

Angiotensin-Like Protein 4 Is a High-Density Lipoprotein (HDL) Component for HDL Metabolism and Function in Nondiabetic Participants and Type-2 Diabetic Patients

Long-Yan Yang, PhD;* Cai-Guo Yu, MD;* Xu-Hong Wang, MD; Sha-Sha Yuan, MD; Li-Jie Zhang, MS; Jia-Nan Lang, MS; Dong Zhao, MD; Ying-Mei Feng, MD, PhD

Background—ANGPTL4 (angiotensin-like protein 4) is a LPL (lipoprotein lipase) inhibitor and is present in high-density lipoprotein (HDL). However, it is not defined whether ANGPTL4 in HDLs could affect HDL metabolism and function in type 2 diabetes mellitus (T2DM).

Methods and Results—ANGPTL4 levels in the circulation and HDLs were quantified in nondiabetic participants (n=201, 68.7% females) and T2DM patients (n=185, 66.5% females). HDLs were isolated from nondiabetic controls and T2DM patients to assess cholesterol efflux or subjected to endothelial lipase (EL)-overexpressed HEK293 cells for EL hydrolysis in vitro. The association between ANGPTL4 in HDLs and HDL components and function was analyzed in nondiabetic participants or diabetic patients, respectively. Plasma or HDLs of ANGPTL4+/+ and ANGPTL4−/− mice was subjected for cholesterol efflux or EL hydrolysis, respectively. ANGPTL4 levels in the plasma and HDLs were 1.7- and 2.0-fold higher in T2DM patients than nondiabetic controls, respectively ($P<0.0001$). Multivariable analysis demonstrated that per 1 doubling increase of ANGPTL4 levels in HDLs, the changes amounted to +0.27% cholesterol efflux ($P=0.03$), +0.06 $\mu\text{g}/\text{mL}$ apolipoprotein A-I ($P=0.09$) and −9.41 $\mu\text{g}/\text{L}$ serum amyloid A ($P=0.02$) in nondiabetic controls. In T2DM patients, the corresponding estimates were −0.06% cholesterol efflux ($P=0.10$), −0.06 $\mu\text{g}/\text{mL}$ apolipoprotein A-I ($P=0.38$), and +3.64 $\mu\text{g}/\text{L}$ serum amyloid A ($P=0.72$). HDLs isolated from ANGPTL4−/− mice showed accelerated hydrolysis by EL and reduced cholesterol efflux compared with ANGPTL4+/+ littermates.

Conclusions—Physically, ANGPTL4 in HDLs protected HDLs from hydrolysis. Resulting from increased circulating ANGPTL4 levels in T2DM, ANGPTL4 levels in HDLs were elevated but with compromised inhibitory effect on EL, leading to increased HDL hydrolysis and dysfunction. (*J Am Heart Assoc.* 2017;6:e005973. DOI: 10.1161/JAHA.117.005973.)

Key Words: angiotensin-like protein 4 • cholesterol efflux • diabetes mellitus • endothelial lipase • high-density lipoproteins

ANGPTL4 (angiotensin-like protein 4) belongs to the angiotensin-like protein family, which are secreted glycoproteins for angiogenic regulation.^{1,2} So far, the origins of ANGPTL4 production include hepatocytes,^{3,4} adipocytes,^{5,6} skeletal muscle,⁷ glia cells⁷, and macrophages.⁸ Once secreted, the full-length ANGPTL4 is processed by proprotein convertases at the linker region, releasing its N-terminal

region and the monomeric C-terminal portion of ANGPTL4. The N-terminal region is responsible for its assembly to form oligomerization, which mediates the inhibition of lipoprotein lipase LPL (lipoprotein lipase). In contrast, the C-terminal region interacts with integrins and extracellular matrix proteins for cell adhesion and migration.² Given that ANGPTL4 is produced by a variety of cells upon different stimuli and carries multiple functions through its N- and C-terminal regions, it is not surprising that the biological function of ANGPTL4 could be complicated.

As a potent LPL inhibitor, ANGPTL4 disrupts LPL dimerization, converting this enzyme into inactive form.^{9,10} Because of increased LPL activity, ANGPTL4-null mice are featured as having lower triglycerides in the blood.¹¹ The inverse correlation between ANGPTL4 and high-density lipoprotein-cholesterol HDL-c (high-density lipoprotein-cholesterol) has been reported in European and American populations.^{12,13} In line with these findings, ANGPTL4 variants have been identified in European subjects.^{14–17} For instance, E40K carriers, who have loss-of-function mutation of ANGPTL4, display higher HDL-c with

From the Beijing Key Laboratory of Diabetes Prevention and Research, Department of Endocrinology, Lu He Hospital, Capital Medical University, Beijing, China.

*Dr. Yang and Dr. Yu contributed equally to this study.

Correspondence to: Ying-Mei Feng MD, PhD, or Dong Zhao, MD, Beijing Key Laboratory of Diabetes Prevention and Research, Department of Endocrinology, Lu He Hospital, Capital Medical University, Beijing 101149, China. E-mails: yingmeif13@ccmu.edu.cn; yingmeif13@sina.com, zdoc66@126.com Received February 28, 2017; accepted May 11, 2017.

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Clinical Perspective

What Is New?

- Our study demonstrates that ANGPTL4 (angiotensin-like protein 4), as a component of HDL (high-density lipoprotein), modulates HDL metabolism and function. As circulating angiotensin-like protein 4 levels increase in type 2 diabetes mellitus patients, HDL-associated angiotensin-like protein 4 increases but with skewed inhibition of endothelial lipase activity, leading to HDL hydrolysis and dysfunction.

What Are the Clinical Implications?

- This study suggests that targeting angiotensin-like protein 4 for improved HDL function might confer reduction of cardiovascular risk and improve glycemic control in patients with type 2 diabetes mellitus.

reduced risk of coronary heart disease when compared with noncarriers.^{14–17} Recently, plasma levels of ANGPTL4 have been found to be increased in patients with type-2 diabetes mellitus (T2DM) and subjects with metabolic syndromes; both of them are featured as having reduced HDL-c.⁸ These data suggest an intimate link between ANGPTL4 and HDLs.

It is well known that HDL function is critically determined by the components in HDLs such as apoA-I (apolipoprotein A-I).^{18–20} Pathological alteration of HDL metabolism imbalances HDL components and thus abrogates the protective effects of HDLs in cardiovascular events.^{18–20} Previously, we demonstrated that ANGPTL3, another family member of ANGPTL4, is positively associated with apolipoprotein A-I and HDL function in nondiabetic individuals.²¹ Interestingly, ANGPTL4 is present in HDLs isolated from mice and human plasma.²² However, limited data have determined whether and how ANGPTL4, as a component of HDLs, has any direct impact on HDL metabolism and its function. Therefore, in the study, we tested the hypothesis that ANGPTL4 in HDLs could directly regulate HDL metabolism and function. Under the diabetic condition, the levels of ANGPTL4 in HDLs might alter following the elevated concentration of ANGPTL4 in the circulation, resulting in abnormal HDL metabolism and impaired HDL function.

Methods

Human Subjects, Sample Size, and Clinical Measurements

Because of limited data on ANGPTL4 expression in Asian subjects, we quantified circulating ANGPTL4 expression in a small number of nondiabetic participants and patients with T2DM. Based on the pilot experiment, we calculated the sample size by the standard formula: (<http://powerandsa>

mplesize.com/Calculators/Compare-2-Means/2-Sample-1-Sided), in which $1-\beta$ is equal to 0.80 and α is equal to 0.01.

The formula used to compute sample size is

$$N = (\sigma_A^2 + \sigma_B^2/\kappa) \left(\frac{Z_{1-\alpha} + Z_{1-\beta}}{\mu_A - \mu_B} \right)^2$$

In which, σ_A and σ_B are SDs of ANGPTL4 levels in nondiabetic and T2DM subjects, respectively; μ_A and μ_B are means of ANGPTL4 levels in nondiabetic and T2DM subjects, respectively; $\kappa=1$; $\alpha=0.01$; $\beta=0.80$.

After calculation, the minimum sample size was 91 for each group. From January 2016 until May 2016, 204 nondiabetic subjects and 186 T2DM patients were recruited to the Department of Endocrinology at Lu He Hospital in Beijing. All the participants were Han people from the same region. The criteria of T2DM diagnosis were adapted from that of the World Health Organization and the American Diabetes Association as follows: (1) fasting glucose level ≥ 7 mmol/L; or (2) 2-hour oral glucose tolerance test ≥ 11.1 mmol/L; or (3) random glucose level ≥ 11.1 mmol/L and accompanied by typical DM symptoms such as polydipsia, polyuria, increased food intake, and loss of body weight. We excluded 3 nondiabetic subjects and 1 T2DM patient because either ANGPTL4 levels ($n=2$) or total cholesterol values ($n=2$) were deviated more than 3 SDs from the mean. Thus, the number of the analyzed subjects totaled 386.

After a 12-hour overnight fast, venous blood samples were obtained to measure total cholesterol, triglyceride, HDL-c, plasma glucose, insulin, and serum creatinine by the central laboratory in the hospital. LDL-c (low-density lipoprotein-cholesterol) was computed from serum total cholesterol and HDL-c and serum triglycerides by the Friedewald equation.²³ Body mass index (BMI) was measured as described before.²¹ Pancreatic β -cell function and insulin resistance were computed by Homeostasis Model of Assessment (Homa-B and HOMA-IR; <http://www.dtu.ox.ac.uk/homacalculator/>), using fasting insulin and glucose in the subjects. To assess kidney function, estimated glomerular filtration rate was calculated according to the formula.²⁴

The study complied with the Helsinki Declaration for investigation of human subjects. The entire study obtained ethical approval from the competent Institutional Review Boards of Capital Medical University. All participants provided written informed consent.

Mice and Genotype

C57BL/6 mice at the age of 10 to 12 weeks old were used in the study. ANGPTL4^{-/-} mice were kindly provided by Prof. Guo-Qing Liu (Institute of Cardiovascular Sciences and Key Laboratory of Molecular Cardiovascular Sciences, Beijing

Table 1. Primer Sequences of ANGPTL4 Wild-Type and ANGPTL4 Knockout Mice

Primer Name	Sequence (5' to 3')	PCR Product
ANGPTL4-wt-F	CCAGCAGCAGAGATACCT	240 bp
ANGPTL4-wt-R	GTCTTGTCTACTCCATTGTCT	
ANGPTL4-ko-F	GGTGAATAAGAGGAGGTTGC	1000 bp
ANGPTL4-ko-R	CCGATTGTCTGTTGTGCC	

ANGPTL4 indicates angiotensin-like protein 4; PCR, polymerase chain reaction.

University Health Science Center, China). To obtain stable expansion, ANGPTL4^{-/-} were first crossed back with ANGPTL4^{+/+} mice to generate ANGPTL4^{+/-} mice and then ANGPTL4^{-/-} pups were screened out from ANGPTL4^{+/-} couples for further breeding. The phenotype of ANGPTL4^{-/-} mice was determined by 2 pairs of primers (Table 1). Ethical approval for the entire study was obtained from the competent Institutional Review Boards of Capital Medical University.

Lipoprotein Isolation

Plasma samples were separated by density gradient ultracentrifugation in a swing-out rotor described by Chapman et al.²⁵ Based on the density, very low-density lipoprotein/intermediate-density lipoprotein ($g < 1.019$ g/mL), LDL (1.019 g/mL $< g < 1.063$ g/mL), and HDLs (1.063 g/mL $< g < 1.21$ g/mL) fractions were isolated. After being dialyzed in 1 mmol/L EDTA overnight, fractions were subjected to Western blot. Thereafter, HDL fractions were concentrated by centrifugation at 3220g for 20 minutes to assess cholesterol efflux or to evaluate the inhibition of ANGPTL4 on endothelial lipase (EL) activity in vitro.

HDL Fractionation and HDL Component Quantification

To quantify HDL components, plasma samples were precipitated by very low-density lipoprotein/LDL precipitation buffer to obtain an HDL fraction, following the manual's instruction (Biovision, CA). The concentrations of apoA-I, phospholipid, and serum amyloid A in HDL fraction were quantified following the instruction, respectively (MLBio, Shanghai, China). Triglyceride concentration in HDL fractions was determined (BioSino Biotechnology).

Total Cholesterol and Triglyceride Determination in Mice

After overnight fasting, mice were bled and plasma levels of cholesterol and triglyceride in ANPTL4^{+/+} and ANGPTL4^{-/-} mice were measured (BioSino Biotechnology).

Enzyme-Linked Immunosorbent Assay

The levels of ANGPTL4 in the plasma and HDL fraction were determined by Human ANGPTL4 assay kit according to the manufacturer's instruction (Catalogue number 27749, IBL International GMBH, Japan) as described before.²⁶ After overnight fasting, plasma levels of ANGPTL4 in ANPTL4^{+/+} and ANGPTL4^{-/-} mice were quantified by enzyme-linked immunosorbent assay (SEB019Mu, Cloud-Clone Corp).

Cholesterol Efflux Assay

Cholesterol efflux assay was performed following the manual's instruction (Catalog K582-100, Biovision) as described before.²¹ Briefly, RAW264.7 macrophages were plated at the density of 1×10^5 cells/well in a 96-well plate and maintained in DMEM plus 10% fetal bovine serum (Sigma-Aldrich) for 2 hours. The adherent cells were first loaded with labeled cholesterol for 16 hours and then exposed to 2.5 μ L plasma or 100 μ g/mL HDLs for 4 hours.^{21,27} When using plasma as cholesterol acceptor, they were treated with Serum Treatment Reagent prior to loading (Catalog K582-100, Biovision). The supernatant was transferred to a 96-well plate to measure the fluorescence (Ex/Em=482/515 nm). The adherent cells were solubilized by cell lysis buffer to measure the fluorescence (Ex/Em=482/515 nm). Cholesterol efflux %=(fluorescence intensity of the media/(fluorescence intensity of the cell lysate+media) $\times 100$).²⁸

Cell Culture, Transfection, and Fluorescence-Activated Cell Sorting

HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. When cells reached 80% confluence, they were transfected with plasmid construction containing GFP-flag using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions. After transfection, cells were cultured for 48 hours. To obtain pure EL-expressing HEK 293 cells, cells were harvested and sorted by its GFP expression. After sorting, purified cells were expanded in vitro for EL hydrolysis assay.

EL Hydrolysis Assay

HDLs were concentrated by centrifugation, and HDL components (cholesterol, phospholipid, and triglyceride) were determined as aforementioned. EL-expressing HEK293 cells were exposed to serum-free medium containing HDLs (100 μ g/mL) and incubated at 37°C. After 4 hours of incubation, the medium was collected to quantify the concentrations of cholesterol, phospholipid, and free fatty

acid using commercially available kits (MLBio, Shanghai, China). The absolute amounts of phospholipid and cholesterol before and after EL assay were calculated to obtain the amounts of metabolized cholesterol and phospholipid. The hydrolyzed triglyceride was expressed by the ratio between the amount of free fatty acid generated after EL hydrolysis assay and the amount of triglyceride loaded before the assay.

Immunoprecipitation and Western Blot

HDL fractions (10 μ L) were separated using 10% SDS-PAGE. After transfer, nitrocellulose membranes were probed with anti-ANGPTL4 antibody 1/1000 (Abcam, Cambridge, UK) overnight and then incubated with horseradish peroxidase-conjugated IgG secondary antibody 1/3000 (Amersham Biosciences, Piscataway, NJ) for 1 hour. Signals were detected by ECL chemiluminescence (Thermo Fisher Scientific, Rockford, IL).

To further confirm the presence of ANGPTL4 on HDL fractions, HDLs (400 μ L) and lysis buffer (400 μ L) were incubated with anti-ANGPTL4 antibody (Santa Cruz, Dallas, TX) or IgG (Cell Signaling Technology, Beverly, MA) coupled with protein G plus/protein A-agarose (Santa Cruz, Dallas, TX) overnight. After washing, the immunoprecipitated proteins were processed for Western blotting as described above.

HEK 293 cells transfected with plasmid containing EL cDNA (GFP-EL-flag) were lysed in 1 mL ice-cold lysis buffer (10 mmol/L HEPES, 50 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L benzamidine, and 0.5% Triton X-100 [pH 7.4]). The lysates were analyzed by Western blotting with anti-flag antibody (Medical and biological laboratories, Nagoya, Japan) and anti-GAPDH antibody (Santa Cruz, Dallas, TX).

Statistical Analysis

The statistical analysis was performed using the SAS system, version 9.4 (SAS Institute Inc, Cary, NC). For untransformed and logarithmically transformed variables, we expressed the central tendency and spread of the distributions by the arithmetic mean and SD and the geometric mean and interquartile range, respectively. To achieve normal distribution, logarithmic transformation was performed in ANGPTL4 levels in plasma and HDL fractions, serum triglyceride, fasting insulin in blood, Homa-B, Homa-IR, and phospholipid and triglyceride levels in HDLs. We compared means by the large-sample z-test and proportions by Fisher exact test. Pearson's correlation was used for univariate association analysis. Multivariate analysis was performed by adjusting for age, sex, BMI, mean arterial pressure, and use of lipid-lowering drugs. When analyzing the kinetics of EL hydrolysis, Dunnett's

multiple comparison test was used for subjects with medium- or high-ANGPTL4 levels in HDLs versus ones with low ANGPTL4 levels in HDLs. In the mice study, unpaired *t*-test was used to compare the difference between ANGPTL4+/+ and NAGPTL4-/- mice. Significance was a 2-tailed α -level endothelial lipase of 0.05 or less.

Results

General Characterization of the Study Subjects

Table 2 lists the characteristics of nondiabetic participants and T2DM patients. In 201 nondiabetic participants, age averaged 53.1 years and the proportion of women was 68.7%. In 185 T2DM patients, age averaged 55.9 years and the proportion of women was 66.5%. All T2DM patients were received antidiabetic treatment; among them 58 (31.8%) used metformin and 19 (10.3%) took insulin injection. Among all T2DM patients, 78 subjects were just diagnosed diabetic during enrollment. Compared with nondiabetic controls, T2DM patients were featured as having a higher prevalence of hypertension, increased systolic blood pressure, plasma glucose and serum triglyceride, but reduced HDL-c (Table 2). To further compare the HDL components between the 2 groups, HDLs were fractionated. In addition to decreased HDL-c, the concentration of apoA-I was 1.3-fold higher in nondiabetic subjects than T2DM patients ($P<0.0001$). By contrast, the concentrations of serum amyloid A and phospholipid were 1.4- and 1.5-fold increase in T2DM patients when compared with controls, respectively ($P<0.0001$ for both).

Circulating ANGPTL4 in the Study Subjects

Consistent with previous reports,^{8,12} plasma level of ANGPTL4 was 1.7-fold higher in T2DM patients than controls (74.4 pg/mL versus 132.1 pg/mL, $P<0.0001$). The distribution of plasma ANGPTL4 levels in nondiabetic participants and T2DM patients is shown in Figure 1A and 1B, respectively. When comparing by sexes, plasma ANGPTL4 level in women was lower than in men in nondiabetic controls (87.9 pg/mL versus 66.2 pg/mL, $P=0.0003$). When compared with male and female controls, ANGPTL4 level was 1.5- and 1.9-fold increased in male and female T2DM patients, respectively (Figure 1C).

As ANGPTL4 is critically involved in metabolic syndrome,¹² the association between circulating ANGPTL4 level and the individual metabolic parameter was examined in nondiabetic participants and T2DM patients (Table 3). Based on Pearson correlation, circulating ANGPTL4 levels were positively correlated with BMI ($r=0.176$, $P=0.017$), systolic blood pressure ($r=0.188$, $P=0.010$), serum triglyceride ($r=0.153$, $P=0.171$),

Table 2. General Characteristics of Study Participants

	Nondiabetic	Type 2 Diabetic	P Value
N	201	185	
Female (%)	138 (68.7%)	123 (66.5%)	0.649
Hypertension (%)	63 (16.3%)	73 (18.9%)	0.030
Coronary heart disease (%)	6 (1.6%)	11 (2.9%)	0.157
Medications (%)			
Lipid-lowering drugs (0, 1)	3 (0.8%)	17 (4.4%)	0.001
Antihypertensive (0, 1)	25 (12.4%)	33 (17.8%)	0.224
Insulin (0, 1)	NA	19 (4.9%)	NA
Metformin (0, 1)	2	58 (15.0%)	<0.0001
Mean of characteristics			
Age, y	53.1 (9.4)	55.9 (7.4)	0.001
Body mass index, kg/m ²	26.2 (3.8)	26.8 (4.0)	0.094
Systolic blood pressure, mm Hg	128.6 (18.3)	135.0 (18.9)	0.001
Diastolic blood pressure, mm Hg	77.8 (11.0)	79.4 (10.4)	0.144
Fasting blood glucose, mg/dL	5.0 (0.5)	9.8 (3.1)	<0.0001
Insulin, pmol/L	51 (34–71)	52 (27–85)	0.42
Homa-B, %	93 (72–112)	29 (13–47)	<0.0001
Homa-IR, %	0.9 (0.7–1.3)	1.1 (0.6–1.9)	0.24
eGFR, mL/min per 1.73 m ²	124.3 (45.6)	103.4 (45.5)	<0.0001
Total cholesterol, mmol/L	5.04 (1.03)	5.21 (1.16)	0.130
Triglyceride, mg/dL	114 (81–183)	158 (92–246)	<0.0001
LDL-c, mmol/L	3.04 (0.88)	3.19 (0.88)	0.102
HDL-c, mmol/L	1.36 (0.34)	1.27 (0.32)	0.007
HDL components			
Apolipoprotein A-I, µg/mL	8.8 (2.3)	7.0 (3.6)	<0.0001
Serum amyloid A, µg/L	657 (462–831)	892 (537–1129)	<0.0001
Phospholipid, mg/dL	460 (291–744)	678 (518–904)	<0.0001
Triglyceride, mg/dL	17 (10–22)	17 (13–22)	0.08

eGFR, estimated glomerular filtration rate calculated according to the CKD-EPI formula. Hypertension was a blood pressure of ≥140 mm Hg systolic, or ≥90 mm Hg diastolic, or use of antihypertensive drugs. Pancreatic β-cell function (Homa-B) and insulin resistance (Homa-IR) were computed by Homeostasis Model Assessment (Homa-B and HOMA-IR; <http://www.dtu.ox.ac.uk/homacalculator/>), using fasting insulin and glucose in the subjects. For untransformed and logarithmically transformed variables, we expressed the central tendency and spread of the distributions by the arithmetic mean and SD and the geometric mean and interquartile range, respectively. HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; NA, not applicable.

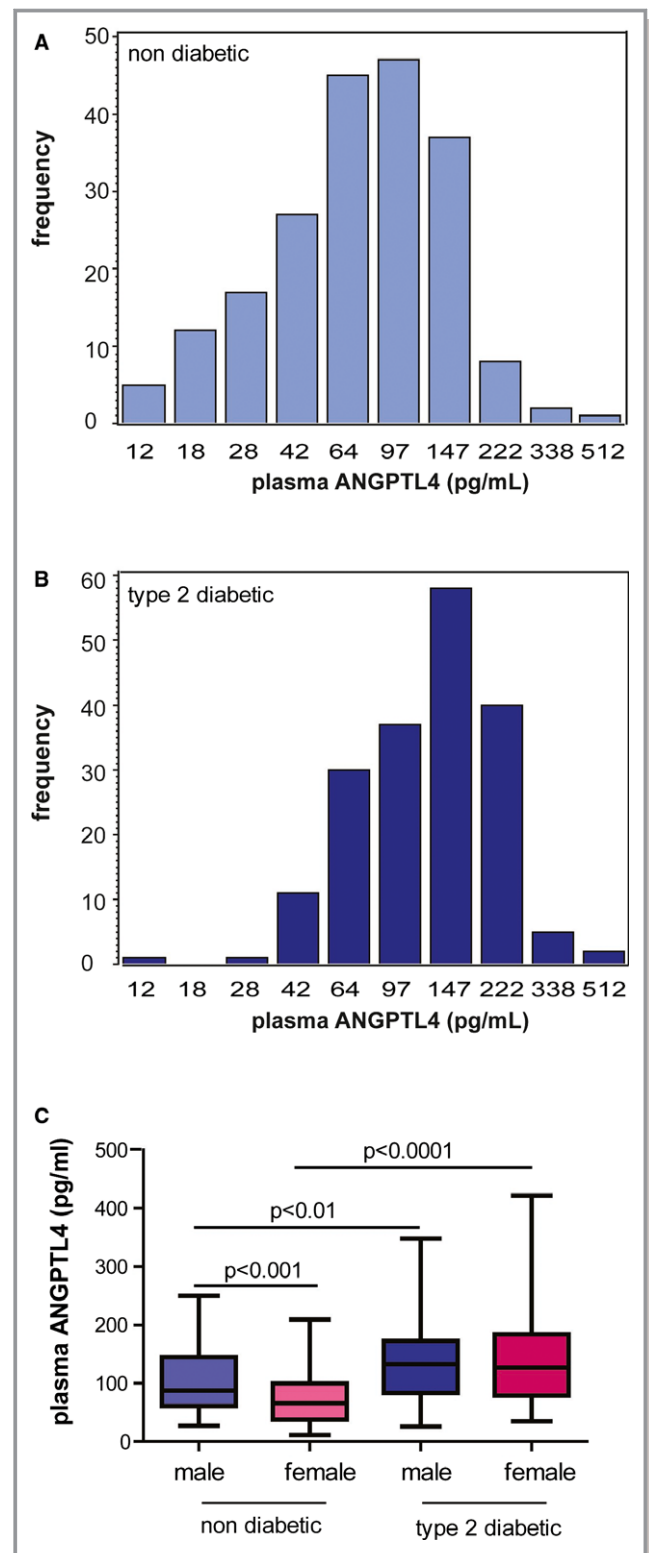


Figure 1. Circulating ANGPTL4 levels in the study subjects. The distribution of circulating ANGPTL4 in 201 nondiabetic subjects (A) and 185 diabetic patients (B) is shown. C, Plasma levels of ANGPTL4 in male and female nondiabetic controls and type 2 diabetic patients are shown. ANGPTL4 indicates angiotensin-like protein 4.

and insulin resistance index (Homa-IR) ($r=0.181$, $P=0.014$) but negatively correlated with HDL-c ($r=-0.176$, $P=0.017$) in T2DM patients. Different from T2DM patients, circulating ANGPTL4 did not correlate with any of these parameters mentioned above in controls ($P>0.17$ for all) (Table 3).

ANGPTL4 in HDLs

As ANGPTL4 was identified as a component of HDLs in humans,²² hereby, we investigated whether the concentration of ANGPTL4 in HDLs was changed in T2DM patients. Firstly, by Western blot, ANGPTL4 was found present in HDLs isolated both from nondiabetic controls and T2DM patients, which was further confirmed by immunoprecipitation followed by Western blot (Figure 2A and 2B).

Next, HDLs were fractionated from plasma and the concentration of ANGPTL4 in HDLs was measured by enzyme-linked immunosorbent assay. ANGPTL4 was distributed in HDLs and peripheral blood in a linear mode in both groups (Figure 2C and 2D). Similar to its expression level in the blood, the concentration of ANGPTL4 in HDLs was 22.9 pg/mL (5th to 95th percentile interval: 2.0, 87.4 pg/mL) in controls but increased to 46.3 pg/mL (5th to 95th percentile interval: 8.5, 106.2 pg/mL) in T2DM patients ($P<0.0001$) (Figure 2E and 2F). When comparing ANGPTL4

level by sexes, the concentrations of ANGPTL4 in HDLs was 1.5- and 1.8-fold higher in male and female T2DM patients compared with male and female controls, respectively ($P<0.001$ for both, Figure 2G).

Impact of ANGPTL4 on Other HDL Components

By unadjusted analysis, ANGPTL4 levels in HDLs were significantly correlated with apoA-I but inversely associated with SAA in HDLs of nondiabetic controls (apoA-I: $r=0.156$, $P=0.027$; SAA: $r=-0.169$, $P=0.016$) but not in T2DM patients (apoA-I: $r=-0.083$, $P=0.26$; SAA: $r=-0.039$, $P=0.59$) (Table 4). Cholesterol level in HDLs was negatively associated with ANGPTL4 components in T2DM patients ($r=-0.169$, $P=0.022$) but displayed a trend of association in controls ($r=-0.135$, $P=0.056$).

When adjusting for age, sex, BMI, mean arterial pressure, and lipid-lowering drugs, an inverse correlation between cholesterol and ANGPTL4 in HDLs was seen in both nondiabetic controls and T2DM patients (controls: $\beta=-0.32$, $P=0.044$; T2DM: $\beta=-0.72$, $P=0.041$). The inverse associations between ANGPTL4 and phospholipid and SAA in HDLs remained in nondiabetic controls (phospholipid: $\beta=-0.10$, $P=0.044$; SAA: $\beta=-31.4$, $P=0.021$) (Table 4). A trend of positive association between ANGPTL4 and apoA-I in HDLs was observed in controls ($\beta=0.06$, $P=0.09$). Multivariate-adjusted analysis supported the above findings in T2DM patients (apoA-I: $\beta=-0.06$, $P=0.38$; SAA: $\beta=3.64$, $P=0.72$; phospholipid: $\beta=0.04$, $P=0.45$) (Table 4).

Table 3. Unadjusted Analysis of the Relationship Between ANGPTL4 and Metabolic Parameters in the Study Subjects

	Nondiabetic		Type-2 Diabetic	
	R	P Value	r	P Value
Number, n	201		185	
Age, y	-0.001	0.99	0.071	0.34
Body mass index, kg/m ²	0.060	0.40	0.176	0.017
Systolic blood pressure, mm Hg	0.033	0.65	0.188	0.010
Diastolic blood pressure, mm Hg	0.071	0.32	0.054	0.47
Plasma glucose, mmol/L	0.084	0.24	0.108	0.15
Homa-B, %	-0.033	0.64	0.059	0.42
Homa-IR, %	-0.003	0.96	0.181	0.014
Serum triglyceride, mg/dL	0.024	0.74	0.153	0.037
Total cholesterol, mmol/L	0.094	0.19	0.101	0.17
LDL-c, mmol/L	0.097	0.17	0.069	0.35
HDL-c, mmol/L	-0.07	0.34	-0.176	0.017

Pancreatic β -cell function and insulin resistance were computed by Homeostasis Model of Assessment (Homa-B and HOMA-IR; <http://www.dtu.ox.ac.uk/homacalculator/>), using fasting insulin and glucose in the subjects. ANGPTL4 indicates angiotensin-like protein 4; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol.

Effect of ANGPTL4 on HDL Metabolism and Function in Mice

To directly assess the role of ANGPTL4 in HDL metabolism, ANGPTL4^{-/-} mice were obtained (Figure 3A). As reported earlier, ANGPTL4^{-/-} mice were featured as having low levels of cholesterol and triglyceride in the peripheral blood compared with their wild-type littermates (Figure 3B). Plasma levels of ANGPTL4 were quantified by enzyme-linked immunosorbent assay to further confirm the phenotype (Figure 3C). EL is a member of the LPL family and mediates cholesterol hydrolysis in HDLs.²⁹ To further determine whether ANGPTL4 in HDLs acted on EL and thus led to the reduced HDL-c in T2DM, HEK 293 cells were transfected with the plasmid encoding human EL cDNA and then purified by fluorescence-activated cell sorting. The sorted cells were expanded in vitro and EL expression was checked by Western blot (Figure 3D). When HDLs were isolated from plasma and subjected to EL-expressing 293 cells, both cholesterol and triglyceride content were metabolized faster in ANGPTL4^{-/-} mice than ANGPTL4^{+/+} mice (Figure 3E and 3F). Consequently, cholesterol efflux was 15% reduced in ANGPTL4^{-/-}

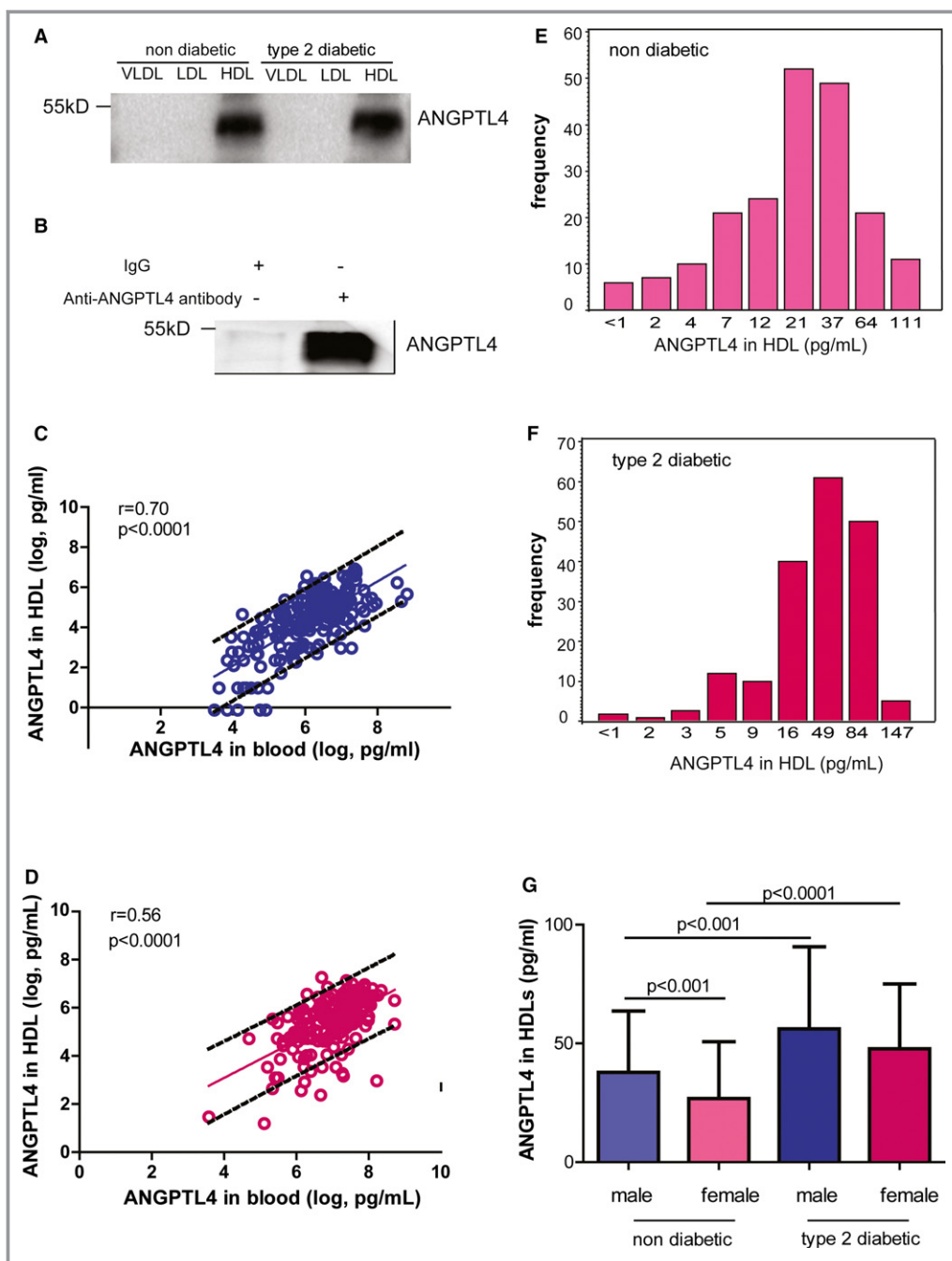


Figure 2. ANGPTL4 in human HDLs. The presence of ANGPTL4 in HDLs by Western blot (A) and immunoprecipitation followed by Western blot (B). n=5 to 6. The correlation is shown between circulating ANGPTL4 in the circulation and HDLs of nondiabetic controls (C) and diabetic patients (D). The distribution of ANGPTL4 in HDLs of nondiabetic controls (E) and diabetic patients is shown (F). ANGPTL4 levels in HDLs of nondiabetic controls and diabetic patients (G). ANGPTL4 indicates angiotensin-like protein 4; HDL, high-density lipoprotein; IgG, immunoglobulin G; LDL, high-density lipoprotein; VLDL, very low-density lipoprotein.

mice compared with wild-type mice (Figure 3G). Distinct from cholesterol and triglyceride, phospholipid hydrolysis was similar between the 2 groups ($P=0.38$). Altogether, these data indicate that ANGPTL4 in HDLs protect HDLs from EL-mediated hydrolysis and maintain HDL function.

ANGPTL4-Containing HDL on EL Activity in Human Subjects

We then determined how ANGPTL4, as a component of HDLs, influenced HDL metabolism in humans. HDLs isolated from

Table 4. Relationship Between ANGPTL4 in HDLs and Other Components in HDLs

	Nondiabetic Participants		T2DM Patients	
	Univariate	Multivariate	Univariate	Multivariate
	<i>R</i>	Estimate (95% CI)	<i>r</i>	Estimate (95% CI)
Number	201	201	185	185
Cholesterol, mmol/L	−0.135	−0.37 (−0.71, −0.03)*	−0.169*	−0.72 (−1.40, −0.04)*
apoA-I, μg/mL	0.156*	0.06 (0.01, 0.12)	−0.083	−0.06 (−0.19, 0.07)
Serum amyloid A, μg/L	−0.169*	−9.41 (−17.32, −1.50)*	−0.039	3.64 (−16.49, 23.77)
Phospholipid, mg/dL	−0.137	−0.93 (−1.00, −0.87)*	0.041	1.03 (0.96, 1.10)
Triglyceride, mg/dL	0.130	1.04 (0.99, 1.10)	0.113	1.06 (0.99, 1.12)

All associations were adjusted for age, sex, body mass index, mean arterial pressure, and lipid-lowering drugs. Estimates given with 95% CI express the difference in HDL components associated with 1 doubling increase of ANGPTL4 in HDLs. ANGPTL4 indicates angiotensin-like protein 4; apoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; T2DM, type 2 diabetes mellitus.

Significance: * $P < 0.05$.

nondiabetic participants and T2DM patients were subjected at the final concentration of 100 μg/mL onto EL-overexpressed 293 cells. The hydrolysis of cholesterol, phospholipid, and triglyceride in the medium was assessed.

Following the increase of ANGPTL4 levels in HDLs, the percentage of hydrolyzed cholesterol did not differ in nondiabetic controls and T2DM patients with low-, medium- and high-level of ANGPTL4 in HDLs ($P > 0.11$ for both) (Figure 4A). After EL hydrolysis, the percentage of metabolized phospholipid in HDLs was not different among nondiabetic subjects with low-, medium-, and high-level of ANGPTL4 in HDLs ($n = 36$, $P = 0.62$). However, the percentage of metabolized phospholipid increased 2.6-fold ($P = 0.0001$) and 2.1-fold ($P = 0.015$) in T2DM patients with medium- and high-level of ANGPTL4 in HDLs compared with those with low level of ANGPTL4 in HDLs, respectively (Figure 4B). Similar to the kinetics of phospholipid hydrolysis, the yield of free fatty acid was comparable among nondiabetic controls with the tertile levels of ANGPTL4 in HDLs ($P = 0.44$) but was enhanced in the T2DM patients with medium- and high-levels of ANGPTL4 when compared with ones with low ANGPTL4 levels in HDLs ($P = 0.037$) (Figure 4C). Altogether, these data suggest that ANGPTL4 in HDLs of nondiabetic participants and T2DM patients had different inhibitory effect on EL and therefore influenced HDL metabolism differently.

ANGPTL4 and HDL Function

Next, the relationship between ANGPTL4 and HDL function was studied. After isolation, HDLs were loaded to macrophages that were preloaded with fluorescently labeled cholesterol. HDL function was assessed as its capacity of cholesterol efflux. By Pearson correlation analysis, the percentage of cholesterol efflux was positively associated with ANGPTL4 levels in both peripheral blood and HDLs of

nondiabetic participants (Figure 5A and 5B). The percentage of cholesterol efflux was positively associated with circulating ANGPTL4 levels but not with ANGPTL4 levels in HDLs in T2DM patients (Figure 5C and 5D).

Multivariable analysis confirmed the positive association between ANGPTL4 levels and the percentage of cholesterol efflux in nondiabetic controls (circulating ANGPTL4: $\beta = 1.70$, $P = 0.008$; ANGPTL4 in HDLs: $\beta = 0.91$, $P = 0.03$). Distinct from nondiabetic controls, after adjusting for the covariables, the percentage of cholesterol efflux was neither associated with circulating ANGPTL4 levels nor with the concentrations of ANGPTL4 in HDLs in T2DM patients (circulating ANGPTL4: $\beta = 1.74$, $P = 0.10$; ANGPTL4 in HDLs: $\beta = -0.19$, $P = 0.81$).

Taken together, per 1 doubling increase of ANGPTL4 level in HDLs, the changes amounted to +0.27% cholesterol efflux (95% CI: 0.03–0.51%; $P = 0.03$), −0.37 mmol/L cholesterol (95% CI: −0.71 to −0.03 mmol/L; $P = 0.044$), +0.06 μg/mL apoA-I (95% CI: −0.01 to 0.12 μg/mL; $P = 0.09$), and −9.41 μg/L SAA (95% CI: −17.3 to −1.5 μg/L; $P = 0.018$) and −0.93 mg/dL phospholipid (95% CI: −0.99 to −0.87 mg/dL; $P = 0.044$) in nondiabetic controls. In T2DM patients, the corresponding estimates were −0.06% cholesterol efflux (95% CI: −0.50% to 0.39%; $P = 0.10$), −0.72 mmol/L cholesterol (95% CI: −1.40 to 0.04 mmol/L; $P = 0.041$), −0.06 μg/mL apoA-I (95% CI: −0.19 to 0.07 μg/mL; $P = 0.16$), +3.64 μg/L SAA (95% CI: −16.5 to 23.8 μg/L; $P = 0.72$), and +1.03 phospholipid (95% CI: 0.96–1.10; $P = 0.45$) (Figure 6A through 6D).

Discussion

To our best knowledge, our current study is the first to assess and compare whether and how ANGPTL4, as a component of HDLs, participates in HDL metabolism and function in nondiabetic participants and T2DM patients. The key findings

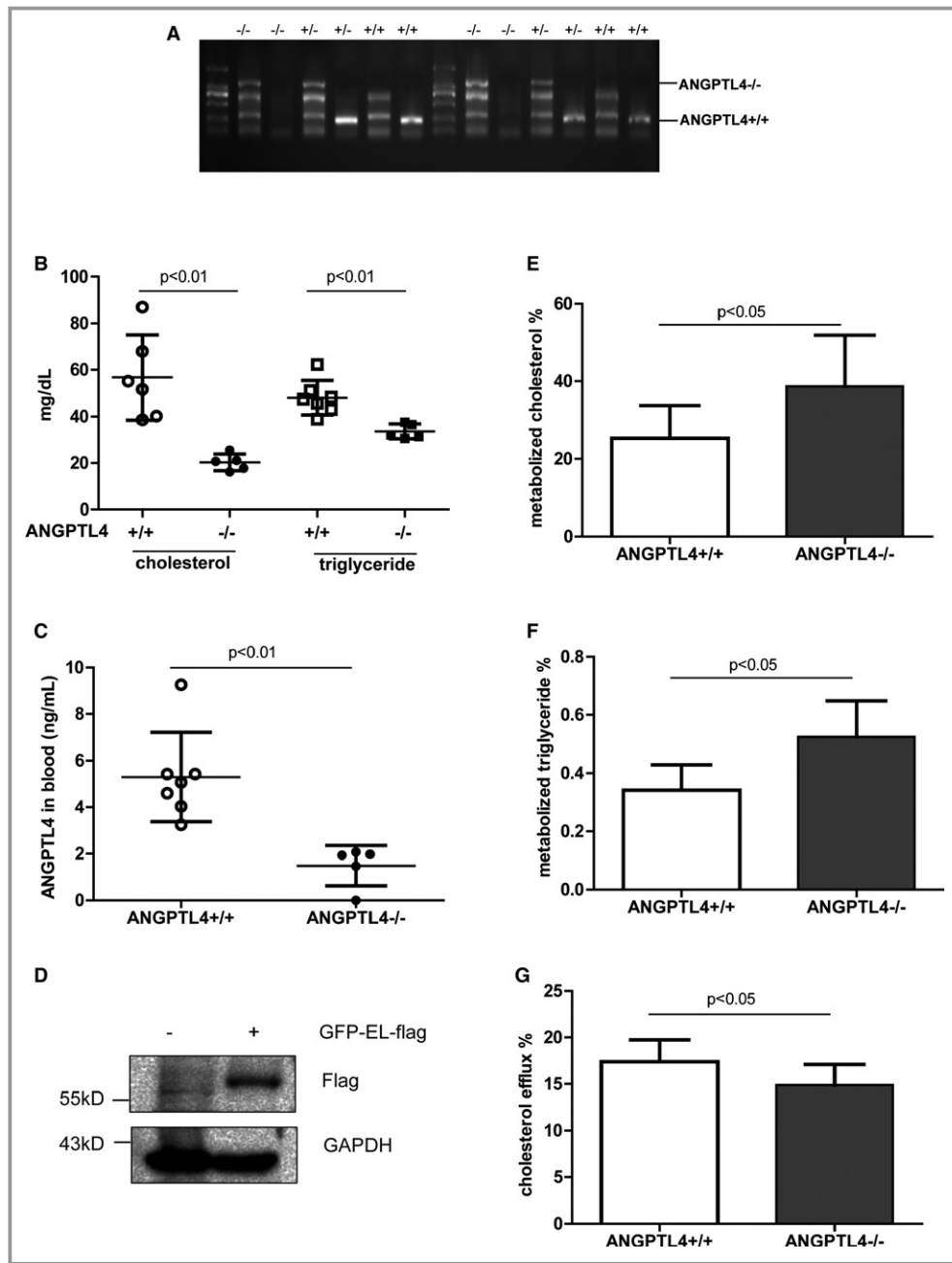


Figure 3. ANGPTL4 as a HDL component for HDL metabolism and function in mice. A, Phenotype determination by RT-PCR. Two pairs of primers were used to screen ANGPTL4^{-/-} mice. B, Total cholesterol and triglyceride levels in the blood of ANGPTL4^{+/+} and ANGPTL4^{-/-} mice. n=5 to 7. C, Plasma levels of ANGPTL4. n=5 to 7. The filled circles and squares represent ANGPTL4^{+/+} mice and the open circles denote ANGPTL4^{-/-} mice. HDLs were isolated from ANGPTL4^{+/+} and ANGPTL4^{-/-} mice and subjected at a final concentration of 100 μg/mL onto endothelial lipase (EL)-expressing 293 cells for hydrolysis. Human EL expression in purified HEK 293 cells transfected with the plasmid containing human EL cDNA (D). The percentage of metabolized cholesterol is shown in (E) and the ratio between the amount of free fatty acids produced by EL lysis vs total amount of triglyceride input before EL lysis is shown in (F). n=6 to 7. G, The percentage of cholesterol efflux against plasma of ANGPTL4^{+/+} and ANGPTL4^{-/-} mice. n=8 to 18. ANGPTL4 indicates angiotensin-like protein 4; HDL, high-density lipoprotein; RT-PCR, reverse transcription polymerase chain reaction.

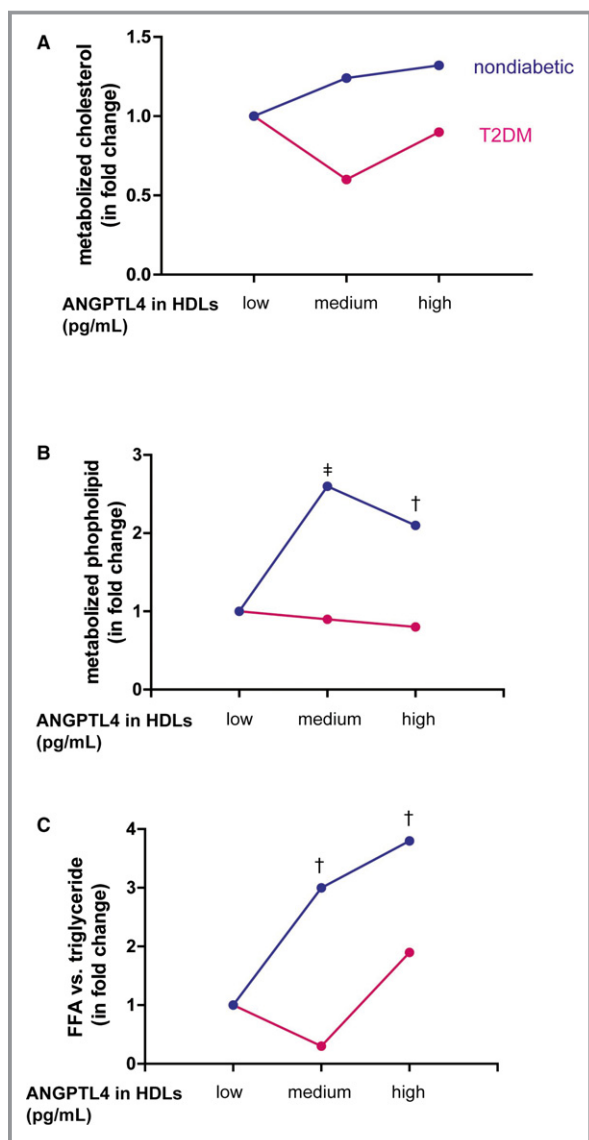


Figure 4. The influence of ANGPTL4 in HDLs on endothelial lipase hydrolysis in humans. Equal amount of HDLs were loaded into endothelial lipase (EL)-expressing cells for hydrolysis for 4 hours. Before loading, cholesterol, phospholipid, and triglyceride in HDLs were quantified. After loading, medium was collected to measure cholesterol, phospholipid, and free fatty acid. The absolute amounts of metabolized cholesterol and phospholipid and triglyceride were calculated among the subjects with low-, medium-, and high-levels of ANGPTL4 in HDLs in both groups. Metabolized cholesterol (A) and phospholipid (B) were expressed as the fold change when the subjects with medium- and high-levels of ANGPTL4 compared with those having low ANGPTL4 levels in HDLs. $n=11$ to 12 per subgroup. C, To assess triglyceride metabolism, the ratio of the amount of free fatty acid after hydrolysis vs the amount of triglyceride loaded was calculated. Data are expressed as the fold change when comparing the subjects with the low ANGPTL4 levels in HDLs. $n=11$ to 12 per subgroup. Significance: [‡] $P<0.0001$; [†] $P\leq 0.01$. ANGPTL4 indicates angiotensin-like protein 4; FFA, free fatty acid; HDL, high-density lipoprotein; T2DM, type 2 diabetes mellitus.

can be summarized as follows: (1) the levels of ANGPTL4 in the circulation and HDLs were increased in T2DM patients compared with nondiabetic controls; (2) after adjusting for age, sex, BMI, and use of lipid-lowering drugs, 1 doubling increase of ANGPTL4 in HDLs was associated with $+0.06$ $\mu\text{g}/\text{mL}$ increase of apoA-I and 0.27% increase of cholesterol efflux but 0.37 mmol/L decrease of cholesterol, 0.93 $\mu\text{g}/\text{mL}$ decrease of phospholipid, and 9.41 $\mu\text{g}/\text{L}$ reduction of SAA in HDLs in nondiabetic controls; (3) by contrast, multivariable analysis only illustrated the inverse correlation between cholesterol and ANGPTL4 in HDLs in T2DM patients; (4) in mice, the absence of ANGPTL4 in HDLs accelerated HDL hydrolysis and reduced HDL function; and (5) compared with mice, the role of ANGPTL4 in HDL metabolism was more complicated in human subjects, as evidenced by minor effect on HDL metabolism in nondiabetic controls and dramatic phospholipid and triglyceride hydrolysis in HDLs in T2DM patients when the increased concentrations of ANGPTL4 in HDLs were exposed to EL in vitro.

The incidence of cardiovascular diseases is high in diabetic patients.³⁰ Population studies elucidated HDLs as an independent inverse predictor of cardiovascular disease.^{30,31} These epidemiologic findings were confirmed in vitro and in animal models, as supported by promotion of cholesterol efflux from cells or tissues,³² inhibition of vascular inflammation³³, and improved endothelial cell function.³⁴ Whereas most studies reported the relationship between circulating ANGPTL4 levels and HDL-c, they mainly recruited Europeans,^{8,14} American,¹² or Hispanic subjects.³⁵ Few studies addressed the question of whether the concentration of ANGPTL4 in HDLs was associated with HDL-c in Chinese subjects, nor did anyone evaluate whether and how ANGPTL4 directly affected HDL metabolism and/or function in T2DM patients.

Apart from these reports, it has been revealed that HDLs become glycosylated under hyperglycemia and thus render its atheroprotective properties.³⁶ ANGPTL4 plays an important role in lipid metabolism via its inhibition of LPL and is present in HDLs in mice and humans. Thus, it becomes very crucial and relevant to investigate whether ANGPTL4, as a HDL component, modifies HDL metabolism and function in Chinese nondiabetic participants and T2DM patients.

In the literature, the relationship between circulating ANGPTL4 and cholesterol seems controversial. On the one hand, reports showed that plasma ANGPTL4 levels were inversely correlated with HDL-c but not associated with LDL-c or triglyceride in the British population.¹³ Circulating ANGPTL4 levels were negatively associated with HDL-c and positively associated with triglyceride in Americans.¹² On the other hand, no correlation was seen between circulating ANGPTL4 levels with any of these aforementioned parameters in Finnish subjects.³⁷ In the study, we did not observe any significant association between circulating ANGPTL4 levels

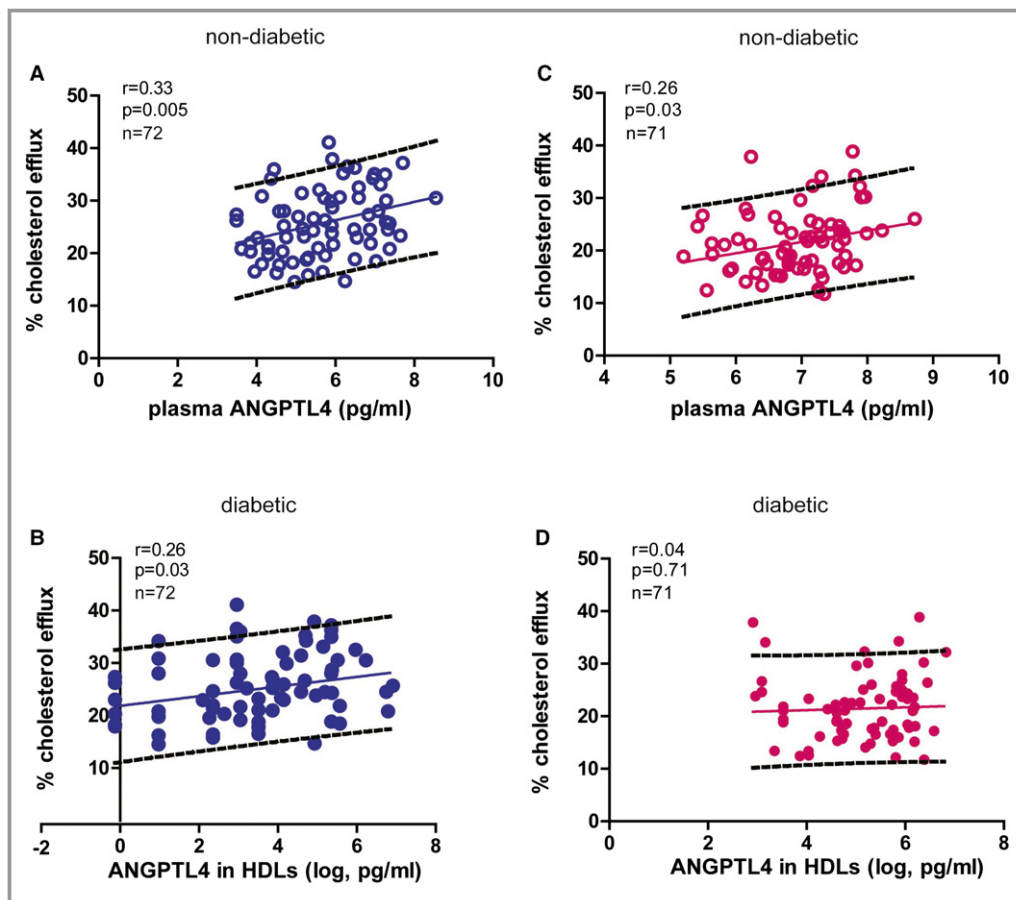


Figure 5. The relationship between ANGPTL4 and cholesterol efflux. Unadjusted association between circulating ANGPTL4 levels and the percentage of cholesterol efflux in nondiabetic controls (A) and diabetic patients is shown (B). Univariate association between ANGPTL4 in HDLs and the percentage of cholesterol efflux in nondiabetic controls (C) and diabetic patients is shown (D). ANGPTL4 indicates angiotensin-like protein 4; HDL, high-density lipoprotein.

and HDL-c, LDL-c, or triglyceride in nondiabetic participants. Nevertheless, multivariate-adjusted analysis delineated an inverse correlation between ANGPTL4 and cholesterol in HDLs in both controls and T2DM patients, refining a more specific relationship between ANGPTL4 and cholesterol in HDLs.

We then explored the impact of ANGPTL4 on HDL hydrolysis. As a member of the LPL family, EL catabolizes cholesterol and phospholipid in HDLs.²⁹ Because EL shares a high degree of homology with LPL and hepatic lipase, it tends to be more specific at hydrolyzing phospholipids than LPL and hepatic lipase.³⁸ Furthermore, serum EL activity was increased in T2DM patients.³⁹ In the study, we explored whether ANGPTL4 could have any inhibitory impact on EL as it does for LPL. We first investigated the impact of ANGPTL4 on HDL metabolism and function in a simplified model (ie, ANGPTL4+/+ and ANGPTL4-/- mice). The absolute absence of ANGPTL4 led to prompt cholesterol and triglyceride hydrolysis when exposed to EL, which contributed to

decreased HDL function. These data hint at the importance of ANGPTL4 for protecting HDLs from EL-mediated hydrolysis and maintaining HDL function.

As always, human data are more complex than those of mice. Our data demonstrated that physical levels of ANGPTL4 in HDLs have a minor effect on cholesterol, phospholipid, and triglyceride metabolism in HDLs when exposed to EL. Different from controls, phospholipid and triglyceride were metabolized faster in T2DM patients with high levels of ANGPTL4 in HDLs when compared with ones with low ANGPTL4 levels in HDLs. These data imply that as circulating ANGPTL4 levels were increased in T2DM patients, the distribution of ANGPTL4 in HDLs was elevated but accompanied by reduced inhibition on EL, leading to the accelerated hydrolysis of phospholipid and triglyceride in HDLs and enhanced free fatty acid production. Despite dramatic hydrolysis of triglyceride and phospholipid in HDLs, cholesterol metabolism was similar among T2DM patients with different levels of ANGPTL4 in HDLs. Further studies are

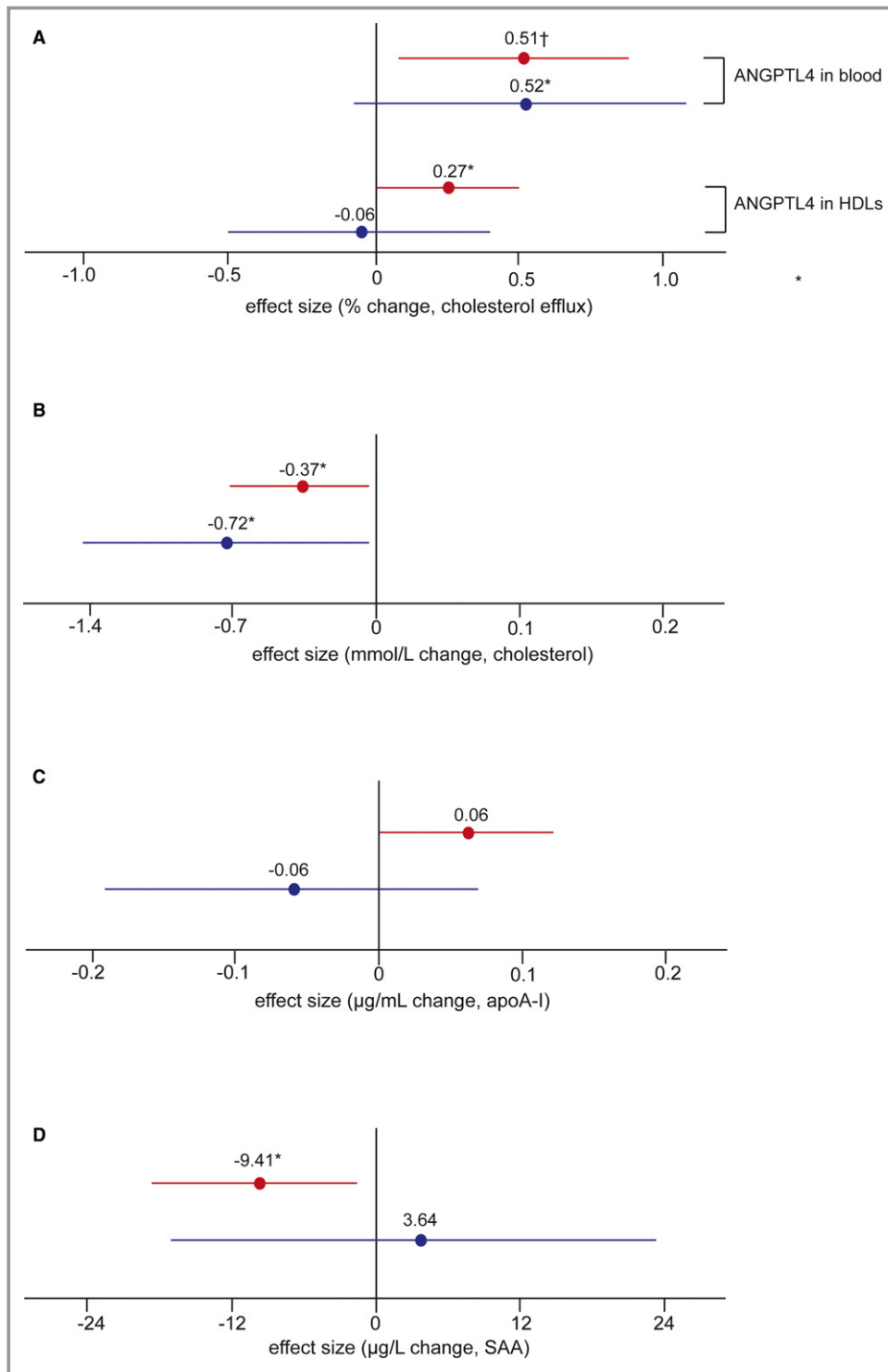


Figure 6. Multivariable analysis of the association between ANGPTL4 and HDL components and function. When adjusting for age, sex, body mass index, mean arterial pressure, and lipid-lowering drugs, the association between the percentage of cholesterol efflux and circulating ANGPTL4 and ANGPTL4 levels in HDLs in both groups (A) is shown. The correlation between ANGPTL4 and cholesterol (B), apoA-I (C), and SAA (D) in HDLs is shown. Red and blue lines present nondiabetic controls and T2DM patients, respectively. Data are expressed as the change per 1 doubling increase of ANGPTL4 in the circulation (A) or HDLs (A through D). Nondiabetic: n=201; T2DM: n=185. Significance: † $P<0.01$; * $P<0.05$. ANGPTL4 indicates angiotensin-like protein 4; apoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; SAA, serum amyloid A; T2DM, type 2 diabetes mellitus.

needed to determine how elevated ANGPTL4 levels in HDLs have reduced inhibitory property of EL or other lipases in the setting of diabetes mellitus.

Although the phospholipid levels in HDLs were observed to be higher in T2DM patients than that of controls (Table 2), phospholipid in HDLs of T2DM was hydrolyzed more than controls in EL-expressing cells in vitro (Figure 4B). The contradictory observations might be explained as the following: (1) EL expression and activity are different between nondiabetic controls and T2DM patients,³⁹ which could not be exactly examined in vitro; (2) we could not exclude the regulation of ANGPTL4 on other LPL members for HDL metabolism; and (3) we speculate that other HDL components might regulate ANGPTL4 activity. However, our in vivo and in vitro data verified that ANGPTL4 was critically involved in the diverse HDL metabolism patterns between nondiabetic controls and T2DM patients, all of which substantially translate to different HDL function.

ANGPTL4 is expressed in multiple tissues, and cleavage of its N-terminal and C-terminal of this protein could not only inhibit LPL activity but also affect vasculature.² Thus, ANGPTL4 comprises multifunctional secreted proteins with different targets. ANGPTL4 induced in adipocytes promotes α -cell proliferation.⁴⁰ The sialylation form of ANGPTL4 produced in podocytes interacts with $\alpha v \beta 5$ integrin in glomerular endothelial cells to govern glomerulus structure and stability.⁴¹ Blockade of this interaction could postpone the recovery from peak proteinuria in mice models of kidney diseases.^{41,42} Furthermore, on the one hand, ANGPTL4 promotes endothelial cell integrity and improves angiogenesis for cerebral protection in ischemic stroke; on the other hand, upregulation of ANGPTL4 in hypoxic Müller cells accelerates abnormal angiogenesis and retinopathy.⁴³ Collectively, ANGPTL4 is a double-edged sword in different diseases.

The present study must be interpreted within the context of some other potential limitations. First, we did not compare LPL and EL activity in the peripheral blood of nondiabetic controls and T2DM patients, nor did we evaluate the effect of ANGPTL4 on LPL-mediated HDL hydrolysis because HDLs were our main focus in the study. Second, we did not assess the angiogenic function of ANGPTL4 in the study subjects because it was not our focus. Third, we were not able to explore how ANGPTL4 levels were differently regulated in nondiabetic subjects and T2DM patients.

Metformin is the initial treatment for diabetes mellitus. Nevertheless, about 39.7% of T2DM patients could achieve HbA1c less than 7.0% in clinical practice.⁴⁴ Consistent with the current status, fasting glucose levels of T2DM were much higher than controls in our study. Therefore, a stepwise intensified strategy for pharmaceutical interventions from metformin monotherapy to dual or triple therapies is recommended and being optimized at present.⁴⁵

In conclusion, our data illustrated that ANGPTL4 in HDLs physically protects HDLs from hydrolysis and sustains HDL function. As circulating ANGPTL4 levels were increased in T2DM patients, the concentration of ANGPTL4 was elevated in HDLs in diabetic patients but with skewed inhibition of EL activity, which contributes substantially to HDL hydrolysis and dysfunction.

Acknowledgments

We express our sincere appreciation to Prof Guo-Qing Liu for providing ANGPTL4^{-/-} mice for the study and Prof Jan A. Staessen for his guidance on statistical analysis using SAS software.

Sources of Funding

The study received support from the National Science Funding in China (#81470566 and #81670765), the Beijing Municipal Science & Technology Commission (#Z131100006813018), the Health and Research Bureau of Tongzhou District (#R5; KJ2016CX030; KJ2016CX025; KJ2016CX037-08), Capital Health Development Research Project (2014-3-7081), and Lu He Hospital Funding (LH201504).

Disclosures

None.

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