Short Communication

Angiopoietin-like protein 3: 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 3 (Angptl3) in human blood, and investigate its levels in healthy volunteers and donors suffer from metabolic syndrome and familiar hypercholesterolemia. We developed and evaluated the sandwich ELISA method for the quantitative determination of human Angptl3 in serum samples. We conducted also the pilot study on individuals with metabolic syndrome or familiar hypercholesterolemia and healthy probands. The following parameters were measured: blood pressure, waist circumference, Angptl3 serum levels, serum cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, insulin, glucose, A-FABP, and BMI and Quicki insulin sensitivity index was calculated. In the study on 93 healthy volunteers we demonstrated that sex or age is not the determinant for Angptl3 serum values. Futhermore, 118 individuals with metabolic syndrome and 200 patients with familiar hypercholesterolemia were tested and it was found that probands with metabolic syndrome or familiar hypercholesterolemia had higher Angptl3 values than healthy individuals from the first study (medians 289.5 *vs*. 277.1 *vs*. 224.8 ng/ml, $p < 0.01$).

All of groups did not differ in sex or age. Angptl3 values correlated with the systolic blood pressure, LDL and A-FABP ($p < 0.05$). No connection of Angptl3 with triglycerides was found (presumably influences of statins, fibrates *via* PPARs, etc). However, we performed stepwise regression and found A-FABP and Angptl3 serum values as the independent markers for metabolic syndrome presence only (F ratio 29, $p < 0.01$). Then we adjusted Angptl3 to A-FABP (reputable metabolic syndrome marker) and recognised that Angptl3 is the A-FABP-independent marker.

The pilot study supports the hypothesis about the role of Angptl3 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, CAD and different medication in order to assess Angptl3 value as a risk predictor of accelerated atherosclerosis.

Key words: Angiopoietin-like protein 3 — ELISA — Metabolic syndrome — Triglycerides

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Angiopoietin-like protein 3 (Angptl3) is a member of the angiopoietin-like family of proteins (Angptl). Angptl3 and 4 are the only two members of this superfamily that inhibit lipoprotein lipase (LPL) activity. 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).

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).

Angptl3 was recently presented as a novel thyroid receptor β target gene and provided a new potential mechanism to explain the hypotriglyceridemic properties of thyroid receptor β agonists *in vivo* (Fugier et al. 2004).

Similar study presented that elevated Angptl3 by leptin or insulin resistance is attributed to increased plasma triglycerides and free faty acids concentrations in obesity (Shimamura et al. 2004).

Furthermore, Angptl3 activated the lipolysis to stimulate the release of free faty acids and glycerol from adipocyte, it seems that Angptl3 is a liver-derived lipolytic factor targeting on adipocyte (Kersten 2005).

We established and evaluated the immunoassay for quantitative determination of human Angptl3 in human serum and plasma.

The sandwich ELISA employs specific rabbit polyclonal anti-human Angptl3 antibody provided by Biovendor-Laboratory Medicine (Czech Republic) and coated in microtiter wells (Corning Costar, High Binding type): 100 μl/well, 3 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 binding sites were blocked with 250 μl/well 1%BSA (w/v) in TBS-Tw for 30 min at 25°C. After aspiration, diluted samples (serum or plasma samples diluted 10-fold with TBS (0.05 mol/l Tris-HCl; 0.15 mol/l NaCl; pH 7.2); 0.1% BSA (w/v); 0.01% Thimerosal) 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 rabbit polyclonal antibody (provided by Biovendor-Laboratory Medicine) was added and the plate was incubated for 1 h at 25°C. Following three washes, 100 μl/well of streptavidin-HRP conjugate (Research Diagnostic), 0.025 μg/ml in 0.5% BSA (w/v) in TBS (pH 7.2); 0.01% Thimerosal was added and the plate was incubated for 30 min at 25°C. After washing, 100 μl/well of TMB substrate (KPL) was then added and the plate was incubated for another 10–15 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 Angptl3 provided by Biovendor–Laboratory Medicine. The protein content of recombinant Angptl3 was determined by BCA method (Sigma-Aldrich) and its purity confirmed by SDS PAGE (Fig. 1A). Standards were prepared at concentrations of 400, 200, 100, 50, 25, 12.5 and 6.25 ng/ml (Fig. 1B) in TBS (pH 7.2); 0.5% BSA (w/v); 0.01% Thimerosal and 100 μl directly pipetted into the wells.

The specificity of the assay was confirmed by testing the cross-reactivity with human recombinant angiopoietin-like protein 4 (Angptl4) provided by Biovendor–Laboratory Medicine; when no signal was observed as well as for sera of several mammal species (mouse, rat, rabbit, horse, cow, sheep, goat and pig).

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 with baseline Angptl3 levels of 166 and 126 ng/ml were spiked with increasing amounts of recombinant protein (+100, +25 and +12.5 ng/ml) and assayed. The mean recovery was 94.3%. Moreover, we tested human serum samples from another two subjects with baseline Angptl3 levels of 308.9 and 276.3 ng/ml for dilution linearity. The mean recovery was 107.4%.

The limit of detection of the assay was 0.6 ng/ml; the intraassay and interassay coefficient of variation were always less than 9%.

Our pilot study involved the group of healthy volunteers, and the group of donors suffering from metabolic syndrome (MS) and familiar dyslipidemia. We determined Angptl3 levels by using ELISA presented above. The study was arranged as follows: in the study on healthy volunteers (n = 93, body mass index (BMI) < 24, normal values of triglycerides, without therapy) we demonstrated no sex and the age significant difference (abnormal distribution, men *vs.* women; medians 227.6 *vs.* 218.9 ng/ml, p = 0.53, sampling in the morning after 8 h of fasting) (Table 1). Furthermore, we tested 118 individuals with MS (NCEP criteria), treated only with inhibitors of angiotensine converting enzyme (ACE) and acetylsalicylic acid (100 mg *per* day) and 200 probands with familiar hypercholesterolemia (FH), treated with combination of atorvastatine, fibrates and ezetimibe. In addition to Angptl3, we determined blood pressure,

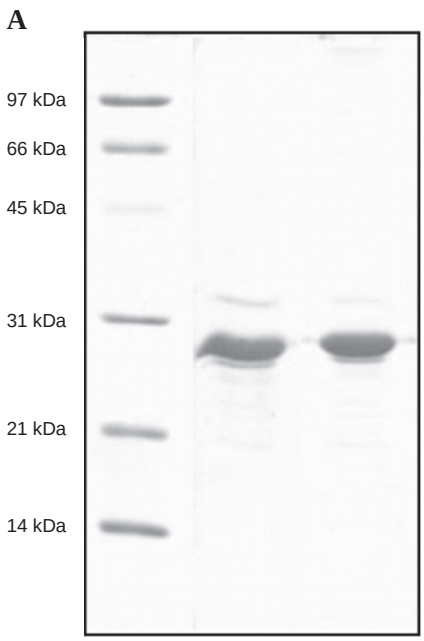

Figure 1A. Recombinant (purity of recombinant) human angiopoietin-like protein 3 (Angptl3) was analyzed in SDS PAGE under reducing and non-reducing conditions (12% homogenous gel, Laemli method); 3.5 μg/lane. In both lanes was used the same amount of sample.

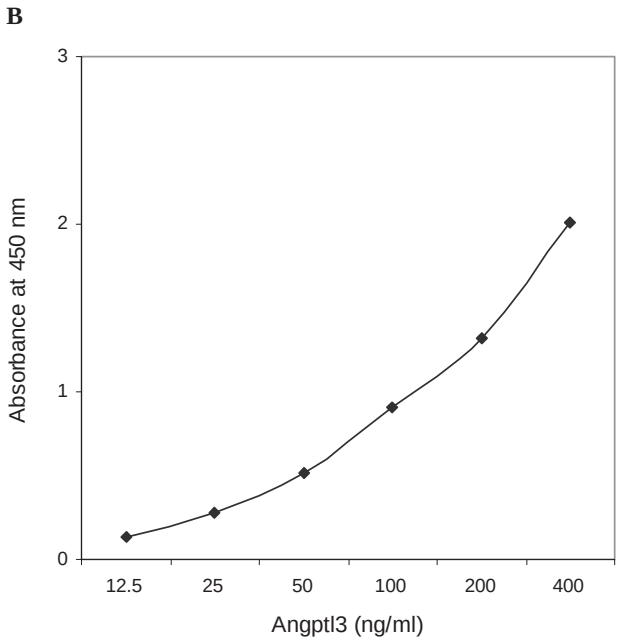

Figure 1B. The standard curve was constructed by plotting the absorbance at 450 nm of standards against *log* of the known concentration of standards, using the four-parameter algorithm. Standard curve for human angiopoietin-like protein 3 (Angptl3) is plotted as a proportion of Angptl3 concentration and absorbance at 450 nm.

waist circumference (WC), BMI and the quantity of serum adiponectin, cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, insulin, glucose, A-FABP and calculated insulin sensitivity index Quicki. Probands with history of MS and FH had higher Angptl3 serum values than healthy but their Angptl3 values did not differ asunder (abnormal distribution, medians 289.5 *vs*. 277.1 *vs*. 224.8 ng/ml in MS, FH and healthy groups, respectively; $p < 0.01$). All of groups did not differ in sex or age. Angptl3 correlated with the systolic blood pressure ($r = 0.43$, $p < 0.01$), A-FABP ($r = 0.21$, $p = 0.03$), and LDL ($r = 0.33$, $p < 0.01$) (not shown). No significant correlation was found between serum Angptl3 and triglycerides, BMI, WC or Quicki (not shown) – presumably influences of statins, fibrates *via* PPARs, etc). However, we performed stepwise regression and found A-FABP and Angptl3 as and independent markers for MS presence only

Table 1. Sex differences of serum angiopoietin-like protein 3 (Angptl3) and significance of serum Angptl3 differences between defined subgroups

Parameter	Units	Mean	Median	SD	Normality	*p* (FH-MS)	*p* (MS-H)	*p* (FH-H)	*p*
Angptl3 (men)	ng/ml	256.2	227.6	105.1	no				
Angptl3 (women)	ng/ml	244.8	218.9	95.2	no				0.53
Angptl3 (FH)	ng/ml	308.4	277.1	103.1	no				
Angptl3 (MS)	ng/ml	297.2	289.5	97.5	no	0.54	<0.01	<0.01	
Angptl3 (H)	ng/ml	234.4	224.8	88.9	no				
Angptl3 (FH)	years	56.0	56.2	12.7	yes				
Angptl3 (MS)	years	57.5	58.4	11.4	yes	0.69	0.56	0.62	
Angptl3 (H)	years	55.2	53.1	8.7	yes				

FH, familiar hypercholesterolemia ; MS, metabolic syndrome; H, healthy; *p,* values of probability.

(F ratio 29, $p < 0.01$). Then we adjusted Angptl3 to A-FABP (reputable MS marker) and recognised that Angptl3 is the A-FABP-independent marker (not shown).

Therefore we believe that serum Angptl3 is a new class of lipid metabolism modulator which regulates VLDL triglyceride levels through the inhibition of LPL activity and connected with MS presence.

Angptl3 serum values could be a new key predictor and prognostic factor of MS. These results were confirmed recently presented information about actuality that Angptl3 is closely associated with arterial wall thickness in human subjects (Hatsuda et al. 2007). Relation between Angptl3 and triglycerides don´t need to be well-marked because of therapy PPARs regulators.

In summary, presented results demostrated the analytical competence of the ELISA Angptl3 assay and showed its usefulness for the study of MS.

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