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Quantitation of serum angiopoietin-like proteins 3 and 4 in a Finnish population sample

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Abstract **We have developed and validated quantitative ELISAs for human angiopoietin-like (ANGPTL)3 and 4 and correlated their serum levels with parameters of lipid and carbohydrate metabolism. For this study, we used a random subsample of the Health 2000 Health Examination Survey consisting of 125 men and 125 women, aged 30–94 years. The anthropometric and biochemical parameters of subjects were characterized in detail. ANGPTL 3 and 4 levels were determined using the developed ELISAs. The intra- and inter-assay coefficients of variation for the assays were less than 15%. The average serum concentration of ANGPTL3 was 368 ± 168 ng/ml (mean ± SD) and for ANGPTL4 it was 18 ± 23 ng/ml (mean ± SD). ANGPTL4 serum levels displayed high variability between individuals ranging from 2 to 158 ng/ml. In post-heparin plasma, both ANGPTL 3 and 4 were increased. Low levels of ANGPTL3 were associated with decreased HDL-cholesterol and increased triglyceride levels. ANGPTL4 levels were positively correlated with FFAs (P = 0.044) and waist-hip ratio (P = 0.016). The developed ELISAs will be important tools to clarify the role of ANGPTL 3 and 4 in human energy metabolism and partitioning of triglycerides between sites of storage (adipose tissue) and oxidation (skeletal and cardiac muscle).**—Robciuc, M. R., E. Tahvanainen, M. Jauhiainen, and C. Ehnholm. **Quantitation of serum angiopoietin-like proteins 3 and 4 in a Finnish population sample.** ***J. Lipid Res.*** **2010.** 51: **824–831.**

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Angiopoietin-like (ANGPTL) proteins 3 and 4 belong to a family of proteins that share a common modular structure consisting of an N-terminal signal sequence, a coiled-coil domain and a large fibrinogen/angiopoietin-like domain (1, 2). Recently, ANGPTL 3 and 4 have emerged as important modulators of lipid metabolism (3).

Dysregulation of lipid metabolism is closely related with a number of pathological states including atherosclerosis, obesity, and insulin resistance. An extensive amount of data relates elevation of cholesterol and triglycerides (TG) in apolipoprotein (apo)B-containing particles such as VLDL and LDL to increased risk for atherosclerosis. Epidemiological and clinical studies have provided strong evidence that a low level of cholesterol in HDL is also a major risk factor for the development of atherosclerosis (4, 5). LPL is a key molecule involved in the hydrolysis of TG in VLDL and chylomicrons and in the clearance of these particles from circulation (6). In vitro observations as well as animal models have firmly established ANGPTL 3 and 4 as potent inhibitors of LPL (7, 8). These studies are supported by genetic studies in humans that have associated mutations in these two proteins with circulating TG levels (9–11). Endothelial lipase (EL), a member of the triglyceride lipase gene family, has been demonstrated to influence the levels of HDL in circulation (12). Recently, it was shown that ANGPTL3 can inhibit EL and thereby increase the levels of HDL (13).

Epidemiological studies clearly demonstrate that obesity has increased worldwide. Obesity increases the risk of diabetes, heart disease, fatty liver, and even some forms of cancer. It is therefore important to better understand the biological basis of obesity in order to aid its prevention and treatment. Several studies in mice have shown that ANGPTL4 can act as a powerful signal from adipose and other tissues to prevent fat storage and stimulate fat mobilization (14, 15). There is a large body of evidence demonstrating that ANGPTL 3 and 4 play major roles in rodent energy metabolism. However, there is limited information on the physiological roles of ANGPTL 3 and 4 in humans.

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Abbreviations: ANGPTL, angiopoietin-like; apo, apolipoprotein; BMI, body mass index; CRP, c-reactive protein; EL, endothelial lipase; Hcy, homocysteine; HOMA IR, homeostasis model assessment for insulin resistance; PHP, post-heparin plasma; TC, total cholesterol; TG, triglyceride.

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In order to increase our knowledge concerning the roles of ANGPTL 3 and 4 in human physiology, we developed methods to measure circulating levels of these proteins. The present study reports on the measurements of serum ANGPTL 3 and 4 in a normal Finnish population sample and correlates their plasma levels to parameters of lipid and carbohydrate metabolism.

MATERIALS AND METHODS

Subjects

For this study we used a random subsample of the Health 2000 Health Examination Survey carried out in Finland consisting of 250 subjects (125 men and 125 women), age range 30–94 years (16, 17). Serum samples (1.5 ml aliquots) were stored at –70°C before analysis. To evaluate the effect of heparin on the levels of ANGPTL 3 and 4, seven volunteers received a heparin injection (100 IU/kg body weight) and post-heparin plasma was collected after 15 min. ANGPTL 3 and 4 were measured in pre- and post-heparin plasma. For kinetic studies, three volunteers received a heparin injection (100 IU/kg body weight) and plasma samples were collected at 0, 5, 10, 15, 30, and 60 min after injection and levels of ANGPTL4 were assessed. Each study subject gave written informed consent before participating in the study. The samples were collected in accordance with the Helsinki Declaration and ethics committees of the participating centers approved the study design.

General procedures

Protein concentration was determined by the method of Lowry et al. (18). ApoA-I, apoA-II, and apoB concentrations were measured by immunoturbidometric methods using commercial kits (Orion Diagnostica, Espoo, Finland; Boehringer-Mannheim, Mannheim, Germany) using an automatic clinical chemistry analyzer (Olympus Diagnostica GmbH). Serum total cholesterol (TC) and TG were analyzed using fully enzymatic methods (Olympus Diagnostica). Serum FFAs were quantified using a kit from Zen-Bio, Inc. (Research Triangle Park, NC). HDL- and LDL-cholesterol (HDL-C, LDL-C) were measured using direct enzymatic methods (Roche Diagnostics GmbH). Plasma glucose concentration was analyzed by a hexokinase method (Olympus Diagnostica), and insulin was analyzed by a microparticle enzyme immunoassay (Abbott Diagnostics Division, Axis-Shield, Oslo, Norway). Concentration of C-reactive protein (CRP) was determined by an immunoturbidometric method (Orion Diagnostica). Serum homocysteine (Hcy) was measured using the high-pressure liquid chromatographic method (19). The homeostasis model assessment for insulin resistance (HOMA IR) was calculated from the fasting plasma glucose and serum insulin concentrations as follows: fasting insulin (μU/ml) × fasting glucose (mmol/l)/22.5 (20). Physical activity of the subjects was assessed by questionnaire and divided in four categories: ideal, sufficient, uncertain, and insufficient.

Determination of ANGPTL3 concentration

We developed a noncompetitive ELISA to determine the concentration of human serum/plasma ANGPTL3. As a capture antibody, we used a rabbit polyclonal antibody raised against recombinant ANGPTL3 (aa 17-223) (BioVendor, Czech Republic) and as a detection antibody, a biotinylated sheep IgG raised against human recombinant ANGPTL3 (aa 17-460) (RnD Systems, Minneapolis, MN). The capture antibody was used to coat 96-well plates, 100 μl/well (1 μg/ml in PBS) overnight at 4°C. The plate was washed four times with PBS-Tw (50 mM potassium phosphate 150 mM NaCl, 0.1% Tween 20; pH 7.4) on the Wellwash AC (Thermo Scientific). Nonspecific binding sites were blocked with 300 μl/well of 0.5% BSA (w/v) in PBS for 2 h at room temperature. After aspiration, samples and standards were diluted with TBS (0.05 mM Tris-HCl, 0.15 mM NaCl, pH 7.6 containing 0.1% BSA (w/v) and 0.1% Tween 20) and were pipetted in duplicates (100 μl/well). The plate was incubated for 1 h at room temperature. After washing four times with 350 μl/well PBS-Tw, detection antibody was added 100 μl/well (0.2 μg/ml) and the plate was incubated for 1 h at room temperature. Following four washes, streptavidin-HRP (streptavidin conjugated to horseradish-peroxidase), diluted 1: 200 in PBS, 1% BSA (w/v) was added (100 μl/well) and the plate was incubated for 30 min at room temperature. After washing, 3, 3′, 5, 5′ tetramethylbenzidine (TMB) substrate (Sigma) was added (100 μl/well) and the plate incubated for another 4–6 min at room temperature. The reaction was stopped with 1 N sulfuric acid, 100 μl/well. The developed color was determined by reading the plate on the microplate reader Victor^2 Multilabel Counter (Wallac, Turku, Finland) at a wavelength of 450 nm. Standards were prepared at concentrations of 1.25, 2.5, 5, 10, 20, and 40 ng/ml. For standardization, we used a human serum quantified for ANGPTL3 with a commercially available kit from BioVendor following the manufacturer's instructions. The standardized serum was stored at –70°C in small aliquots and for every measurement a new aliquot was thawed. The serum samples were diluted 50-fold. The standard curve was constructed by plotting the concentration (X) of standards against the mean absorbance (Y) of standards at 450 nm (Fig. 1A). A second order polynomial equation was used to fit the standards and calculate the sample concentrations.

Determination of ANGPTL4 concentration

To measure the concentration of human ANGPTL4 in circulation we have developed a noncompetitive direct ELISA using the DuoSet Elisa hANGPTL4 (RnD Systems) and the protocol described by Kersten et al. with some modifications (21). We used the goat anti-human ANGPTL4 as a capture antibody to coat 96-well plates, 100 μl/well (1.6 μg/ml in PBS) overnight at 4°C. The plate was washed four times with PBS-Tw (50 mM potassium phosphate 150 mM NaCl, 0.1% Tween 20; pH 7.4) on the Wellwash AC (Thermo Scientific). Nonspecific binding sites were blocked with 300 μl/well of 0.5% BSA (w/v) in PBS for 2 h at room temperature. After aspiration, samples and standards (human recombinant ANGPTL4) diluted with TBS (0.05 mM Tris-HCl, 0.15 mM NaCl, pH 7.6) containing 0.1% BSA (w/v) and 0.05% Tween 20 were pipetted in duplicates (100 μl/well). The plate was incubated for 1 h at room temperature. After four washes with PBS-Tw, 100 μl/well (0.2 μg/ml) of detection antibody (biotinylated goat anti-human ANGPTL4) was added and the plate was incubated for 1 h at room temperature. Following four washes, 100 μl/well of streptavidin-HRP diluted 1:200 in PBS, 1% BSA (w/v) was added and the plate was incubated for 30 min at room temperature. After washing, 100 μl/well TMB substrate was added and the plate was incubated for another 4–6 min at room temperature. The reaction was stopped with 100 μl/well of 1 N sulfuric acid. The plates were analyzed using the microplate reader Victor^2 Multilabel Counter (Wallac) at 450 nm. Standards were prepared at concentrations of 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, and 25 ng/ml. Serum samples were diluted 1: 10 and samples exceeding the highest absorbance of the standard curve were diluted further 1: 20 or 1: 40. The standard curve was constructed by plotting the concentration (X) of standards against the mean absorbance (Y) of standards at 450 nm (Fig. 1B). A second order polynomial equation was used to fit the standards and calculate the sample concentration.

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Expression of ANGPTL4 in cultured cells

Human ANGPTL4 cDNA cloned into pcDNA3.1 vector under the control of the cytomegalovirus (CMV) promoter-enhancer was a kind gift from Professor Sander Kersten (Wageningen University, The Netherlands). Human hepatocellular carcinoma cell line Huh7 (ATCC CCL-185) was transfected with pcDNA3.1 (mock) or pcDNA3.1-ANGPTL4 using Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions. Huh7 cells were seeded at 90% confluence in 12-well plates a day before the transfection and grown in DMEM in the presence of 10% FBS. Cells were transiently transfected with 1.6 μg of expression plasmids and 4 μl of transfection reagent per well. Five h after the addition of transfection complexes, medium was replaced with serum free medium. Medium was collected after 48 h and centrifuged to remove cellular debris. Cells were washed with PBS (pH 7.4) and then lysed in 175 μl of RIPA buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (v/v) sodium deoxycholate, 0.1% SDS, and complete mini EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)]. Cell lysate was transferred to 1.5 ml tubes prior to centrifugation for 10 min at 15000 *g*. Aliquots from medium and cell lysate were subjected to SDS-PAGE and Western blot analysis. To evaluate the effect of heparin on the release of ANGPTL4, cells were incubated with 100 IU/ml heparin for 1, 2, and 3 h. For Western blot analyses, cells were maintained after transfection in serum free medium containing 10 IU/ml of heparin for 48 h. One h before cell harvest, another 10 IU/ml of heparin were added to boost up the release. The viability and attachment of the cells were carefully evaluated by light microscopy and no effect of heparin at the concentrations used was observed.

SDS-PAGE and immunoblot analysis

Equivalent amounts of protein (25μg) from the cell lysate and medium (50 μl medium, concentrated by evaporation) were mixed with the reducing sample buffer and boiled in water for 5 min. Samples were loaded on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated in TBST buffer (1 × TBS [pH 7.4] and 0.05% Tween 20) with 5% fat free milk for 1 h at room temperature before addition of the primary antibodies. Primary antibodies were diluted in TBST buffer (1: 1000) and incubated with membranes for 40 min at room temperature. Membranes were washed 4 × 15 min with TBST buffer. As a primary antibody, a rabbit affinity purified IgG raised against the region 26–229 of human ANGPTL4 (BioVendor) was used. HRP-conjugated donkey antigoat IgG (Santa-Cruz) or goat anti-rabbit IgG (BioRad) were diluted (1: 10000 and 1: 2000 respectively) in TBST-5% fat free milk and incubated with membranes for 40 min at room temperature. Membranes were washed 4 × 15 min with TBST and visualized using chemiluminescence assay (GE Healthcare, Buckinghamshire, UK).

Heparin chromatography

Human plasma (115 ml) was recycled (flow rate, 1 ml/min) overnight at + 4°C in a 250-ml HiTrap heparin affinity chromatography column (Amersham Biosciences). The column was washed with 25 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA at a flow rate of 5 ml/min. The bound material was eluted with sequential steps using buffers containing 0.5 M, 1 M, and 2 M NaCl (flow rate, 10 ml/min/fraction). The fractions were analyzed for ANGPTL 3 and 4 using the developed ELISAs as described above.

Statistical analysis

All statistical analyses were performed using SPSS version 16.0 for Windows (SPSS, Inc.) and GraphPad Prism 4.03 (GraphPad Software, Inc.). Selected parameters were logarithmically transformed before they were used in statistical analyses. To determine the relationship between serum levels of ANGPTL 3 and 4 and other measured parameters, the Pearson correlations test, Spearman test, partial correlations, and Mann Whitney test were used.

RESULTS

Validation of ANGPTL 3 and 4 ELISAs

The specificity, sensitivity, and accuracy of the ANGPTL 3 and 4 assays were determined. The standard curves for ANGPTL 3 and 4 ELISAs are presented in **Fig. 1A** and B, respectively. The use of human serum or plasma resulted in similar values for both assays. The specificity of the assays was confirmed by testing the cross-reactivity with human recombinant ANGPTL 4 (RnD Systems) using ANGPTL3 ELISA and with human recombinant ANGPTL3 (BioVendor) using ANGPTL4 ELISA. No cross-reactivity was observed. Sera derived from different mammalian species, mouse, rabbit, and bovine did not react in these assays. The detection limit of the assay was 1 ng/ml for ANGPTL3 and 0.1 ng/ml for ANGPTL4. Intra- and inter-assay coefficients of variation were less than 10%. Serum samples from two

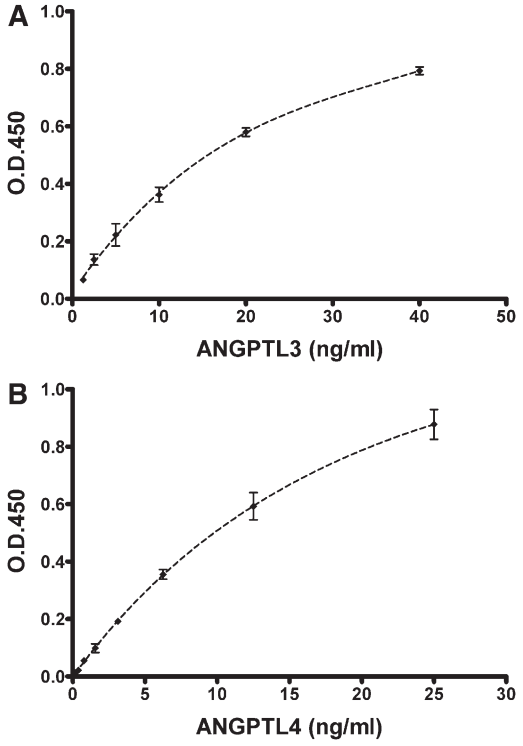

Fig. 1. Standard curves for ANGPTL 3 and 4 ELISAs. A: Standard curve for ANGPTL3 ELISA was constructed using a standardized human serum with a concentration of 400 ng/ml of ANGPTL3. The standard serum was diluted to obtain the following concentrations 1.25, 2.5, 5, 10, 20, and 40 ng/ml of ANGPTL3. All analyzed serum samples fell within the standard curve range when diluted 1:50. B: standard curve for ANGPTL4 ELISA was constructed using human recombinant protein at concentrations of 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, and 25 ng/ml. Serum samples were diluted 1: 10, 1: 20, or 1: 40 in order to fall within the range of the curve. A second order polynomial equation fits best for both standard curves (r^2>0.99).

subjects were tested for dilution linearity (5- to 50-fold dilution range) and the mean coefficient of variation was 14.1% for ANGPTL3 assay and 9.6% for ANGPTL4 assay. When increasing amounts of recombinant protein were added to serum, the average recovery was 113% for ANGPTL3 and 104% for ANGPTL4. No significant differences of ANGPTL 3 and 4 concentrations were observed after three to six freezing/thawing cycles for two different human plasma samples. To further verify the ANGPTL3 ELISA, we have quantified 18 serum samples with our method and with the commercially available kit from BioVendor. The values obtained with our method (325 ± 114 ng/ml, mean ± SD, n = 18) and commercial ELISA (418 ± 136 ng/ml, mean ± SD, n = 18) were highly correlated (r = 0.96, P < 0.0001, data not shown).

Characteristics of the study population

The study population consisted of 125 male and 125 female subjects with the mean age of 55 years. The age, body mass index (BMI), TG, LDL-C, apoB, insulin, HOMA-IR, CRP, and Hcy values did not differ significantly between genders. A significant gender difference was observed for waist-hip ratio (WHR), FFA, TC, HDL-C, apoA-I, and glucose concentrations (for all, P < 0.05). Serum ANGPTL3 levels demonstrated high variability in the population with an average value (± SD) of 368 ± 168 ng/ml. The distribution in the population was skewed to the left and normalized after logarithmic transformation. Also, ANGPTL4 serum levels were highly variable between individuals with values ranging from 2 to 158 ng/ml. No gender differences were observed for ANGPTL 3 or 4 (**Table 1**).

Correlation of ANGPTL 3 and 4 with clinical and biochemical parameters

As depicted in **Table 2**, bivariate correlations revealed a positive association of serum ANGPTL3 with age (r = 0.292, P < 0.001), HDL-C (r = 0.224, P < 0.001), and apoA-I (r = 0.144, P = 0.023) and a negative correlation with TG (r = -0.182, P = 0.004) and apoB/apoA-I ratio (r = -0.192, P = 0.002). The observed correlations remained significant after adjustment for age, gender, and BMI. However, when HDL-C and apoA-I levels were used as control variables, the correlation of ANGPTL3 with triglycerides was completely lost (r = -0.029, P = 0.649). Furthermore, we have divided the study population in quartiles for TG levels and for HLD-C levels. When analyzing quartiles, a significant decrease (P < 0.0001) of ANGPTL3 levels in subjects with high TG and low HDL-C (75th TG/25th HDL, n = 36) compared with subjects with low TG and high HDL-C (25th TG/ 75th HDL, n = 33) (**Fig. 2**) was observed.

Serum ANGPTL4 levels were positively correlated with age (r = 0.178, P = 0.013), WHR (r = 0.155, P = 0.016), FFA (r = 0.129, P = 0.044), and CRP (r = 0.177, P = 0.002) and negatively with Hcy (r = -0.136, P = 0.034) (Table 1). Because the ANGPTL4 distribution was still slightly skewed after the logarithm transformation, we also used nonparametric tests to verify the results. The Spearman test revealed the significant correlations for the same parameters that were obtained with the Pearson test.

TABLE 1. Characteristics of the study population

Parameter	Males, n = 125 (mean ± SD)	Females, n = 125 (mean ± SD)	p
Age (years)	55.29 ± 15.29	54.94 ± 15.13	0.855
BMI (kg/m^2)	25.62 ± 3.11	26.58 ± 4.72	0.060
WHR	0.95 ± 0.05	0.85 ± 0.05	**<0.001**
FFA (μmols/l)	392.17 ± 203.65	575.32 ± 256.70	**<0.001**
TC (mmol/l)	5.45 ± 1.07	5.80 ± 0.93	**0.006**
TG (mmol/l)	1.54 ± 1.31	1.40 ± 0.65	0.276
LDL-C (mmol/l)	3.52 ± 0.91	3.69 ± 0.81	0.123
apoB (g/l)	1.12 ± 0.26	1.15 ± 0.24	0.368
HDL-C (mmol/l)	1.25 ± 0.35	1.47 ± 0.38	**<0.001**
apoA-I (g/l)	1.47 ± 0.27	1.67 ± 0.28	**<0.001**
apoB/apoA-I	0.78 ± 0.22	0.70 ± 0.19	**0.003**
Glucose (mmol/l)	5.61 ± 0.82	5.32 ± 0.62	**0.002**
Insulin (mU/l)	7.94 ± 8.34	7.45 ± 4.85	0.572
HOMA IR	2.03 ± 2.29	1.85 ± 1.49	0.469
CRP (mg/l)	2.85 ± 7.73	2.09 ± 5.44	0.399
Hcy (μmol/l)	12.47 ± 5.23	12.45 ± 4.86	0.976
ANGPTL3 (μg/l)	347.88 ± 159.43	388.60 ± 204.12	0.080
ANGPTL4 (μg/l)	20.70 ± 25.77	16.33 ± 20.62	0.142

P-values under 0.05 were considered significant and highlighted in the table using bold font.

To test the relationship between ANGPTL4 and BMI, we performed partial correlation analyses using as control variables age, WHR, waist circumference, FFA, CRP, and Hcy, all related to serum levels of ANGPTL4. When the data was then adjusted for age, FFA, and waist circumference, serum ANGPTL4 displayed inverse correlations with BMI (r = -0.172; P = 0.008). In accordance with this, we observed that in subjects in the age range of 30–45 years the levels of ANGPTL4 were significantly decreased (P = 0.03) in overweight subjects (8.8 ± 1.3 ng/ml, mean ± SEM, n = 23) as compared with values obtained in normal-weight subjects (17.5 ± 2.9 ng/ml, mean ± SEM, n = 41)

TABLE 2. Correlations of ANGPTL 3 and 4 with clinical and biochemical parameters

Parameter	Correlations with ANGPTL3		Correlations with ANGPTL4	
	Pearson Correlation	Significance (2-tailed)	Pearson Correlation	Significance (2-tailed)
Age (years)	**0.292**	**<0.001**	**0.178**	**0.005**
BMI (kg/m^2)	−0.071	0.263	0.061	0.343[a]
WHR	−0.077	0.231	**0.155**	**0.016**
FFA (μmols/l)	0.099	0.118	**0.129**	**0.044**
TC (mmol/l)	0.040	0.527	−0.046	0.471
TG (mmol/l)	**-0.182**	**0.004**[b]	−0.049	0.441
LDL-C (mmol/l)	0.040	0.527	−0.046	0.471
apoB (g/l)	−0.122	0.054	−0.092	0.152
HDL-C (mmol/l)	**0.224**	**<0.001**	0.048	0.457
apoA-I (g/l)	**0.144**	**0.023**	0.011	0.860
apoB/apoA-I	**-0.192**	**0.002**	−0.081	0.208
Glucose (mmol/l)	−0.006	0.931	0.046	0.470
Insulin (mU/l)	0.083	0.191	−0.028	0.664
HOMA IR	0.070	0.270	−0.037	0.560
CRP (mg/l)	−0.002	0.978	**0.177**	**0.008**
Hcy (μmol/l)	−0.105	0.096	**-0.136**	**0.034**

P-values under 0.05 were considered significant and highlighted in the table using bold font.

[a] When the data was adjusted for age, waist circumference, and FFA a negative correlation of serum ANGPTL4 and BMI was observed (r = −0.172; P = 0.008).

[b] If HDL-C and apoA-I levels were used as control variables the observed correlation of ANGPTL3 with triglycerides is completely lost (r = −0.029, P = 0.649).

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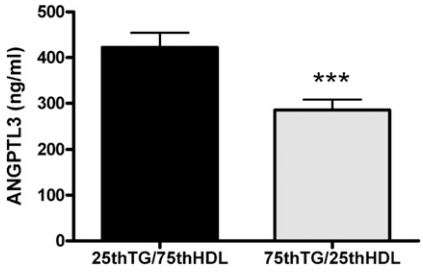

Fig. 2. Serum ANGPTL3 concentration in subjects classified according to HDL-C and TG levels. The study population was divided in quartiles based on TG and HDL-C levels. Levels of ANGPTL3 were compared in subjects with high TG and low HDL-C (75th TG/25th HDL, n = 36) and in subjects with low TG and high HDL-C (25th TG/ 75th HDL, n = 33). The difference in ANGPTL3 levels between these two groups was highly significant ($P < 0.0001$) as measured by the Mann Whitney test. The values represent mean ± SEM.

(**Fig. 3**). No differences in physical activity between the normal-weight subjects (37.2% had ideal and sufficient physical activity) and overweight subjects (39.2% had ideal and sufficient physical activity) were observed. No significant differences were observed in the other age groups (group 2: 45–60 years, $P = 0.62$; group 3: 60–94 years, $P = 0.28$).

No correlation between the serum levels of ANGPTL 3 and 4 was evident.

Effect of heparin on human ANGPTL 3 and 4 in vivo and in vitro

To test whether ANGPTL 3 and 4 interact with the endothelial surface, we quantified both proteins in pre- and post-heparin plasma. Kinetic measurements revealed that 5–15 min after heparin injection are optimal to assess the release of ANGPTL4 (**Fig. 4A**). Injection of 100 IU/kg body weight heparin increased the levels of both ANGPTL 3 and 4 in post-heparin plasma from all subjects studied (Fig. 4B, C).

To test whether ANGPTL4 interact with the surface proteoglycans in vitro, we tested the effect of adding heparin to the growth medium of Huh7 cells overexpressing ANGPTL4. Addition of 100 IU/ml heparin to the culture medium induced an increase in the release of ANGPTL4 to the culture medium and a concomitant decrease of cellular ANGPTL4 (**Fig. 5A**, B). Western blot analyses showed

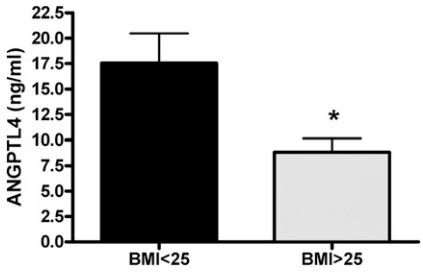

Fig. 3. ANGPTL4 concentration in normal and overweight subjects. ANGPTL4 levels were compared in subjects, age 30–45 years, with normal weight (BMI < 25, n = 41) or overweight (BMI > 25, n = 23). The values represent mean ± SEM. Significance was tested using the Mann Whitney test ($P = 0.03$).

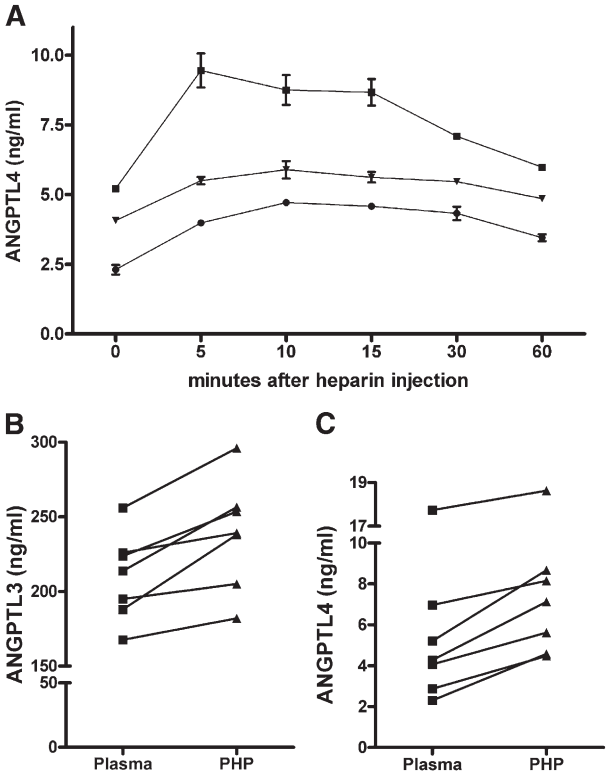

Fig. 4. ANGPTL 3 and 4 in pre- and post-heparin plasma. ANGPTL4 was measured in plasma collected at baseline (0 minutes) and 5, 10, 15, 30, and 60 min after a heparin injection (100 IU/kg body weight) from three subjects (A). ANGPTL3 (B) and ANGPTL4 (C) were measured in preheparin plasma (Plasma) and in post-heparin plasma (PHP) collected 15 min after the injection of heparin (100 IU/kg body weight) from 7 different subjects.

that the transient overexpression of ANGPTL4 in Huh7 cells for 48 h resulted in the release of full length, C- and N-terminal fragments of ANGPTL4 into the growth medium (Fig. 5B). Addition of heparin to the growth medium resulted in increased release of all three forms of ANGPTL4 to the medium after 48 h (Fig. 5C). Following shorter incubation times (1, 2, and 3 h) no indication of ANGPTL4 cleavage, neither in the medium nor in the cells, could be demonstrated using Western blot (data not shown).

To further verify the interaction between ANGPTL 3 and 4 with heparin, we analyzed human plasma using heparin affinity chromatography. Human plasma containing 83 ng/ml ANGPTL3 and 4.6 ng/ml ANGPTL4 were recycled overnight on the column at + 4°C. Using the ELISAs, we quantified ANGPTL 3 and 4 in the nonbound fraction and fractions eluted with 0.5M, 1M and 2M NaCl. The percentage of recovery was 98% for ANGPTL3 (10% in the nonbound fraction and 88% in 0.5 M NaCl fraction) and 64% for ANGPTL4 (12% in the nonbound fraction, 50% in 0.5 M NaCl fraction and 3% in 1 M NaCl fraction) (**Fig. 6**).

DISCUSSION

The present study reports on the serum concentrations of ANGPTL 3 and 4 in a Finnish human population sample using quantitative ELISAs. Our measurements were in

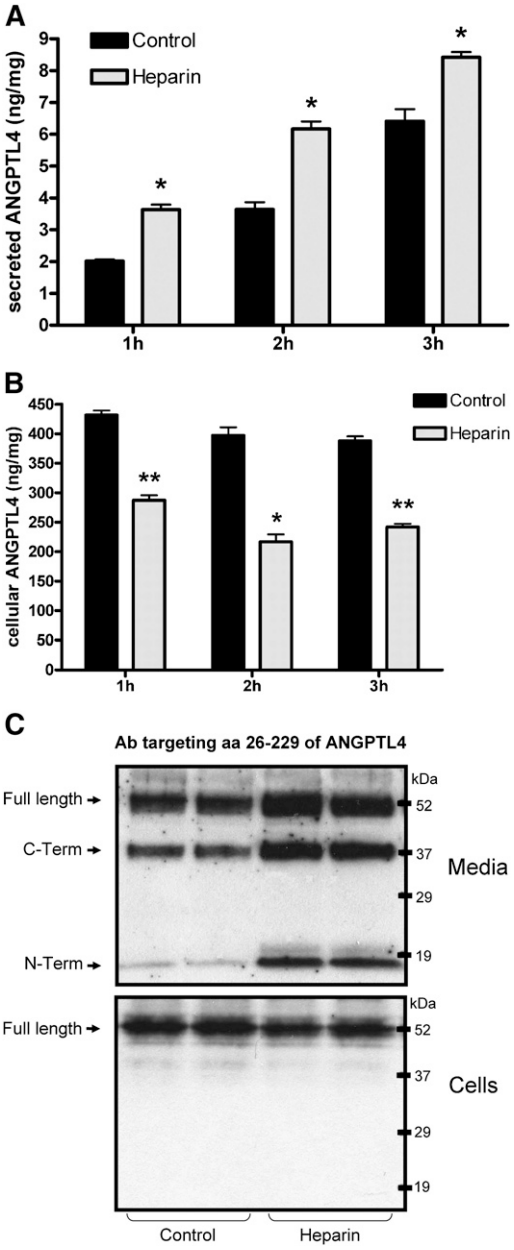

Fig. 5. Effect of heparin on the release of ANGPTL4 from Huh7 cells. The release of ANGPTL4 from Huh7 cells overexpressing human ANGPTL4 was quantified by ELISA in the medium (A) and cells (B) grown in the absence (Control) or presence of 100 IU/ml heparin (Heparin) for 1, 2, and 3 h. Bars represent mean ± SD of triplicate samples. C: Analysis of growth medium and cells by immunoblot to evaluate the effect of heparin after 48 h on the release of ANGPTL4 from Huh7 cells overexpressing human ANGPTL4 protein using a polyclonal anti-ANGPTL4 antibody.

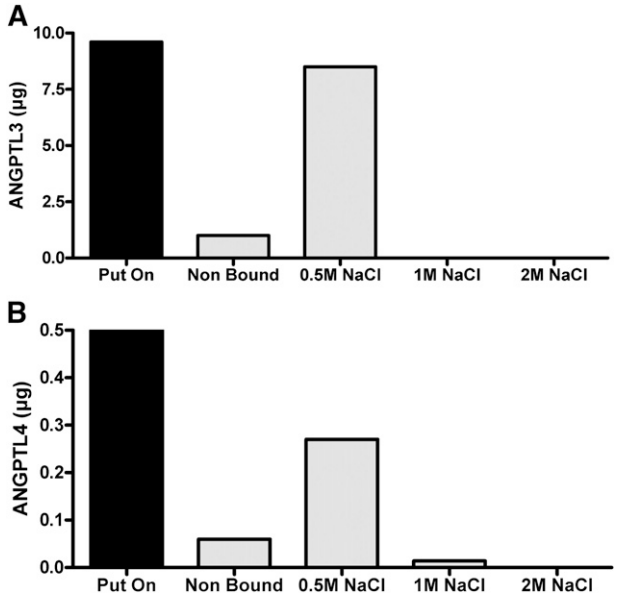

Fig. 6. Interaction of ANGPTL 3 and 4 with heparin. Human plasma was subjected to heparin affinity chromatography and stepwise eluted with buffer containing increasing concentrations of NaCl. ANGPTL3 (A) and ANGPTL4 (B) were measured by ELISA in plasma loaded to the column (Put On), in the nonbound fractions (Non Bound) and in elution fractions with increasing salt concentrations (0.5M NaCl, 1M NaCl and 2M NaCl). Bars represent the total amount of ANGPTL 3 and 4.

good agreement with previous reports showing that ANGPTL3 is present in the circulation at a higher concentration (224 ng/ml) compared with ANGPTL4 (1.73 ng/ml) (22, 23). Although some differences exist in the values reported for ANGPTL 3 and 4, these probably are due to differences in method standardization, population samples, and/or the antibodies used. Based on our ANGPTL3 and 4 determinations, major findings of our study are: *a*) low levels of ANGPTL3 are associated with high circulating TG and low HDL-C, *b*) low levels of ANGPTL4 are associated with increased body weight, and *c*) both ANGPTL 3 and 4 are increased in post-heparin plasma.

Genetic and in vitro studies have suggested an inhibitory effect of human ANGPTL 3 and 4 on LPL activity and thereby, have an increasing effect on circulating TG levels (9–11). One therefore could expect that high levels of ANGPTL 3 and 4 in the circulation would be associated with high TG levels. Our observation that serum ANGPTL 3 and 4 are not positively correlated with fasting TG concentration was therefore somewhat surprising. However, this cannot exclude that ANGPTL 3 and 4 would not inhibit LPL in humans. A possible explanation for the lack of correlation between ANGPTL 3 and 4 and TG levels could be that the functional fractions of these proteins do not reside in the circulation but may exist bound to the endothelial surface that is the actual site for LPL-mediated lipolysis of TG-rich lipoproteins (24). LPL is synthesized and secreted via adipocytes, macrophages, and muscle cells and thereafter associated with the vascular endothelium through heparin sulfate proteoglycans and the glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 from where it can be released in circulation with heparin treatment (25, 26). We demonstrated here that intravenous injection of heparin also induces release of ANGPTL 3 and 4 and that the levels of circulating and endothelial bound ANGPTLs may differ between individuals. There are several basic residue-rich regions in ANGPTL 3 and 4, both in N- and C-terminal regions that could mediate the binding of these proteins at the sites important for LPL activity. For ANGPTL3,

mutations in these residues have been associated with a loss-of-function in vivo and in vitro (11, 27). The ANGPTL 3 and 4 binding capacity to negatively charged residues was confirmed by heparin affinity chromatography. The affinity to negatively charged residues was also observed in cultured hepatocytes where the addition of heparin to culture medium of cells transfected with human recombinant ANGPTL4 increased the release of protein. Our results are in agreement with the data previously reported (28). Further studies are needed to verify whether the levels of ANGPTL 3 and 4 released after heparin treatment relate with LPL activity and fasting TG levels.

Increased levels of TG and low levels of HDL-C are two important features of the metabolic syndrome and also strong predictors of coronary heart diseases (29, 30). Despite intensive effort, to date there is still an unsolved question regarding the mechanism by which these two parameters are linked. In the present study, we observed that ANGPTL3 is negatively correlated with TG and positively correlated with HDL-C. Although it is surprising that ANGPTL3 is actually inversely correlated with TG, an explanation can be found after HLD-C and apoA-I are used as control variables to test this association. Partial correlations in this setting showed a complete loss of correlation between ANGPTL3 and TG, suggesting that this correlation is dependent on the effect of ANGPTL3 on HDL levels. This connection is probably facilitated via the inhibition of EL (13). When the levels of ANGPTL3 in subjects with high TG and low HDL-C were compared with those in subjects with low TG and high HDL-C, we observed a highly significant difference between these two groups. Accordingly, the data suggest that ANGPTL3 may be a factor that links TG and HDL-C metabolisms in humans. However, it is worth note that correlation does not mean causality.

The role of ANGPTL4 in obesity was reported in several studies performed in mouse models. Germ free mice are protected against diet-induced obesity, an effect due to an increased expression of intestinal ANGPTL4 that normally is suppressed by gut microbiota (14). Also, ANGPTL4 has been reported to be a powerful signal from fat and other tissues to prevent fat storage and stimulate fat mobilization (15). We now report that in humans also there is an inverse association of serum ANGPTL4 levels with body weight, especially in a younger population (i.e., 30–45 years old). This difference could not be explained by differences in physical activity. Furthermore, a decreased serum ANGPTL4 in overweight subjects was also observed in another population sample of young Finnish adults (data to be published). This association can be related to increased LPL activity in adipose tissue. Although the effect of ANGPTL4 on LPL activity is well established, this effect probably only partially accounts for the variations in adiposity for at least two reasons. First, we did not observe any correlations between ANGPTL4 and TG levels and second, mutations affecting the ability of ANGPTL4 to inhibit LPL activity do not reflect any variation of body weight (11). Another interesting mechanism by which low levels of ANGPTL4 can induce an increase in adiposity would be the effect of ANGPTL4 on lymphatic blood vessels in adipose tissue. Indeed, ANGPTL4 is required for a normal development of lymphatic system in mice (31). It will be of major interest to study whether elevated levels of ANGPTL4 maintain the integrity of lymphatic vessels and stimulate drainage of lipids from adipose tissue.

Additionally to the aforementioned points, careful data analyses reveal a significant correlation of ANGPTL4 with age, WHR, plasma FFA, CRP, and Hcy. The positive association between FFA and plasma ANGPTL4 was previously reported (21, 23). Our data confirm these results and strengthen them via the observed association with WHR. The strong relationship of circulating FFA with upper-body obesity is well known (32). Furthermore, we report that ANGPTL4, as well as ANGPTL3, increase with age. This is very interesting because it is recognized that there is a selective decrease in the β-adrenergic lipolytic capacity with aging (33). We cannot exclude the possibility that an increased synthesis of ANGPTL3 and 4 in adipose tissue would compensate for the decreased mobilization of fat in older individuals. In fact, this could explain why there is no significant decrease of ANGPLT4 in overweight subjects over 45 years old as observed in overweight subjects under 45 years old. The inverse relationship of ANGPTL4 with Hcy is not surprising as ANGPTL4 was shown to decrease key enzymes in the methionine/Hcy metabolic cycle (34). The positive correlation with CRP suggests that inflammatory processes may be connected to increased ANGPTL4 levels in humans.

Although our study provides important and novel information, it is also subjected to limitations because our sample size was small, we did not determine the tissue-specific protein levels, and we did not measure the concentrations of N- and C-terminal fragments for ANGPTL 3 and 4.

To conclude, we have developed and validated two quantitative ELISAs to measure human ANGPTL 3 and 4. Using these methods, we have demonstrated that a decrease of serum ANGPTL3 is associated with a decrease in HDL-C and an increase in TGs whereas low serum ANGPTL4 is associated with increased body weight. These observations further strengthen the important role of ANGPTL 3 and 4 in the modulation of serum TG and HDL levels as well as in human obesity.

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