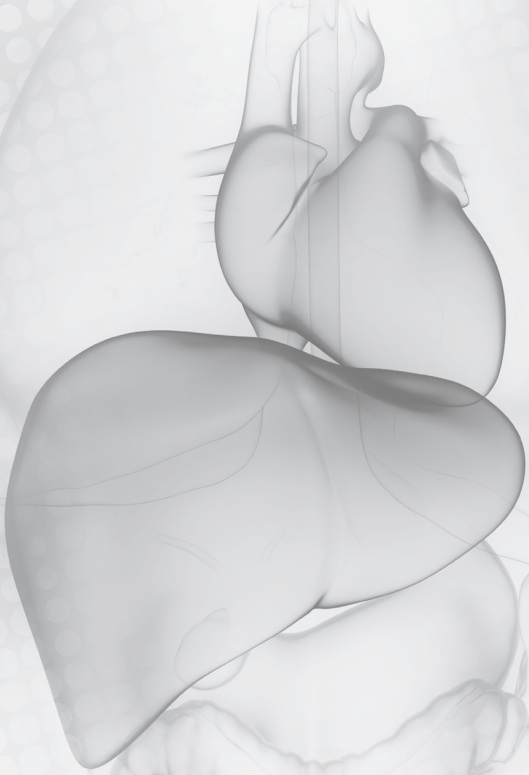
Chapter 9

Dietary modulation of plasma angiopoietin-like protein 4 levels in healthy volunteers and in patients with type 2 diabetes



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ABSTRACT

Background

Angiopoietin-like protein 4 (Angptl4) has been identified as an inhibitor of lipoprotein lipase. Preliminary data suggest that plasma non-esterified fatty acids (NEFA) raise plasma Angptl4 levels in humans. The aim of this study is to assess plasma Angptl4 levels after various nutritional interventions that increase NEFA levels, in healthy subjects and patients with type 2 diabetes mellitus.

Methods

We studied 4 groups, both at baseline and after 3 days of either fasting (n=22 healthy males), very low calorie diet (VLCD, n=10 healthy males and n=10 diabetes patients) or high fat high energy diet (HFED, n=15 healthy males), respectively. Plasma Angptl4, NEFA and triglyceride (TG) levels were measured.

Results

In healthy males, VLCD increased Angptl4 from 13.2 (interquartile range 8.1-24.2) at baseline to 18.2 (16.7-33.4) ng/mL ($P<0.05$), fasting increased Angptl4 from 10.6 (7.6-17.6) to 28.0 (23.1-35.0) ng/mL ($P<0.05$), and HFED increased Angptl4 from 13.9 (8.2-22.0) to 17.2 (11.2-23.6) ng/mL ($P<0.05$). In males with diabetes, VLCD also increased Angptl4 from 10.9 ± 2.4 to 19.2 ± 3 ng/mL ($P<0.05$). All interventions significantly increased plasma NEFA in both healthy males and patients with diabetes. The change in Angptl4 is positively correlated with the change in NEFA levels (ß=0.048, $P<0.001$).

Conclusion

Three days of either fasting, VLCD or HFED in healthy males all increase plasma Angptl4 levels, associated with increased plasma NEFA levels. Similarly, VLCD in patients with diabetes increased Angptl4 levels, associated with increased NEFA levels.

INTRODUCTION

Angiopoietin-like protein 4 (Angptl4) belongs to the family of angiopoietins and angiopoietin-like proteins that are characterized by the presence of an N-terminal coiled domain and a C-terminal fibrinogen-like domain (1). Angptl4 is involved in a variety of functions including angiogenesis and lipoprotein metabolism. Evidence abounds indicating that Angptl4 serves as a potent inhibitor of the enzyme lipoprotein lipase (LPL) that hydrolyzes triglycerides (TG) from apoB-containing lipoproteins, chylomicrons and VLDL. Via this mechanism, Angptl4 suppresses release of non-esterified fatty acids (NEFA) and their subsequent uptake by underlying tissues such as adipose tissue, skeletal muscle and the heart (2-6). In addition, Angptl4 increases intracellular lipolysis of TG within adipocytes, thereby raising plasma NEFA levels (6-8). Expression of Angptl4 in a variety of tissues has been shown to be governed by the lipid sensing peroxisome proliferator-activated receptors (PPAR) α, ß and γ, and is stimulated *in vitro* by fatty acids and *in vivo* by fasting.

Data on the determinants of plasma Angptl4 levels in humans are still scarce as there were no commercial assays for determination of Angptl4 available until recently. Limited evidence indicates that fasting may increase plasma Angptl4 levels (9). In addition to its functions in normal physiology, Angptl4 also seems to play a relevant role in the metabolic syndrome and type 2 diabetes mellitus, both of which are associated with dyslipidemia, including hypertriglyceridemia and high levels of NEFA. In db/db diabetic mice, overexpression of Angptl4 induces hypertriglyceridemia and hepatic steatosis, while at the same time reducing blood glucose and hepatic glucose output (10). Recent studies found that intracerebroventricular administration of Angptl4 in mice suppresses food intake and decreases body weight. Accordingly, Angptl4-deficient mice have greater food intake after a fast than controls (11). To our knowledge, the effect of increased NEFA levels on Angptl4 levels in patients with diabetes is still unknown.

We hypothesized that an increase in plasma NEFA levels would increase plasma Angptl4 levels in healthy subjects. Therefore the objective of the present study is to determine the effects of nutritional interventions, resulting in increased plasma NEFA levels, on Angptl4 levels in healthy subjects. To that end, we nutritionally intervened with very low calorie diet (VLCD), fasting, or high fat high energy diet (HFED), which cause either a moderate (VLCD, HFED) or large (fasting) increase in plasma NEFA levels. In addition, we aimed to study whether a VLCD in patients with diabetes, that results in increased plasma NEFA levels, also increases plasma Angptl4.

METHODS

Study A

We included 22 healthy males (Table 1). Blood samples were drawn at baseline and after 3 days of fasting (only drinking of water was allowed). Baseline measurements were performed while subjects used their regular diet.

Study B

Ten males out of the 22 males who participated in study A also underwent a third study day (12). They were additionally studied after a 3-day very low calorie diet (VLCD) (Table 1). The VLCD consisted of 471 kcal/day (Modifast Intensive, Nutrition & Santé Benelux, Breda, The Netherlands). The order of the 3-day VLCD and the 3-day fasting period was determined by balanced assignment, with a minimum of 14 days between the interventions.

Study C

Fifteen healthy males were included (13, Table 1). Volunteers were studied on two occasions. The first visit was at baseline on a regular diet with abstination from alcohol for 3 days (mean intake 2100 kcal/day). The second visit was after a 3-day high fat, high energy diet (HFED). This consisted of the baseline diet, with the addition of 800 mL cream per day (mean intake 4732 kcal/day with 69% fat).

Study D

Ten males with well-controlled type 2 diabetes mellitus were included (14, Table 1). In the original protocol 11 patients were included, but from one patient blood samples were missing for present analysis. All patients were on stable doses of metformin and glimepiride; patients using other anti-diabetic drugs were excluded. Other exclusion criteria were a history of, or present cardiac disease, any other endocrine disease, or hepatic or renal disease. Patients were studied on two study days, with a minimum of 14 days between the study days. Baseline measurements were performed, while patients used their regular diet with abstination from alcohol during 3 days. Secondly, patients were studied after a 3-day VLCD (471 kcal/ day, Modifast Intensive, Nutrition & Santé Benelux, Breda, The Netherlands).

Inclusion criteria for studies A, B and C were: healthy males, age >18 years and body mass index (BMI) <30 kg/m^2. Exclusion criteria were: smoking, any renal, hepatic or endocrine disease, any abnormal laboratory results found during screening and any use of medication (except NSAIDs). Studies B, C and D were previously described in detail (12-14) and are summarized here. Women were excluded from these intervention studies, to exclude any effect of hormonal status or contraceptive use on lipid metabolism. All volunteers in these studies signed written informed consent and all studies have been approved by the local ethical committee.

Table 1. Study summaries and baseline characteristics

Study	A	B	C	D
n	22 males	10 males	15 males	10 males
Health status	healthy	healthy	healthy	diabetes type 2
Intervention	fasting (3d)	VLCD (3d)	HFED (3d)	VLCD (3d)
Age (years)	23 ± 1	24 ± 2	25 ± 2	58 ± 2
BMI (kg/m^2)	23.0 ± 0.5	23.6 ± 0.9	23.4 ± 0.7	26.7 ± 1.0
HbA1c (%)	4.8 ± 0.1	4.4 ± 0.1	4.4 ± 0.1	6.1 ± 0.2

Data are mean ± SEM. HFED, high fat, high energy diet; VLCD, very low calorie diet; 3d, 3 days

Assays for plasma glucose, insulin, triglycerides and non-esterified fatty acid levels

The last meal was used at least four hours before blood sampling and data collection. Plasma concentrations of glucose and triglycerides were measured on a fully automated P800 analyzer (Roche, Almere, The Netherlands). Insulin concentrations were measured using an Immulite 2500 random access analyzer with a chemo luminescence immunoassay (DPC, Los Angeles, CA, USA). Non-esterified fatty acids were measured by a commercial kit (NEFA-C; Wako Chemicals, Neuss, Germany). Coefficients of variation were <2% (glucose and TG), <5% (insulin), and <8% (NEFA).

Angptl4 assay

Angptl4 was measured as detailed previously (9). Briefly, 96-well plates were coated with anti-human Angptl4 polyclonal goat IgG antibody (AF3485, R&D Systems) and incubated overnight at 4°C. Plates were washed extensively between each step. After blocking, 100 µl of 20-fold diluted human plasma was applied, followed by 2 h incubation at room temperature. A standard curve was prepared using recombinant human Angptl4 (3485-AN, R&D Systems) at 0.3 to 2.1 ng/well. Next, 100 µl of diluted biotinylated anti-human Angptl4 polyclonal goat IgG antibody (BAF3485, R&D Systems) was added for 2 h, followed by addition of streptavidin-conjugated horseradish peroxidase for 20 min, and tetramethyl benzidine substrate reagent for 6 min. The reaction was stopped by addition of 50 µl of 10% H_2SO_4, and the absorbance was measured at 450 nm.

Statistical analyses

Data are expressed as means ± standard error of the mean (SEM) or median (interquartile range) if not normally distributed. In studies A and C comparisons between baseline and post-intervention measurements were performed with paired t-tests or Wilcoxon signed rank tests, depending on the distribution. In study B comparisons between the three measurements were analyzed with a general linear model for repeated measures, with time as within-subject factor. LSD post-hoc tests were used in case of a significant F-ratio. For correlation analysis, Pearson's correlation coefficients were used. A random effects model was used to analyze the pooled data from study A, B and C and to adjust for clustering at the patient

level. The plots were created with GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, USA). All statistical analyses were performed using SPSS 17.0 (SPSS Inc. Chicago, Illinois, USA). A P-value of 0.05 or less was considered statistically significant.

RESULTS

The baseline characteristics of all participants in the studies are described in Table 1.

Study A

Complete fasting in healthy subjects decreased plasma glucose levels from 4.5 ± 0.1 to 3.8 ± 0.1 mmol/L (P<0.05) and decreased insulin levels from 6 (3-7) to 2 (2-3) mU/L (P<0.05) (Table 2). Fasting increased plasma NEFA levels from 0.54 ± 0.06 to 1.50 ± 0.13 mmol/L (P<0.05) and increased Angptl4 levels from 10.6 (7.6-17.6) to 28.0 (23.1-35.0) ng/mL (P<0.05), without changing TG levels.

Study B

VLCD in healthy subjects decreased plasma glucose levels from 5.0 ± 0.1 to 4.3 ± 0.1 mmol/L (P<0.05 vs. baseline, Table 2), but did not significantly change insulin levels. VLCD increased plasma NEFA levels from 0.59 ± 0.11 to 1.23 ± 0.41 mmol/L (P<0.05 vs. baseline) and increased Angptl4 levels from 13.2 (8.1-24.2) to 18.2 (16.7-33.4) ng/mL (P<0.05 vs. baseline). VLCD decreased triglyceride levels (1.3 ± 0.1 to 0.9 ± 0.1, P<0.05 vs. baseline).

Study C

The HFED did not significantly change plasma glucose levels (Table 2). HFED increased plasma insulin levels from 9 ± 1 to 21 ± 2 mU/L (P<0.05) and NEFA levels increased from 0.54 ± 0.07 to 0.92 ± 0.08 mmol/L (P<0.05). HFED increased plasma TG levels from 1.3 ± 0.1 to 2.9 ± 0.3 mmol/L (P<0.05), and plasma Angptl4 from 13.9 (8.2-22) to 17.2 (11.2-23.6) ng/mL (P<0.05).

Study D

VLCD in patients with diabetes decreased plasma glucose (6.2 ± 0.4 to 5.2 ± 0.3 mmol/L; P<0.05), insulin (7 ± 1 to 3 ± 1 mU/L; P<0.05) and triglyceride levels (2.2 ± 0.4 to 1.3 ± 0.2 mmol/L; P<0.05). VLCD increased plasma NEFA levels from 0.56 ± 0.09 to 0.90 ± 0.14 mmol/L (P<0.05) and increased plasma Angptl4 levels from 10.9 ± 2.4 to 19.2 ± 3.2 ng/mL (P<0.05).

Table 2. Effects of dietary interventions on plasma parameters in healthy volunteers (A, B and C) and in patients with type 2 diabetes (D)

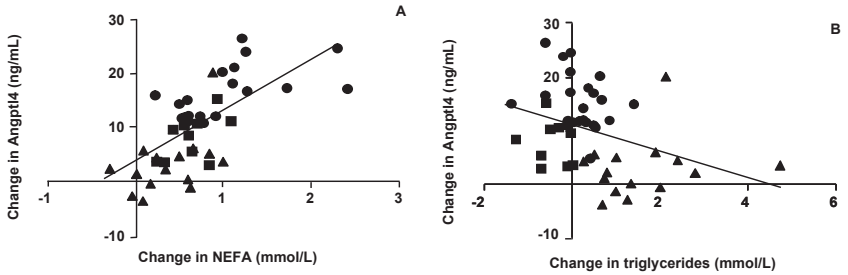
	Study A		Study B		Study C		Study D	
	Baseline	Fasting (3d)	Baseline	VLCD (3d)	Baseline	HFED (3d)	Baseline	VLCD (3d)
Glucose (mmol/L)	4.5 ± 0.1	3.8 ± 0.1*	5.0 ± 0.1	4.3 ± 0.1*	4.9 ± 0.1	5.0 ± 0.1	6.2 ± 0.4	5.2 ± 0.3*
Insulin (mU/L)	6 (3-7)	2 (2-3)*	10 ± 2	8 ± 1	9 ± 1	21 ± 2*	7 ± 1	3 ± 1*
NEFA (mmol/L)	0.54 ± 0.06	1.50 ± 0.13*	0.59 ± 0.11	1.23 ± 0.41*	0.54 ± 0.07	0.92 ± 0.08*	0.56 ± 0.09	0.90 ± 0.14*
Triglycerides (mmol/L)	1.1 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	0.9 ± 0.1*	1.3 ± 0.1	2.9 ± 0.3*	2.2 ± 0.4	1.3 ± 0.2*
Angptl4 (ng/mL)	10.6 (7.6-17.6)	28.0 (23.1-35.0)*	13.2 (8.1-24.2)	18.2 (16.7-33.4)*	13.9 (8.2-22.0)	17.2 (11.2-23.6)*	10.9 ± 2.4	19.2 ± 3.2*

Data are mean ± SEM or median (interquartile range). VLCD, very low calorie diet; HFED, high fat, high energy diet; 3d, 3 days. * P<0.05 vs. baseline

Pooled data of studies A, B and C

We pooled the data of nutritional intervention in healthy males (studies A, B and C) and observed a significant positive correlation between the change in plasma Angptl4 and plasma NEFA levels as induced by the three short-term interventions (ß=0.048, P<0.001, Figure 1A). In contrast, we observed a negative correlation between the change in plasma Angptl4 and plasma TG levels (ß=-0.051, P=0.01, Figure 1B).

Figure 1. Correlations between changes in plasma Angiopoietin-like protein 4 (Angptl4) and changes in plasma non-esterified fatty acids (NEFA) and plasma triglycerides in healthy males



Pooled analysis of changes in plasma NEFA and Angptl4 (adjusted ß=0.048, P<0.001) (A) and changes in plasma triglycerides and Angptl4 (adjusted ß=-0.051, P=0.01) (B) as induced by 3 short-term dietary interventions in healthy males: 3-day fasting (circles), 3-day very low calorie diet (squares) and 3-day high fat high energy diet (triangles)

DISCUSSION

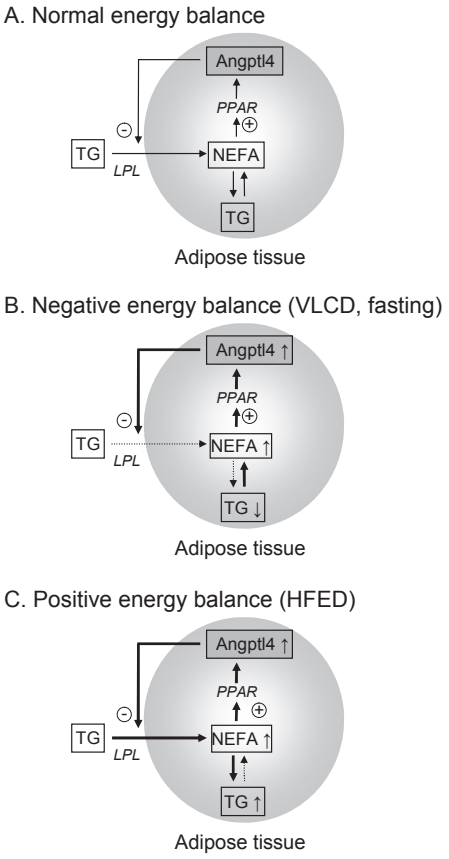
This study shows that three days of fasting in healthy subjects induces a more than 2-fold increase in plasma NEFA levels with a concomitant increase in plasma Angptl4 levels. This extends the recent finding by Kersten et al. (9) who found an increase in Angptl4 after 24 and 48 hours of fasting in 4 healthy subjects. In addition, the current studies indicate that nutritional interventions associated with more modest increases in NEFA levels than fasting also increase Angptl4 levels. A VLCD, compared to 3 days of total fasting, led to lesser but still significant increases in both NEFA and Angptl4 levels. Moreover, we found a positive correlation between the increase in Angptl4 and increase in plasma NEFA after dietary interventions.

A negative energy balance induced by fasting or by VLCD increases the hydrolysis of TG stored within white adipose tissue, thereby liberating NEFA that are subsequently released into the plasma for use as energy source by tissues such as skeletal muscles and heart. Since activation of the PPARs (types α, β and γ) can induce Angptl4 expression in human adipocytes (15), we postulate that increased intracellular fattt acid levels may, via activation of PPARs, stimulate transcription of Angptl4 that is then released into the plasma. Likewise, PPAR activation may also account for induction of Angptl4 expression in tissues where NEFA are subsequently taken up including skeletal muscle and heart (Figure 2) (16).

In mice, overexpression of Angptl4 not only increases plasma NEFA levels, but also increases TG levels, due to a suppressed VLDL-TG clearance (2,4,6). *In vitro* and *in vivo* studies have shown that Angptl4 promotes the conversion of lipolytically active LPL dimers to inactive LPL monomers (5), thereby reducing the enzymatic activity of LPL (4) which can explain the reduction in VLDL-TG clearance. In our study we did not find an increase in plasma TG levels after fasting or VLCD despite the increase in plasma Angptl4 levels. Instead, when we pooled the data from all our dietary intervention studies in healthy men, a negative correlation was found between changes in plasma Angptl4 and changes in plasma TG levels. Previous studies did not show a cross-sectional association between Angptl4 and plasma TG in humans (17,18). It is conceivable that plasma Angptl4 levels, which are determined by the net balance between production and clearance of the protein, may not reflect the actual concentration at the level of LPL and do not provide insight on its biological function as an inhibitor of LPL activity. In addition, dietary interventions aimed at disturbing energy balance evidently influence TG metabolism through a plethora of Angptl4-independent effects that may overshadow the LPL-inhibitory effect of Angptl4.

Next, we assessed whether elevation of NEFA resulting from a different mechanism, i.e. increased exogenous lipid supply by HFED, would also be associated with an increase in Angptl4. A 3-day HFED in healthy volunteers increased plasma NEFA and Angptl4 levels. We hypothesize that the increased supply of exogenous triglycerides, increased the flux of plasma TG-derived NEFA into adipose tissue, as well as other tissues including liver and skeletal muscle, resulting in an increased intracellular NEFA concentration. These NEFA may

Figure 2. Simplified schematic representation of the proposed mechanism underlying increased plasma Angptl4 induced by either a negative or positive energy balance



As compared to normal energy balance (A), a negative energy balance increases intracellular TG hydrolysis in adipose tissue, resulting in increased Angptl4 expression through NEFA-induced PPAR activation (B). A positive energy balance increases the entry of plasma TG-derived NEFA into adipose tissue, also resulting in increased Angptl4 expression (C). See text for further explanation

activate PPARs, resulting in increased expression of Angptl4 that is subsequently transported into plasma, possibly to inhibit further TG-derived NEFA flux into the adipose tissue via attenuation of LPL activity (Figure 2).

Since we found that dietary interventions resulting in increased NEFA levels are correlated with an increase in Angptl4 levels in healthy subjects, we also studied the effect of an increase in NEFA levels on Angptl4 levels in patients with diabetes by intervention with VLCD. We found that patients with diabetes respond similarly as compared to healthy subjects with respect to an increase in Angptl4.

In conclusion, fasting, VLCD and HFED increase Angptl4 levels in healthy volunteers, which is correlated with increases in NEFA levels. Moreover, VLCD increases plasma Angptl4 levels in patients with diabetes, associated with increased plasma NEFA levels. Collectively, dietary interventions that increase NEFA levels also increase Angptl4 levels.

REFERENCE LIST

1. Kim I. Hepatic expression, synthesis and secretion of a novel fibrinogen/angiopoietin-related protein that prevents endothelial-cell apoptosis. Biochem. J. 2000; 346: 603-610
2. Ge H, Yang G, Yu X, Pourbahrami T, Li C. Oligomerization state-dependent hyperlipidemic effect of angiopoietin-like protein 4. J Lipid Res 2004; 45:2071-2079.
3. Lee EC, Desai U, Gololobov G, Hong S, Feng X, Yu XC, Gay J, Wilganowski N, Gao C, Du LL, Chen J, Hu Y, Zhao S, Kirkpatrick L, Schneider M, Zambrowicz BP, Landes G, Powell DR, Sonnenburg WK. Identification of a new functional domain in angiopoietin-like 3 (ANGPTL3) and angiopoietin-like 4 (ANGPTL4) involved in binding and inhibition of lipoprotein lipase (LPL). J Biol Chem 2009; 284: 13735-13745.
4. Lichtenstein L, Berbee JF, van Dijk SJ, van Dijk KW, Bensadoun A, Kema IP, Voshol PJ, Muller M, Rensen PC, Kersten S. Angptl4 upregulates cholesterol synthesis in liver via inhibition of LPL- and HL-dependent hepatic cholesterol uptake. Arterioscler Thromb Vasc Biol 2007; 27:2420-2427.
5. Sukonina V, Lookene A, Olivecrona T, Olivecrona G. Angiopoietin-like protein 4 converts lipoprotein lipase to inactive monomers and modulates lipase activity in adipose tissue. Proc Natl Acad Sci U S A 2006; 103:17450-17455.
6. Yoshida K, Shimizugawa T, Ono M, Furukawa H. Angiopoietin-like protein 4 is a potent hyperlipidemia-inducing factor in mice and inhibitor of lipoprotein lipase. J Lipid Res 2002; 43:1770-1772.
7. Sanderson LM. Peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) but not PPARalpha serves as a plasma free fatty acid sensor in liver. Mol Cell Biol 2009; 29: 6257-6267
8. Mandard S, Zandbergen F, van Straten E, Wahli W, Kuipers F, Muller M, Kersten S. The fasting-induced adipose factor/angiopoietin-like protein 4 is physically associated with lipoproteins and governs plasma lipid levels and adiposity. J Biol Chem 2006; 281:934-944.
9. Kersten S, Lichtenstein L, Steenbergen E, Mudde K, Hendriks HF, Hesselink MK, Schrauwen P, Muller M. Caloric restriction and exercise increase plasma ANGPTL4 levels in humans via elevated free fatty acids. Arterioscler Thromb Vasc Biol 2009; 29:969-974.
10. Xu A, Lam MC, Chan KW, Wang Y, Zhang J, Hoo RL, Xu JY, Chen B, Chow WS, Tso AW, Lam KS. Angiopoietin-like protein 4 decreases blood glucose and improves glucose tolerance but induces hyperlipidemia and hepatic steatosis in mice. Proc Natl Acad Sci U S A 2005; 102:6086-6091.
11. Kim HK, Youn BS, Shin MS, Namkoong C, Park KH, Baik JH, Kim JB, Park JY, Lee KU, Kim YB, Kim MS. Hypothalamic Angptl4/Fiaf is a novel regulator of food intake and body weight. Diabetes 2010; 59:2772-2780.
12. Hammer S, van der Meer RW, Lamb HJ, Schar M, de Roos A, Smit JW, Romijn JA. Progressive caloric restriction induces dose-dependent changes in myocardial triglyceride content and diastolic function in healthy men. J Clin Endocrinol Metab 2008; 93:497-503.
13. van der Meer RW, Hammer S, Lamb HJ, Frolich M, Diamant M, Rijzewijk LJ, de Roos A, Romijn JA, Smit JW. Effects of short-term high-fat, high-energy diet on hepatic and myocardial triglyceride content in healthy men. J Clin Endocrinol Metab 2008; 93:2702-2708.

14. Hammer S, van der Meer RW, Lamb HJ, de Boer HH, Bax JJ, de Roos A, Romijn JA, Smit JW. Short-term flexibility of myocardial triglycerides and diastolic function in patients with type 2 diabetes mellitus. Am J Physiol Endocrinol Metab 2008; 295:E714-E718.

15. Mandard S, Zandbergen F, Tan NS, Escher P, Patsouris D, Koenig W, Kleemann R, Bakker A, Veenman F, Wahli W, Muller M, Kersten S. The direct peroxisome proliferator-activated receptor target fasting-induced adipose factor (FIAF/PGAR/ANGPTL4) is present in blood plasma as a truncated protein that is increased by fenofibrate treatment. J Biol Chem 2004; 279:34411-34420.

16. Lichtenstein L, Kersten S. Modulation of plasma TG lipolysis by Angiopoietin-like proteins and GPIHBP1. Biochim Biophys Acta 2010; 1801:415-420.

17. Smart-Halajko MC, Robciuc MR, Cooper JA, Jauhiainen M, Kumari M, Kivimaki M, Khaw KT, Boekholdt SM, Wareham NJ, Gaunt TR, Day IN, Braund PS, Nelson CP, Hall AS, Samani NJ, Humphries SE, Ehnholm C, Talmud PJ. The relationship between plasma angiopoietin-like protein 4 levels, angiopoietin-like protein 4 genotype, and coronary heart disease risk. Arterioscler Thromb Vasc Biol 2010; 30:2277-2282.

18. Robciuc MR, Tahvanainen E, Jauhiainen M, Ehnholm C. Quantitation of serum angiopoietin-like proteins 3 and 4 in a Finnish population sample. J Lipid Res 2010; 51:824-831.