Short Communication

Angiopoietin-like protein 4: development, analytical characterization, and clinical testing of a new ELISA

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Abstract. The aim of our work was to develop an assay for the determination of angiopoietin-like protein 4 (Angplt4) in human blood, and to investigate its levels in healthy volunteers and donors suffer from metabolic syndrome. We developed and evaluated the sandwich ELISA method for the quantitative determination of human Angplt4 in serum samples. We conducted also the pilot study on individuals with metabolic syndrome or familiar hypercholesterolemia and healthy probands and measured blood pressure, waist circumference, Angplt4 serum levels, serum cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, insulin, glucose, A-FABP and calculate BMI and QUICKI insulin sensitivity index. In the study on 30 healthy volunteers we demonstrated that sex or age is not the determinant for Angplt4 serum values. Futhermore, we tested 115 individuals with metabolic syndrome and found that probands with metabolic syndrome did not differ in Angplt4 values than healthy individuals from the first study (medians 8.7 *vs*. 8.1 ng/ml, $p = 0.6$).

Individuals with metabolic syndrome did not differ in sex or age from healthy. Angplt4 values correlated with the HDL-cholesterol ($r = -0.25$; $p < 0.01$), FGF-21 ($r = 0.23$, $p < 0.01$), glucose ($r = 0.17$; $p = 0.03$), uric acid ($r = 0.17$; $p = 0.49$). lipocalin-2 ($r = 0.23$, $p < 0.01$), triacylglycerols ($r = 0.25$; $p < 0.01$) and number or characters of metabolic syndrome ($r = 0.21$; $p < 0.01$). No significant correlation was found between serum Angplt4 and BMI, WC or QUICKI. However, we performed stepwise regression and we found that Angplt4 was not an independent marker for metabolic syndrome. The patients from the metabolic syndrome group suffering diabetes mellitus ($n = 83$) did not differ in serum Angplt4 from the group of healthy patients, too.

The pilot study supports the hypothesis about the role of Angplt4 as a new class of lipid metabolism modulator. Their values could be a new key predictors of metabolic syndrome. Further research is necessary to confirm our findings in individuals with dyslipidemia, obesity, coronary artery diseases and different medication in order to assess Angplt4 value as a risk predictor of accelerated atherosclerosis.

Key words: Angiopoietin-like protein 4 — ELISA — Metabolic syndrome — Triglycerides

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Angiopoietin-like protein 4 (Angplt4) is a member of the angiopoietin-like family of proteins. Angptl3 and 4 are the only two members of this superfamily that inhibit lipoprotein lipase (LPL) aktivity. However, Angptl3 and 4 are differentially regulated at multiple levels, suggesting non-redundant functions *in vivo* (Ge et al. 2005; Feng et al. 2006; Li 2006). Angptl3 and 4 are proteolytically processed into two halves and are differentially regulated by nuclear receptors. Transgenic overexpression of Angptl4 as well as knockout of Angptl3 or 4 demonstrate that these two proteins play essential roles in lipoprotein metabolism: liver-derived Angptl3 inhibits lipoprotein lipase activity primarily in the fed state (*via* liver X receptors; Inaba et al. 2003), while Angptl4 plays important roles in both fed and fasted states. In addition, Angptl4 regulates the tissue-specific delivery of lipoprotein-derived fatty acids (Li 2006). Angptl4 -/- mice had lower triglyceride levels resulting both from increased very low-density lipoprotein (VLDL) clearance and decreased VLDL production and had modestly lower cholesterol levels (Desai et al. 2007).

LPL is a key regulator of triglyceride clearance. Its coordinated regulation during feeding and fasting is critical for maintaining lipid homeostasis and energy supply. Angptl3-deficient mice displayed hypotriglyceridemia with elevated LPL activity, but these mice showed a greater effect in the fed state. Results of studies show that Angptl3 and Angptl4 function to regulate circulating triglyceride levels during different nutritional states and therefore play a role in lipid metabolism during feeding/fasting through differential inhibition of LPL (Koster et al. 2005).

In patients with type 2 diabetes, serum levels of Angptl4 were significantly lower than those in healthy subjects, suggesting that the decreased Angptl4 could be a causative factor of this disease. These results collectively indicate that Angptl4 exerts distinct effects on glucose and lipid metabolism, and that its beneficial effect on glucose homeostasis might be useful for the treatment of diabetes (Xu et al. 2005). It is probable that Angptl4 expression is activated by ligands of all peroxisome proliferator-activated receptors, (Ge et al. 2005).

Some authors suggest that insulin downregulates Angptl4 mRNA expression *via* PI3K/Foxo1 pathway in 3T3-L1 adipocytes, and that the reduction of Angptl4 mRNA by insulin is attenuated in insulin resistence (Yamada et al. 2006).

Recently was presented that Angtpl4 might elicit its metabolic effects through modulating the mitochondria functions and methionine metabolic cycles in the liver tissue (Wang et al. 2007).

From this reasons, aim of our study was to evaluate fact about significant Angptl4 relations with other markers of metabolit syndrome and possible difference of Angptl4 in individuals with type 2 diabetes or metabolic syndrome from healthy.

We established and evaluated the immunoassay for quantitative determination of human Angptl4 in human serum and plasma.

The sandwich ELISA employs specific sheep polyclonal anti-human Angptl4 antibody provided by Biovendor and coated in microtiter wells (Corning Costar, High Binding type): 100 μl/well, 2 μg/ml in 0.1 mol/l carbonate buffer (pH 9.0) overnight at 4°C. The plate was washed once with TBS-Tw (0.05 mol/l Tris-HCl; 0.15 mol/l NaCl; pH 7.2; 0.05% (w/v) Tween 20) on the washer Columbus (Tecan). Non-specific biding sites were blocked with 300 μl/well blocking buffer (0.05 mol/l Tris-HCl; 0.15 mol/l NaCl; pH 7.2; 0.5% (w/v) bovine serum albumine; 4% sucrose) for 30 min at 25°C. After aspiration, diluted samples (serum or plasma samples diluted 3-fold with StabilZyme-HRP, SurModics Inc.) or standards were pipetted in duplicates at 100 μl/well. The plate was incubated for 1 h at 25°C. After three washes with TBS-Tw, 100 μl/well of biotin-labelled sheep polyclonal antibody (provided by Biovendor; conjugate dilution 1 : 4000 in StabilZyme-HRP) was added and the plate was incubated for 1 h at 25°C. Following three washes, 100 μl/well of streptavidin-HRP conjugate (Amdex), diluted 1 : 7000 in StabilZyme-HRP was added and the plate was incubated for 30 min at 25°C. After washing, 100 μl/well of TMB substrate (KPL, Inc.) was then added and the plate was incubated for another 10 min at 25°C. The reaction was stopped with 100 μl/well of sulfuric acid (0.2 mol/l). The developed colour was determined by reading the plate on the microplate reader MRX II (Dynex) at a wavelength of 450 nm.

As the standard we used a recombinant Angptl4 provided by Biovendor. The protein content of recombinant Angptl4 was determined by BCA method (Sigma-Aldrich) and its purity confirmed by SDS PAGE (Fig. 1A). Standards were prepared at concentrations of 60, 30, 15, 6, 3, 1.5 ng/ml (Fig. 1B) in StabilZyme-HRP and 100 μl directly pipetted into the wells.

The specificity of the assay was confirmed by testing the cross-reactivity with human recombinant Angptl3 provided by Biovendor; when no signal was observed at concentration 100 ng/ml of Anptl3.

To validate the reliability of the assay, we tested the precision and the accuracy of the assay. To analyze the spiking recovery, human serum samples from two subjects were spiked with increasing amounts of recombinant protein (+3, +12 and +24 ng/ml) and assayed. The mean recovery was 125.1%. Human serum samples from another two subjects were tested for dilution linearity. The mean recovery was 94.1%. The intraassay and interassay coefficient of variation were always less than 15%.

Our pilot study was approved by the Ethics Commission of the Hospital Šternberk, Czech Republic. A total of 30 non-obese, healthy individuals and 115 individuals with metabolic syndrome were recruited for our study.

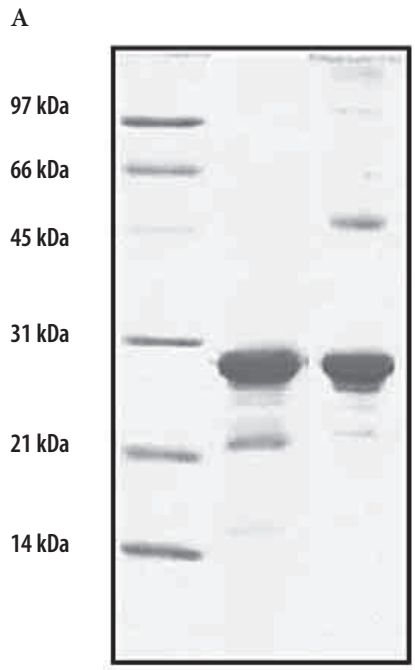

Figure 1A. Purity of recombinant human angiopoitein-like protein 4 (Angplt4) was analyzed in SDS PAGE under reducing and nonreducing conditions (12% homogenous gel, Laemli method); 3.5 μg / lane. In both lanes was used the same amount of sample.

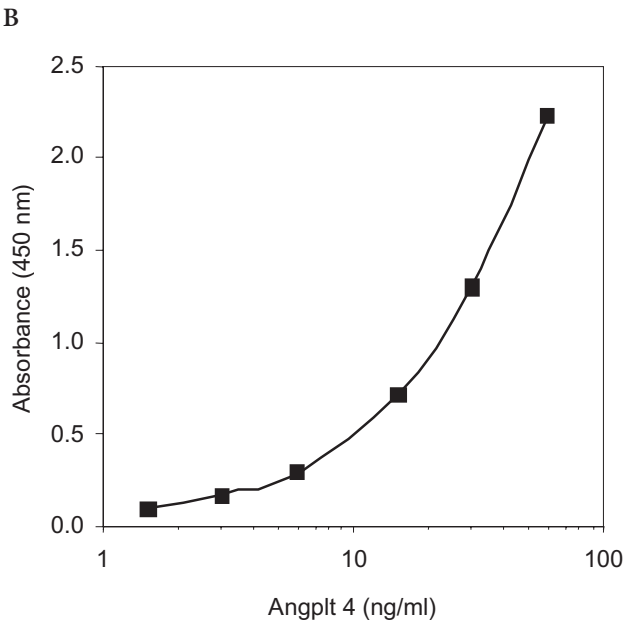

Figure 1B. The standard curve was constructed by plotting the absorbance at 450 nm of standards against *log* of the known concentration of standards, using the four-parameter algorithm. Standard curve for human angiopoietin-like protein 4 (Angplt4) is plotted as a proportion of Angplt4 concentration and absorbance at 450 nm.

The criterion of metabolic syndrome was proposed by the National Cholesterol Education Program (Adult Treatment Panel III) (Grundy et al. 2005). Metabolic syndrome was identified as the presence of three or more of following components: 1. elevated waist circumference (men – equal to or greater than 102 cm, women – equal to or greater than 88 cm); 2. elevated triglycerides (equal to or greater than 1.7 mmol/l); 3. reduced HDL cholesterol (men – less than 1.0 mmol/l, women – less than 1.3 mmol/l); 4. elevated blood pressure (equal to or greater than 130/85 mm Hg or therapy with hypotensives); 5. elevated fasting glucose (equal to or greater than 5.6 mmol/l).

Anthropometric, clinical and laboratory fasting analyses (height, weight, BMI, waist circumference (WC), systolic and diastolic pressures) were performed. Blood samples were drawn under aseptic precautions from vena cubiti, after a several-minute rest in the half-sitting position. Serum sample was separated in a cooled centrifuge at 4°C at 3000 g and subsequently frozen at –80°C. We determined Angplt4 serum levels by using the ELISA presented above (concentrations of Angplt4 were determined after defrosting the sera during the same day). In addition to Angplt4, we determined serum cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, glucose, high sensitivity CRP, creatinine, uric acid, AST, ALT (BioVendor, Advia 1650), adiponectin (BioVendor, ELISA Max002 Dynatech), FGF-21, FGF-19, ZAG, lipocalin-2 (BioVendor, Max002) and insulin (Siemens, Immulite 2000). These parameters were analysed in fresh sera. The quantitative insulin sensitivity check index (QUICKI) was calculated. Sampling was conducted in the morning after 8 h of fasting.

In the study on healthy volunteers we demonstrated no significant sex or age difference (abnormal Angplt4, abnormal distribution, men *vs*. women; medians 8.6 *vs.* 8.0 ng/ml, $p = 0.37$) (Tab. 1). Probands with a history of metabolic syndrome had no different Angplt4 serum values than healthy (abnormal distribution, medians 8.7 *vs*. 8.1 ng/ml; $p = 0.6$ (Tab. 2). Individuals with metabolic syndrome did not differ in sex or age from healthy probands. Angplt4 correlated with the HDL-cholesterol ($r = –0.25$; $p < 0.01$), FGF-21 ($r = 0.23$, $p < 0.01$), glucose ($r = 0.17$; $p = 0.03$), uric acid ($r = 0.17$;

Table 1. Sex differences of serum Angplt4 in healthy probands

	Mean	Standard deviation	Median	Normality	*p*
Men	10.37	5.5	8.6	no	–
Women	9.9	6.9	8.0	no	0.16

Normality tested with Komolgorov–Smirnov. *p*, values of probability.

Table 2. Differences in measured parameters by metabolic syndrome presence

Parameter	Metabolic syndrome	Mean	Standard deviation	Median	Normality	*p*
FGF-21 (*μ*g/l)	yes	0.99	1.77	0.50	no	0.16
	no	0.99	1.50	0.33	no	–
FGF-19 (*μ*g/l)	yes	209.80	156.60	158.60	no	<0.01
	no	478.40	848.70	242.40	no	–
Adiponectin (mg/l)	yes	14.30	14.0	10.80	no	0.69
	no	15.90	12.40	8.90	no	–
Glucose (mmol/l)	yes	7.60	3.0	6.60	no	<0.01
	no	5.60	1.70	5.30	no	–
BMI	yes	30.30	4.0	30.0	no	<0.01
	no	23.90	3.0	23.80	no	–
Total cholesterol (mmol/l)	yes	5.0	0.90	4.80	no	<0.01
	no	5.70	1.10	5.50	no	–
HDL-cholesterol (mmol/l)	yes	1.30	0.30	1.30	no	<0.01
	no	1.80	0.35	1.70	yes	–
LDL-cholesterol (mmol/l)	yes	2.90	0.70	2.80	no	0.01
	no	3.20	0.83	3.20	yes	–
Triglycerides (mmol/l)	yes	1.90	0.90	1.60	no	<0.01
	no	1.40	0.60	1.30	yes	–
Uric acid (*μ*mol/l)	yes	340.0	82.3	333.0	yes	<0.01
	no	304.3	82.1	297.0	yes	–
Insulin (mIU/l)	yes	12.80	7.20	11.50	no	0.17
	no	8.10	4.60	6.90	no	–
QUICKI	yes	0.77	0.78	0.58	no	0.049
	no	1.30	0.75	1.09	no	–
Lipocalin-2 (mg/l)	yes	87.50	36.10	83.50	yes	0.64
	no	90.70	56.70	73.3	no	–
Age (years)	yes	63.20	11.50	63.0	yes	0.88
	no	62.80	13.0	64.0	yes	–
Waist circumpherence (cm)	yes	62.80	13.0	64.0	yes	<0.01
	no	107.50	5.90	109.0	yes	–
Aspartataminotranspherase (*μ*kat/l)	yes	0.56	0.46	0.49	no	0.50
	no	0.53	0.16	0.50	no	–
Alaninaminotranspherase (*μ*kat/l)	yes	0.63	0.68	0.50	no	0.80
	no	0.48	0.25	0.44	no	–
Zinc-A2-glycoprotein (mg/l)	yes	26.20	8.10	24.90	no	0.19
	no	27.40	8.30	27.40	no	–
Creatinine (*μ*mol/l)	yes	109.40	93.70	87.50	no	0.89
	no	116.0	100.20	90.0	no	–
CRP high sensitive (mg/l)	yes	2.50	3.0	1.40	no	<0.01
	no	1.50	3.20	0.60	no	
Angplt4 (*μ*g/l)	yes	10.50	6.50	8.70	no	0.43
	no	9.70	6.90	8.10	no	

Normality tested with Komolgorov–Smirnov. CRP, C reactive protein; *p*, values of probability.

$p = 0.49$), lipocalin-2 ($r = 0.23$, $p < 0.01$), triacylglycerols ($r = 0.25$; $p < 0.01$) and number or characters of metabolic syndrome ($r = 0.21$; $p < 0.01$). No significant correlation was found between serum Angplt4 and BMI, WC or QUICKI (not shown) – presumably influences of medications *via* PPARs, etc. However, we performed stepwise regression and found HDL-cholesterol and BMI as an independent markers for metabolic syndrome presence only (F ratio 68; $p < 0.01$).

Table **3.** Differences in Angplt4 serum values by diabetes mellitus presence

Diabetes mellitus	Mean	Standard deviation	Median	Nornality	*p*
yes	10.9	7.0	8.8	no	–
no	8.20	5.80	7.90	no	0.06

Normality tested with Komolgorov–Smirnov. *p*, values of probability.

Angplt4 was not an independent marker for metabolic syndrome. Then we adjusted Angptl4 to all correlated markers and recognised that Angptl4 is not an independent marker of metabolic syndrome (not shown). The patients from the metabolic syndrome group suffering diabetes mellitus (n = 83) did not differ in serum Angplt4 from the group of healthy patients, too (Tab. 3). When study patients with metabolic syndrome were further stratified according to the number of components of metabolic syndrome, quartiles of Angplt4 serum concentrations did not differ (according to condition; Chi square 0.4; $p = 0.51$).

We believe that serum Angptl4 is a new class of lipid metabolism modulator which regulates VLDL triglyceride levels through the inhibition of LPL activity and connected with metabolic syndrome presence, still and all. Hovewer, we did not verify the diagnostic efficacy of serum Angplt4 as a marker of metabolic syndrome or adiposity. Angplt4 serum values could be a new key predictor and prognostic factor of metabolic syndrome, after all. This fact is supported by recently published paper about induction of FGF-21 through PPAR-α (Lundasen et al. 2007). Such mechanism may explain some of the efects on triglyceride and glucose metabolism reported in response to fibrate therapy in humans.

In summary, presented results demostrated the analytical competence of the ELISA Angplt4 assay and showed its usefulness for the study of metabolic syndrome.

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