistically significant.

RESULTS

We first explored the distribution of ANGPTL3 over lipoproteins by performing ANGPTL3 ELISAs on fractions of plasma of 2 healthy volunteers (Figure 1A and

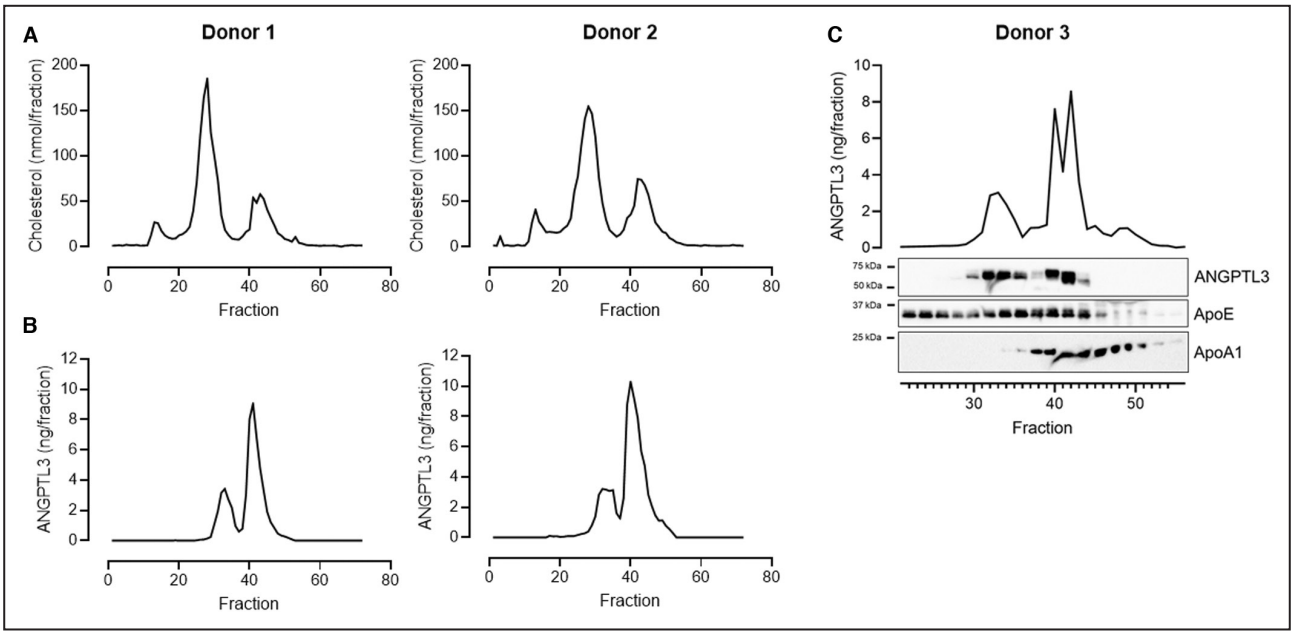

Figure 1. ANGPTL3 predominantly resides on HDL but can also be found on LDL.
Cholesterol (**A**) and ANGPTL3 (**B**) concentrations in plasma fractions of 2 healthy subjects (donor 1 and donor 2). **C**, ANGPTL3 ELISA results and immunoblot analysis in plasma of a healthy subject (donor 3). ANGPTL3 indicates angiopoietin-like protein 3; apoA1, apolipoprotein A1; apoE, apolipoprotein E; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

1B; donor characteristics in Table S2). Approximately 75% of ANGPTL3 was found in HDL-containing FPLC fractions and the remainder was in LDL-containing FPLC fractions. Of interest, ANGPTL3 seems predominantly present on large HDL particles, a subset of HDL particles that is known to be enriched for apolipoprotein E.[29] This was confirmed by immunoblot analysis of the fractionated plasma of a third healthy volunteer (Figure 1C) in which ANGPTL3 coincides with apolipoprotein E in large HDL particles.

To confirm this finding we measured ANGPTL3 on LDL and HDL particles of the first healthy volunteer derived by ultracentrifugation followed by FPLC fractionation. We found that, similar to the results of the initial experiment, ANGPTL3 resides on LDL and HDL particles, although approximately two-thirds of LDL- and HDL-residing ANGPTL3 is lost after ultracentrifugation (Figure 2). We also showed that incubation of recombinant human ANGPTL3 resulted in binding of this protein with both LDL and HDL particles.

Because ANGPTL3 was predominantly found in HDL subfractions, we investigated the presence of ANGPTL3 in a patient with genetic HDL deficiency due to an homozygous C1477R mutation in *ABCA1* and hypothesized that ANGPTL3 would solely be present on LDL particles. Indeed, ANGPTL3 analysis of the

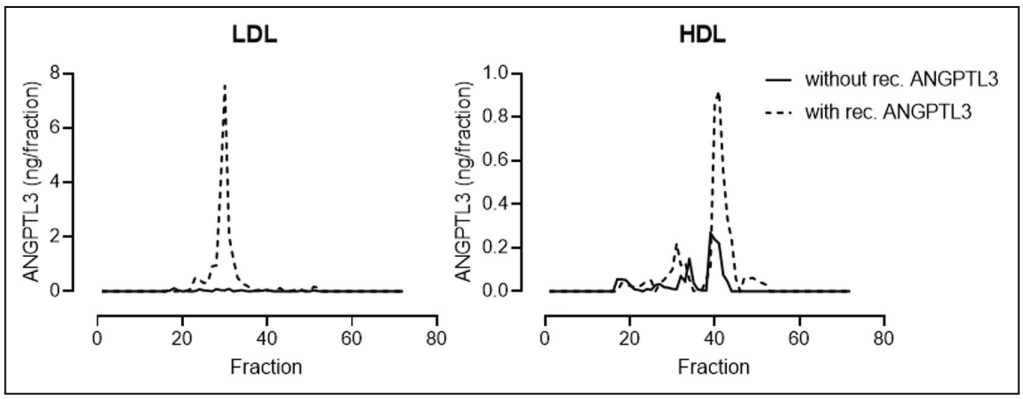

Figure 2. ANGPTL3 can be bound to ultracentrifugation-isolated LDL and HDL.
ANGPTL3 (angiopoietin-like protein 3) ELISA results in fast protein liquid chromatography-fractions from ultracentrifugation-isolated low-density lipoprotein (LDL) and high-density lipoprotein (HDL) incubated with or without recombinant human ANGPTL3.

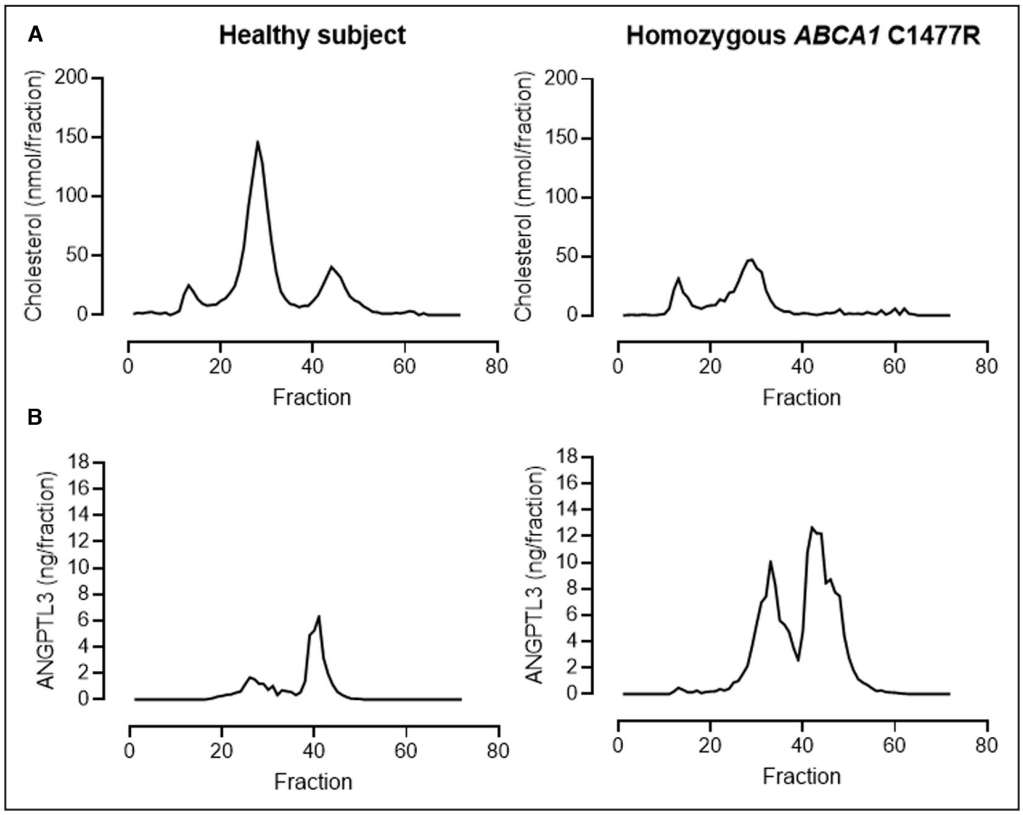

Figure 3. Subject deficient for HDL has more ANGPTL3 residing on LDL.
Cholesterol (**A**) and ANGPTL3 (**B**) concentrations in plasma fractions from plasma from a healthy subject and a subject who is homozygous for the C1477R mutation in *ABCA1*. ABCA1 indicates ATP-binding cassette-A1; ANGPTL3, angiopoietin-like protein 3; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

FPLC-fractionated plasma of this subject (characteristics in Table S3) showed that ANGPTL3 is approximately enriched by 4-fold on LDL compared with LDL obtained of the second healthy volunteer (Figure 3). In addition, we also found ANGPTL3 to be present in the fractions that normally would have contained HDL particles.

We used mature T37i brown adipocytes that are known to express LPL[24] to explore whether binding to HDL or LDL affects the LPL inhibiting capacity of ANGPTL3. For this we used TRLs, LDL, and HDL of a third healthy subject. In the first set of experiments we measured the LPL activity of these cells by means of the FFA concentrations in the medium shortly after addition of TRLs. Because a recent study showed that ANGPTL3 inhibits LPL activity in conjunction with ANGPTL8 in a ratio of 3 ANGPTL3 molecules per 1 ANGPTL8 molecule,[18] cells were incubated with 500 ng/mL ANGPTL3 and 167 ng/mL ANGPTL8 where applicable. Coincubating ANGPTL3 with either HDL or LDL particles leads to the most pronounced ANGPTL3-mediated reductions of LPL activity (ie, less generation of FFA): When ANGPTL3 was added with either HDL or LDL, the average FFA release was reduced by 27.3±13.4% and 26.0±13.8% compared with ANGPTL3-free control, respectively. Incubation with ANGPTL3 in absence of lipoproteins did not affect FFA release (Figure 4A). Of note, in the absence of ANGPTL3, LPL activity was higher in the presence of HDL particles 34.3±10.2% ($P<0.05$), whereas LDL had no significant effect on LPL activity compared with lipoprotein-free control. When the cells were incubated with the different ANGPTL3 and lipoprotein combinations for 1 hour instead of overnight, ANGPTL3 without lipoproteins suppressed LPL activity by 19.1±12.2% ($P<0.05$) (Figure 4B). When administered in conjunction with HDL, ANGPTL3 had no significant effect on LPL activity, but when given with LDL it suppressed LPL activity by 25.5±21.1% ($P<0.05$). Once again, HDL addition increased LPL activity by 41.8±21.1% ($P<0.05$).

Because quantification of LPL activity by FFA release does not take into account fatty acids that are being taken up by the cells, we next measured LPL activity employing ^{3}H-labeled trioleate and determined radioactivity in both the water phase of the medium as well as the cells after ANGPTL3 incubation. In this experimental setting, ANGPTL3 suppressed LPL activity only when given in conjunction with LDL (Figure 4C).

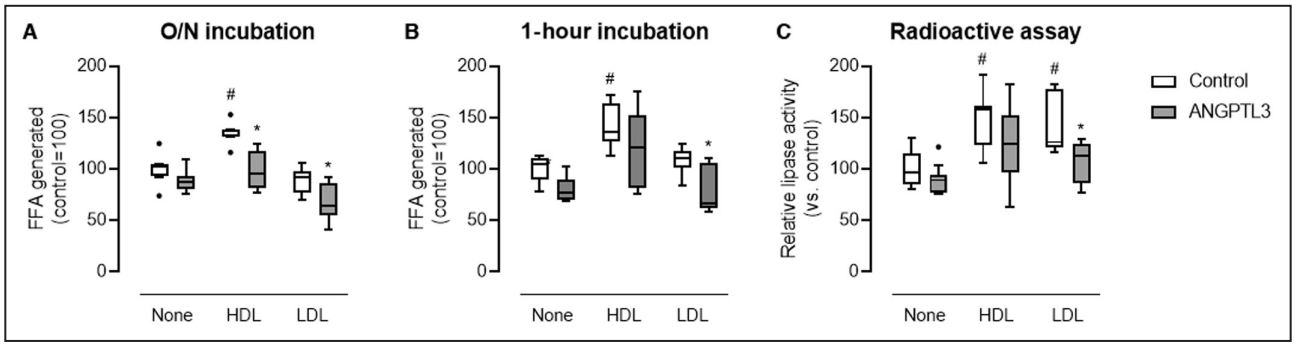

Figure 4. ANGPTL3 has the highest lipase inhibitory activity when residing on LDL.
Triglyceride-rich lipoprotein lipase activity of T37i brown adipocytes incubated with ANGPTL3 in combination with angiopoietin-like 8 in the absence or presence of LDL or HDL overnight (**A**) or 1 hour (**B**). Data are shown as boxplots with results from 2 independent experiments; n=8. **C**, Results of incubating the T37i cells with ^{3}H-trioleate for 30 minutes. Data are shown as boxplots with results from 3 independent experiments; n=12. $^{\#}P<0.05$ vs control (no lipoprotein, no ANGPTL3); $^{*}P<0.05$ vs same lipoprotein condition without ANGPTL3. ANGPTL3 indicates angiopoietin-like protein 3; FFA, free fatty acid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and O/N, overnight.

To investigate the mechanism by which ANGPTL3 inhibits LPL activity, we measured the effect of ANGPTL3 incubations on LPL protein and mRNA expression. Adding ANGPTL3 was found to reduce LPL protein levels on the cell surface of T37i cells, but only in the condition where HDL or LDL particles were added (Figure 5A). ANGPTL3 and the lipoproteins had no effect on the T37i mRNA expression of *Lpl*, the brown adipocyte specific gene *Ucp1*, or adipose-specific ANGPTL3 family-member *Angptl4* (Figure 5B).

To explore the ANGPTL3 lipoprotein binding in a larger human setting, we evaluated the association between plasma ANGPTL3 concentrations and lipoproteins in the nested case–cohort EPIC-Norfolk study (Table 1). Plasma ANGPTL3 concentrations correlated with plasma total cholesterol and HDL cholesterol

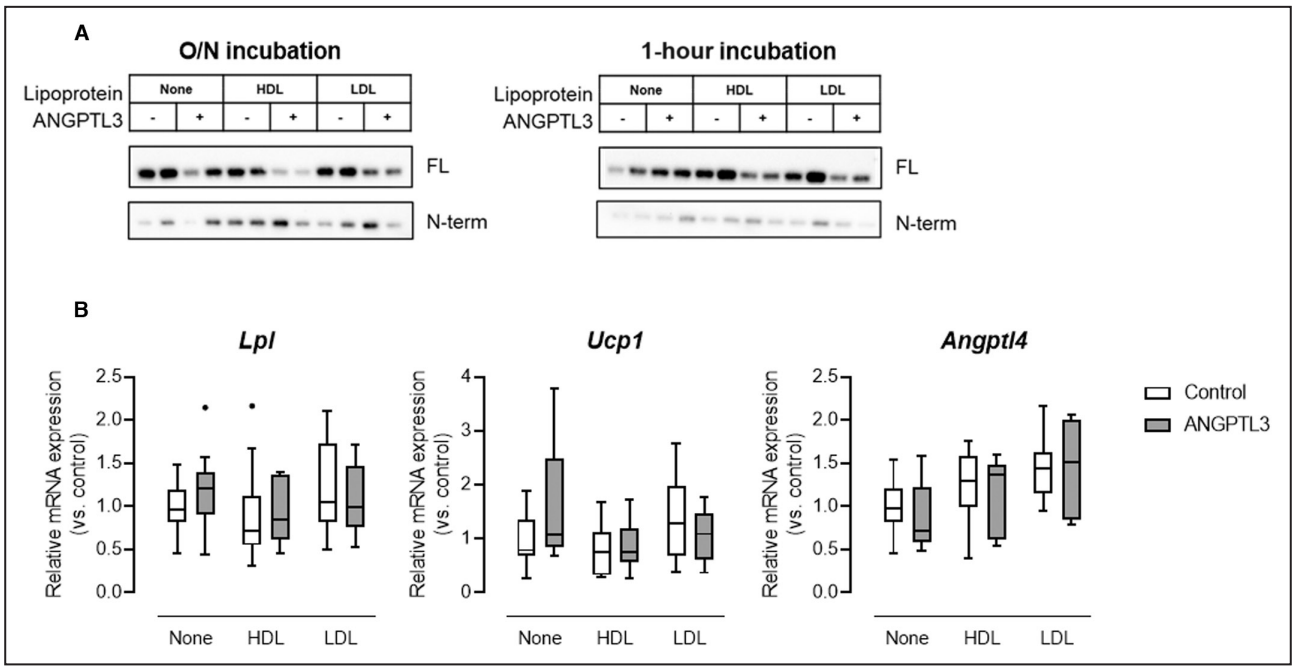

Figure 5. ANGPTL3 reduces LPL protein on the T37i plasma membrane when residing on HDL or LDL.
A, LPL immunoblots of medium of heparin-treated T37i cells incubated with ANGPTL3 in combination with ANGPTL8 (angiopoietin-like 8) in the absence or presence of LDL or HDL overnight (O/N) or 1 hour. **B**, Relative mRNA expression of *Lpl*, *Ucp1,* and *Angptl4* of T37i cells incubated with ANGPTL3 in combination with ANGPTL8 in the absence or presence of LDL or HDL for 24 hours. Relative mRNA expression was normalized to reference genes *Actb* and *Rplp0* with data from cells not incubated with lipoproteins and ANGPTL3 defined as "1." Data are shown as boxplots with results from 3 independent experiments; n=12. Actb indicates beta-actin; ANGPTL3, angiopoietin-like protein 3; Angptl4, angiopoietin-like 4; FL, full-length; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LPL, lipoprotein lipase; N-term, N-terminal; Rplp0, ribosomal protein lateral stalk subunit P0; and Ucp1, uncoupling protein-1.

Table 1. Patient Characteristics of the Nested Case–Cohort EPIC-Norfolk Study

Characteristic	Controls (n=1873)	Cases (n=833)	*P* value
Age, y	65.3±7.7	65.6±7.8	0.420
Female sex, n (%)	704 (37.6)	312 (37.5)	0.982
Current smoking, n (%)	158 (8.5)	127 (15.4)	<0.001
Body mass index, kg/m^2	26.3±3.5	27.3±3.8	<0.001
Systolic blood pressure, mm Hg	139±18	143±19	<0.001
Diabetes, n (%)	32 (1.7)	58 (6.7)	<0.001
Plasma apolipoprotein B, mg/dL	129±31	137±33	<0.001
Plasma total cholesterol, mmol/L	6.3±1.2	6.5±1.2	<0.001
Plasma LDL-cholesterol, mmol/L	4.1±1.0	4.3±1.0	<0.001
Plasma high-density lipoprotein-cholesterol, mmol/L	1.4±0.4	1.3±0.4	<0.001
Plasma triglyceride, mmol/L	1.7 (1.2–2.3)	2.0 (1.4–2.8)	<0.001
Plasma lipoprotein(a), mg/dL	8.4 (6.4–13.6)	9.5 (6.8–24.6)	<0.001
Plasma CRP, mg/dL	1.5 (0.7–3.1)	2.3 (1.1–4.9)	<0.001

Controls were study participants who remained free of any cardiovascular disease during 7.4 years of follow-up. Cases were defined as participants in the subset of the EPIC (European Prospective Investigation Into Cancer) cohort who developed coronary artery disease during follow-up through 2003. Cases were matched in a 2-to-1 ratio to controls by sex, age (within 5 years), and date of visit (within 3 months). Plasma LDL-cholesterol concentrations were calculated by the Friedewald formula.[21] Values are mean±SD or median [interquartile range] for plasma triglyceride, lipoprotein(a), and CRP. CRP indicates C-reactive protein; and LDL, low-density lipoprotein.

concentrations (rho=0.124 and rho=0.235, respectively, both *P*<0.001; Figure 6A) but not with plasma LDL-cholesterol concentrations or other lipid fractions (Figure 6A). All HDL-size subfraction concentrations determined with nuclear magnetic resonance were positively correlated with plasma ANGPTL3 concentrations; the most significant correlation (rho=0.114) was found for large HDL particles (*P*<0.001) (Figure 6B). For LDL, large particles showed a positive, whereas small particles showed a negative correlation with plasma ANGPTL3 concentrations.

The data from the nested case–cohort EPIC-Norfolk study allowed us to explore the association between plasma ANGPTL3 concentrations and CAD. The median ANGPTL3 plasma value in the total cohort was 1103.99 ng/mL (interquartile range, 696.20–1625.23 ng/mL) (Figure 7A). ANGPTL3 levels in the control group were 1040.0 ng/mL (interquartile range, 653.7–1548.6 ng/mL) compared with a median of 1121.4 ng/mL (interquartile range, 664.3–1660.7 ng/mL) in those who developed CAD (Figure 7B). Subjects with plasma ANGPTL3 concentrations above the 80th percentile (1768.94 ng/mL) at baseline had a significantly higher incidence of CAD during a 7.4-year follow-up, adjusted for age and sex (odds ratio [OR], 1.25 [95% CI, 1.01–1.55]; *P*=0.04) (Figure 7C) compared with subjects below the 80th percentile. In a multivariable model that also included systolic blood pressure, LDL-cholesterol, log-transformed triglycerides, diabetes, and current smoking status, the effect was similar albeit not statistically significant (OR, 1.23 [95% CI, 0.98–1.55]; *P*=0.07).

DISCUSSION

The present study shows that ANGPTL3 is largely bound to HDL and LDL particles. We found that unbound circulating ANGPTL3 has a minimal LPL inhibitory effect and that binding to HDL and LDL is a prerequisite for potentiating its effect. This novel finding might be of clinical interest. Instead of targeting ANGPTL3 in its entirety with monoclonal antibodies, compounds directed at selectively blocking the interaction of ANGPTL3 with lipoproteins might accomplish the same effect. In the nested case–cohort EPIC-Norfolk study large-scale primary prevention case–control study of 2706 subjects, we found that ANGPTL3 correlated best with large HDL particles and associated with incident CAD. These data suggest that ANGPTL3 residing on large HDL particles is a driver of the observed positive, albeit weak association between plasma ANGPTL3 concentrations and incident CAD in this study.

Our findings are in line with earlier small cohort studies. Guo et al[30] found a stronger correlation between plasma ANGPTL3 and HDL cholesterol concentrations than between plasma ANGPTL3 and LDL cholesterol concentrations in patients with CAD. Moreover, in patients who were HIV lipoatrophic, plasma ANGPTL3 concentrations were more strongly correlated with HDL cholesterol concentrations and HDL size than LDL cholesterol and size with the most significant and strongest correlation between plasma ANGPTL3 and large HDL particles.[31] The predominant presence of ANGPTL3 on large HDL particles is further underscored by the finding that subjects with enlarged HDL particles (due to deficiency in CETP [cholesterol ester transfer protein]) also have higher ANGPTL3 concentrations in their ultracentrifugation-derived HDL compared with normolipidemic controls.[32] In line, treatment of hypercholesterolemic, treatment-naïve patients with probucol, a compound that increases CETP mass and activity,[33] not only led to a significant reduction of plasma HDL-cholesterol and apolipoprotein A1 concentrations but also resulted in a decrease of plasma ANGPTL3 concentrations.[34] Few HDL proteomic studies report the presence of ANGPTL3 on HDL,[35,36] which might be because a large amount of ANGPTL3

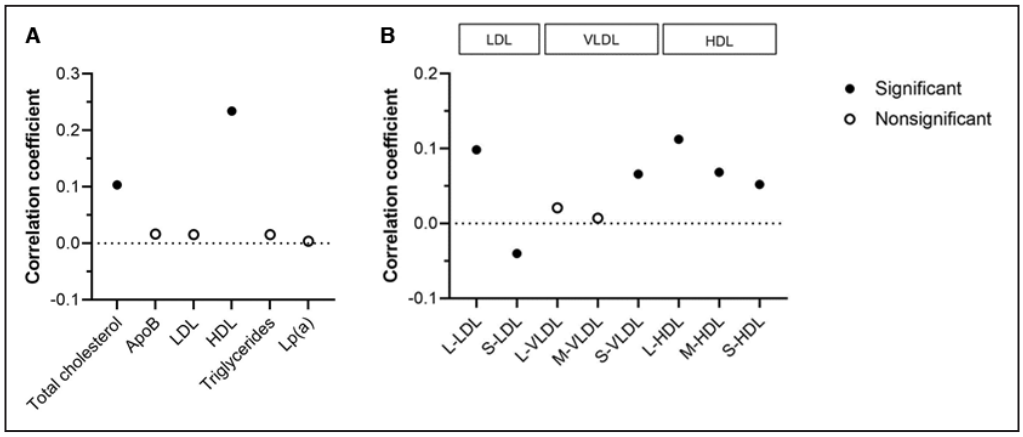

Figure 6. Plasma ANGPTL3 concentrations correlate most strongly with HDL cholesterol and large HDL particles in a human cohort study.
Correlation coefficients of the associations between plasma ANGPTL3 concentrations and plasma lipids (**A**) and the amount of varying sizes of lipoproteins (**B**) in the nested case–cohort EPIC-Norfolk study. ANGPTL3 indicates angiopoietin-like protein 3; ApoB, apolipoprotein B; EPIC, European Prospective Investigation Into Cancer; HDL, high-density lipoprotein; L, large; LDL, low-density lipoprotein; Lp(a), lipoprotein A; M, medium; S, small; and VLDL, very low-density lipoprotein.

dissociates from HDL during the rather harsh ultracentrifugation procedure commonly used to isolate HDL. Collectively, these data suggest that HDL might act as a an inactive storage for ANGPTL3 and that the positive association between plasma ANGPTL3 and HDL cholesterol or particle number might be at least partly due to this buffering effect.

In the patient with a mutation in *ABCA1*, we found ANGPTL3 to be more present in LDL than in healthy controls. In addition, ANGPTL3 was also present in the fractions that normally would have contained HDL. This may in fact not be apolipoprotein A1 containing HDL particles but rather a lipoprotein subfraction that is similar in size to HDL but only contains apolipoprotein E and not apolipoprotein A1.[37–39] In patients with near absence of HDL due to biallelic *ABCA1* variants (Tangier disease), it has been shown that the concentration of this particular subfraction is almost identical compared with normolipidemic subjects.[40] One study showed that hepatocytes derived from patients with Tangier disease secrete more ANGPTL3 resulting in higher plasma ANGPTL3 concentrations compared

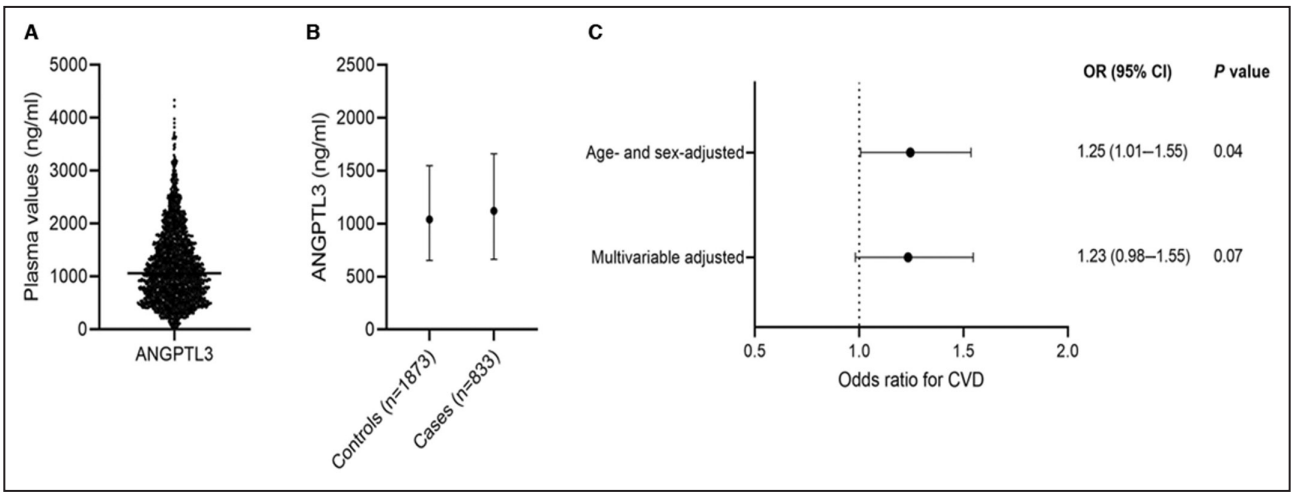

Figure 7. Subjects with the highest plasma ANGPTL3 concentrations have a higher incidence of cardiovascular events.
A, Plasma ANGPTL3 concentrations in the nested case–cohort EPIC-Norfolk study. **B**, Plasma ANGPTL3 values in controls and cases. **C**, Odds ratios for incident future cardiovascular events of subjects within the fifth quintile of plasma ANGPTL3 concentrations compared with the other subjects in the nested case–cohort EPIC-Norfolk study. Upper value is for the age- and sex-adjusted model, lower value is for the multivariable model that also included systolic blood pressure, LDL cholesterol, log-transformed triglycerides, diabetes, and current smoking status. ANGPTL3 indicates angiopoietin-like protein 3; CVD, cardiovascular disease; EPIC, European Prospective Investigation Into Cancer; LDL, low-density lipoprotein; and OR, odds ratio.

with healthy controls.[41] This effect is likely due to disturbances in intracellular cholesterol homeostasis. More research is required to further explore the close link between ANGPTL3 and HDL homeostasis, and studies in patients characterized by abnormalities in HDL metabolism would be critical.

Despite numerous studies on the effects of ANGPTL3 on plasma lipids that suggest a role for LPL, the precise mechanism by which ANGPTL3 inhibits LPL is yet unknown. The data presented in the present study contributes to our understanding. First, we now show that the lipase-inhibiting activity of ANGPTL3 is significantly higher in the presence of lipoproteins, which suggests that the presence of lipoproteins is a prerequisite for LPL inhibition by ANGPTL3 in human physiology. It also shows that targeting circulating unbound ANGPTL3 will probably have a limited effect on LPL activity. While focusing on the relative contribution of LDL and HDL in this potentiating effect, we found that LDL was a minimally stronger inducer of ANGPTL3 activity compared with HDL. Altogether, these results may imply that ANGPTL3 might not be constituently active in in silico models where no lipoproteins are added. As such, we advocate for an approach where lipoproteins are added to mimic the physiological in vivo situation. Second, we show that ANGPTL3 reduces LPL proteins on the cell surface while having no effect on *Lpl* mRNA expression. Although Jin et al[42] reported that ANGPTL3-induced LPL cleavage in HEK293 cells, we did not observe this in the T37i cells. This is likely due to the shorter incubation time used by Jin et al (30–60 minutes), suggesting that the effect of ANGPTL3 on LPL cleavage might be transient. Although the present study already shows that lipoprotein binding has an effect on the ability of ANGPTL3 ability to suppress LPL activity, more dedicated research is required to study the effects of LDL- and HDL-binding on ANGPTL3 physiology in more detail.

Of interest, we found a consistent positive effect of HDL on LPL activity in our experiments. This is in line with various in vitro as well as in vivo studies that report that the presence of HDL stimulates TRL lipolysis,[43–46] most likely due to the effects of the HDL-associated protein apolipoprotein CII.[47] The effects of HDL and LDL on LPL activity has been addressed in the white adipocyte cell line 3T3-L1, in which HDL and LDL both inhibited adipocyte-associated LPL released by heparin as a proxy of LPL activity.[48] This effect of HDL is opposite of what we found in the T37i brown adipocyte cell line. This difference may be due to intrinsic differences between white and brown adipocytes, but more research exploring the potential differential effect of HDL on different adipocytes is needed. Despite the fact that the effects of lipoproteins on brown adipocytes activity has not yet been properly addressed, our data do not support a substantial effect of lipoproteins since neither LDL nor HDL affected mRNA expression of *Ucp1*, that encodes the crucial enzyme uncoupling protein 1 in heat production.[49]

Our studies come with some limitations. First, we describe the effects of binding of recombinant human ANGPTL3 to lipoproteins in an ex vivo setting, which might not reflect the in vivo situation in which ANGPTL3 is not only associated with lipoproteins but also mediates their dynamics. For instance, we show that ANGPTL3 preferentially associates with large HDL particles but subjects deficient for ANGPTL3 have relatively smaller HDL particles.[50] We can therefore not draw any conclusion on the directionality of the association between ANGPTL3 and (large) HDL particles. Second, we evaluated the association between plasma ANGPTL3 concentrations and lipoproteins in the nested case–cohort EPIC-Norfolk study consisting of relatively old subjects with an average age of 65.3 and 65.6 years for controls and cases, respectively. It has been shown that plasma ANGPTL3 concentrations increase with aging,[51,52] thus the relatively old age might have affected the observed associations. Third, here we report the effects of the binding of ANGPTL3 to HDL or LDL on lipase activity in the brown adipocyte cell line T37i. It is not known whether the results in this cell line can be extrapolated to, for instance, white adipocytes and endothelial cells. The use of brown adipocytes is justified by the fact that, in general, brown adipocytes have a higher LPL activity than white adipocytes[53] and that LPL expressed by brown adipocytes and not endothelial cells is crucial for lipid handling of the brown adipose tissue depot of mice.[54]

CONCLUSIONS

In conclusion, ANGPTL3 is present predominantly on large HDL particles and is a potent LPL inhibitor in the presence of HDL and LDL particles.

ARTICLE INFORMATION

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Supplemental Material

Tables S1–S3
Reference[55]

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