



APOH is increased in the plasma and liver of type 2 diabetic patients with metabolic syndrome

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ABSTRACT

Objective: To assess the association of APOH with metabolic and cardiovascular risk markers in type 2 diabetic patients.

Methods: In a cohort of 169 type 2 diabetic subjects, plasma levels of APOH, antibodies anti-APOH, lipoprotein subfractions, oxidation, inflammatory and insulin resistance markers and the Trp316Ser and Val247Leu variations in the *APOH* gene were analyzed. Apo H mRNA levels and protein content were measured in hepatic and adipose tissue (subcutaneous and visceral) samples obtained during bariatric surgery from three diabetics who fulfilled metabolic syndrome (MS) criteria and three non-diabetic, non-MS.

Results: APOH plasma levels were significantly associated with triglycerides ($p < 0.001$), all the components of triglyceride-rich lipoproteins ($p < 0.001$) and RBP4 ($p < 0.001$) levels. APOH was higher in type 2 diabetic patients with MS ($p = 0.003$) and with clinical evidence of macrovascular disease ($p = 0.012$). The Trp316Ser and Val247Leu *APOH* gene variants did not modulate APOH plasma values. Neither Apo H mRNA nor protein was detected in the adipose tissue. Liver from patients with diabetes and MS showed a significant increase of both Apo H mRNA and protein respect to the non-diabetic, non-MS patients.

Conclusion: APOH plasma concentrations are strongly associated to MS alterations and vascular disease in type 2 diabetic patients and could be considered as a clinical marker of cardiovascular risk. The enhanced APOH levels in these patients are due to an increased liver synthesis. If APOH plays a major causal role in macrovascular lesions associated to diabetes and MS need further studies.

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1. Introduction

β_2 glycoprotein I, also known as apolipoprotein H (APOH), is synthesized by the hepatocytes [1] and, circulates mainly in free form although up to 35% is associated with lipoproteins [2]. It is the main autoantigen responsible for negatively charged antiphospholipid antibodies production in the anti phospholipid syndrome (APS). APS has a prevalence of 10% among the general population reaching up to 30% in Systemic Lupus Erythematosus (SLE) patients [3]. The *APOH* Val247Leu variation has been associated with an increased production of anti-APOH antibodies and has also been associated with a worse clinical phenotype in homozygous familial hypercholesterolemia [4]. The Trp316Ser blocks its

binding capacity for negatively charged phospholipids [5]. Regarding the lipoprotein metabolism, APOH seems to play a role in triglyceride-rich lipoprotein (TRL) clearance, likely through activation of lipoprotein lipase [4]. Some data have shown a direct correlation with cholesterol levels in diabetic patients, and a pathogenic role in the accelerated atherosclerosis of these patients has been suggested [6]. Studies performed in healthy individuals have shown correlation between APOH and fasting glucose, lipids and lipoprotein levels [7]. APOH functions are probably downstream of lipoprotein metabolism. Although APOH levels are not increased after an oral fat load test, APOH is associated with adipose tissue disturbances in the postprandial state, and is involved in insulin resistance and changes in body fat mass [8]. However, the liver rather than the adipose tissue is the main source of circulating APOH. APOH co-localizes with CD4 lymphocytes in arteriosclerotic lesions and is present in myocardial infarction areas, suggesting that it could be related to ischemia-mediated immune and inflammatory mechanisms [9,10]. In SLE patients, who have an 8–10% increase in cardiovascular risk compared to the general population

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[11–13], the presence of oxidized LDL/APOH circulating complexes, which stimulate antibodies synthesis, have been observed. The presence of oxidized LDL/anti-APOH antibodies seems to be independent of classical cardiovascular risk factors and intima-media thickness, suggesting an additional immune-mediated mechanism in the arteriosclerosis progression of these patients [14]. Therefore, all evidence supports the relationship between APOH and lipid metabolism, thrombosis and inflammation, leading to the increased risk of CVD. Because all of these conditions are components of the metabolic syndrome (MS), it seems worthwhile to study the role of APOH in patients with complex metabolic disturbances and the mechanisms associated to variations in plasma concentration. In this paper, we have explored the relationship between APOH plasma concentrations and MS components and the presence of macrovascular disease in diabetic patients regarding APOH gene variants. Additionally, we have explored the putative role of liver and adipose tissue in these variations in APOH plasma concentrations.

2. Materials and methods

2.1. Clinical study

We studied 169 non-smoking type 2 diabetic subjects (36–79 years old). Anamnesis clinical examinations, including anthropometrics, blood pressure, and the presence of macro or microvascular diseases, were recorded. The presence of arteriosclerosis, coronary heart disease, stroke or peripheral vascular disease, was assessed by clinical history, EKG, carotid and femoral Eco-Doppler and Ankle-Brachial index. MS was defined by the association to diabetes with at least two of the following characteristics: high triglycerides (>1.69 mmol/l), low HDL-cholesterol (<1.03 mmol/l (men) or <1.29 mmol/l (women)), hypertension (systolic blood pressure/diastolic blood pressure $\geq 130/85$ or pharmacological treatment) or body mass index (BMI >30 kg/m²). Patients with albuminuria (≥ 300 mg/24 h), type 1 diabetes mellitus, secondary diabetes mellitus, morbid obesity (BMI >40 kg/m²), familial hypercholesterolemia, malignancy, liver disorders, and acute or chronic inflammation were not included. Insulin resistance (IR) was estimated using the homeostasis model assessment index (HOMA-IR) [15].

Adipose (subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT)) and liver biopsy samples were collected from six females (33–41 years old) undergoing bariatric surgery (BMI: 41.6–51.3 kg/m²) at the Vall d'Hebron University Hospital. Three of these patients had type 2 diabetes and fulfilled five MS criteria while the others were non-diabetic, non-MS patients.

In order to compare APOH values of type 2 diabetic with non-diabetic subjects we measured APOH in a 137 samples from non-diabetic, non-metabolic syndrome healthy population stored the BioBanc of our center, from the same range of age, gender distribution and geographical area.

All subjects gave written informed consent, and both hospitals ethical committees approved the studies.

2.2. Analytical methods

Lipoproteins (VLDL, IDL, LDL, and HDL) were subfractionated from EDTA-plasma by sequential preparative ultracentrifugation as described previously [16]. The general biochemical parameters, oxidation markers and adipokines were determined by standard methods. The plasma levels of the adipocyte fatty acid binding protein (FABP4) and the retinol binding protein 4 (RBP4) were assessed by commercial ELISA kits (BioVendor Laboratory Medicine Inc., Brno, Czech Republic and AdipoGen Inc., Seoul, Korea). The

antibodies used in the human FABP4 ELISA are highly specific for human FABP4, with no detectable cross-reactivity to human FABP1, FABP2, FABP3 or FABP5. The RBP4 ELISA kit has been previously described, validated [17], and used in different populations including insulin-resistant subjects [18]. The precision of these techniques, as described by coefficient of variation (CV) were all $<8\%$ interassay.

2.3. APOH analyses in plasma

The plasma levels of APOH were determined using a matched-pair antibody set for ELISA of human APOH (Affinity Biologicals Inc., Ancaster, ON, Canada) [19]. The plasma levels of APOH IgG antibodies were assessed by commercial ELISA kit (INOVA Diagnostics Inc., San Diego, CA, USA). Samples with values greater than 20 units were considered positive. The precision of these techniques, as described by coefficient of variation (CV) were $<5\%$ interassay.

2.4. APOH Trp316Ser and Val247Leu variant analyses

Genomic DNA was obtained from leukocytes and extracted with calibrated methods (Qiagen GmbH, Hilden, Germany). The Trp316Ser (rs1801690) and Val247Leu (rs4581) variations in the APOH gene were detected as described previously [20].

2.5. Total RNA preparation and real-time PCR

Total RNA was extracted from tissue samples using TRIzol reagent (Invitrogen SA, Barcelona, Spain) and reverse transcribed to cDNA (Invitrogen). Quantitative real-time PCR using specific primers and Taqman probes for human APOH (Hs00979400.m1; Applied Biosystems) was performed using the gene for β -actin as an endogenous gene expression control (Hs9999903.m1; Applied Biosystems) and Applied Biosystems 7000 equipment. Each sample was assayed in duplicate, and negative controls were included in each experiment.

2.6. Protein extracts and Western blot analysis

Protein was extracted from tissue samples using RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS) supplemented with Complete™ protease inhibitor cocktail (Roche Diagnostics S.L, Barcelona, Spain) at 4 °C, followed by centrifugation (12,000 rpm at 4 °C) for 10 min to obtain total protein extracts.

Protein extracts were used for Western blotting with antibodies against human APOH (ab11733; Abcam Inc., Cambridge, MA, USA) and human β -actin (Calbiochem).

2.7. Statistical analysis

Analysis was performed using SPSS (version 15.0, SPSS Inc., Chicago, IL, USA). All data are presented as the mean $\pm$ S.D. except where otherwise stated. Normal distribution of data was checked with the Kolmogorov–Smirnov test. Log-transformation was performed before analyses when variables were not normally distributed. A comparison of variables between groups was performed using one-way analysis of variance (ANOVA). Univariate linear general models were used to adjust the results of continuous variables for age, gender and BMI. Pearson correlation coefficients between APOH and other variables were determined using a bivariate correlation test. Partial bivariate correlation tests were used to adjust bivariate associations by age, gender and body mass index. Comparisons of ApoH mRNA and protein levels in tissue samples were analyzed using the nonparametric Mann–Whitney U-test. The distribution of genotype frequencies between groups

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