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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.

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 ≥ 130/85 or pharmacological treatment) or body mass index (BMI > 30 kg/m^2). Patients with albuminuria (≥300 mg/24 h), type 1 diabetes mellitus, secondary diabetes mellitus, morbid obesity (BMI > 40 kg/m^2), 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/m^2) 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 transcripted 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^{TM} 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 ± 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

Table 1
Description of clinical characteristic of the type 2 diabetic subjects group in relation to the presence of metabolic syndrome and arteriosclerosis.

	All type 2 diabetic group ($n = 169$)	Type 2 diabetes w/metabolic syndrome ($n = 122$)
Women (%)	53	57
Age (years)	63 ± 9	64 ± 9
BMI (kg/m^2)	30.2 ± 4.3	31.4 ± 4.1
Systolic blood pressure (mmHg)	140 (100–200)	140 (100–200)
Diastolic blood pressure (mmHg)	80 (60–110)	80 (60–110)
Fasting Glucose (mmol/l)	9.6 ± 2.9	9.6 ± 2.7
Insulin (pmol/l)	48.51 (10.44–744.84)	53.14 (11.78–744.84)
HOMA-IR	2.7 (0.5–34.8)	3.2 (0.5–34.8)
HbA_{1c} (%)	7.0 ± 1.1	7.0 ± 1.1
Serum creatinine (μmol/l)	83 (57–191)	84 (57–191)
GFR-MDRD (ml/min/1.73 m^2)	76 ± 17	74 ± 17
Plasma-cholesterol (mmol/l)	4.70 ± 0.77	4.61 ± 0.77
Plasma-triglycerides (mmol/l)	1.52 (0.63–7.05)	1.67 (0.69–7.05)
LDL-cholesterol (mmol/l)	2.81 ± 0.70	2.72 ± 0.69
HDL-cholesterol (mmol/l)	1.10 ± 0.28	1.01 ± 0.25
Diabetes duration (years)	14 ± 8	15 ± 8
Arteriosclerosis (%)	43	47
Hypertension (%)	67	81
Metabolic syndrome (%)	72	100

Data are mean ± S.D., median (range) or frequencies.

were estimated by the nonparametric χ^2 test. The nonparametric Mann–Whitney *U*-test was used to compare APOH mean values between the *APOH* Trp316Ser allele carrier groups. Multiple linear regression analysis, including age, gender, BMI, triglyceride, creatinine, FABP4 and RBP4 plasma levels were performed to find the variables with an independent significant association with APOH in our population. In all cases, a *p* value of less than 0.05 was considered statistically significant.

3. Results

There were no statistically significant differences observed between the APOH values of non-diabetic and diabetic subjects (14.9 ± 3.6 mg/dl versus 14.5 ± 4.0 mg/dl, respectively). Table 1 shows a clinical description of the studied diabetic population which was previously described [21]. APOH plasma levels ranged from 4.6 to 28.6 mg/dl. We did not observe differences in APOH plasma levels between gender (men: 14.3 ± 4.3 mg/dl versus women: 14.6 ± 3.7 mg/dl, $p = 0.608$). Correlations between APOH levels and anthropometry, clinical parameters and lipid profile, and adipokines and other biochemical parameters are shown in Table 2. There is a significant correlation between APOH and components of TRLs: total triglycerides, VLDL-triglycerides, VLDL-cholesterol and VLDL-APOB (Table 2). Also plasma levels of APOH were significantly correlated with plasma-Apo C3 (Table 2).

Hypertriglyceridemic patients had significantly higher APOH values (15.7 ± 4.5 mg/dl versus 13.6 ± 3.5 mg/dl, $p = 0.001$) also after adjustment for age, gender and BMI ($p = 0.002$). HDL-cholesterol values were negatively associated to APOH. Those patients with low HDL-cholesterol had higher APOH although this value remained marginally above the statistical significance after adjustment for confounding variables (14.9 ± 4.2 mg/dl versus 13.7 ± 3.6 mg/dl, $p = 0.082$). Patients with BMI > 30 kg/m^2 had similar APOH values as compared to those with lower BMI (14.6 ± 3.9 mg/dl versus 14.4 ± 4.1 mg/dl, $p = 0.768$). The APOH values were significantly higher in subjects who met the MS criteria (15.0 ± 4.0 mg/dl versus 13.0 ± 3.8 mg/dl, $p = 0.003$) also after adjustment for age, gender and BMI ($p = 0.003$), and values were higher according to the number of MS components. Those patients who had already experienced a major heart, peripheral or cerebral arterial event had significantly higher APOH concentrations compared to those without clinical or subclinical arteriosclerosis (15.2 ± 4.2 mg/dl versus 13.9 ± 3.8 mg/dl, $p = 0.042$), even after adjustment for age, gender and BMI ($p = 0.012$). However, there were no differences observed in relation to microvascular diseases.

APOH was very weakly or not associated to oxidation markers (Table 2). Although a trend towards to a positive correlation between APOH and hsCRP was observed, it did not reach statistical significance (Table 2). APOH was positively associated with RBP4, an adipose tissue molecule involved in insulin resistance (Table 2). No significant correlation was obtained between APOH and adiponectin levels and insulin resistance measured by HOMA-IR (Table 2). There were no differences in APOH levels between type 2 diabetic subjects with and without microalbuminuria (15.0 ± 4.6 mg/dl versus 14.1 ± 3.7 mg/dl, $p = 0.199$).

All bivariate associations observed remained significant after adjustment for age, gender and BMI (Table 2).

Only three subjects (1.8%) had positive anti-APOH antibody levels in plasma.

We did not find any significant expression of APOH mRNA or protein in either subcutaneous or visceral adipose tissue. By contrast APOH was expressed in the liver, and it was significantly higher in obese patients with type 2 diabetes and MS than in those non-diabetic non-MS (265 ± 95 versus 101 ± 22; $p = 0.04$) (Fig. 1a). In addition, a higher APOH protein content was found in the liver from patients with type 2 diabetes and MS than those without diabetes and MS (2.73 ± 0.94 versus 1 ± 0.18) (Fig. 1b).

A total of 20 subjects from the 169 type 2 diabetic group were carriers for the Ser allele in position 316 of the *APOH* gene. Among those 20 subjects, 19 subjects (11.2%) were heterozygous and only one subject (0.6%) was homozygous for the Ser allele. And 149 subjects (88.2%) were carriers of 2 Trp alleles (the common genotype). Regarding the Val247Leu *APOH* gene variant, 92 subjects (54.4%) from the studied type 2 diabetic group had the common genotype (carriers of 2 Val alleles), and 77 subjects were carriers for the Leu allele. Among those 77 subjects, there were 12 subjects (7.1%) homozygous and 65 subjects (38.5%) heterozygous for the Leu allele. There were no differences in the distribution of these genetic variants between MS versus non-MS subjects or between arteriosclerosis versus non-arteriosclerosis subjects. The frequencies of the Ser and Leu alleles were 0.062 and 0.263, respectively, which were not different from those predicted by Hardy–Weinberg distribution. The Trp316Ser and Val247Leu variations in the *APOH* gene did not modulate APOH plasma values in our population (Leu carriers: 14.5 ± 3.2 mg/dl versus non-Leu carriers: 14.4 ± 4.6 mg/dl, $p = 0.943$; and Ser carriers: 13.9 ± 4.3 mg/dl versus non-Ser carriers: 14.5 ± 4.0 mg/dl, $p = 0.360$).

Table 2
APOH correlations.

Variable	All type 2 diabetic group ($n = 169$)		
	r	p	Adjusted p
Age (years)	−0.017	ns	–
BMI (kg/m^2)	0.104	ns	–
Systolic blood pressure (mmHg)[a]	−0.029	ns	–
Diastolic blood pressure (mmHg)[a]	0.071	ns	–
Diabetes duration (years)[a]	0.085	ns	–
Glucose (mmol/l)	−0.035	ns	–
Insulin (pmol/l)[a]	−0.001	ns	–
HOMA-IR[a]	−0.017	ns	–
HbA_{1c} (%)	−0.078	ns	–
Serum creatinine (μmol/l)[a]	0.250	0.001	<0.001
GFR-MDRD (ml/min/1.73 m^2)	−0.255	0.001	<0.001
Plasma-cholesterol (mmol/l)	0.045	ns	–
Plasma-triglycerides (mmol/l)[a]	0.337	<0.001	<0.001
Plasma-Apo A1 (mg/dl)	−0.051	ns	–
Plasma-Apo B (mg/dl)	0.053	ns	–
Plasma-Apo C3 (mg/dl)[a]	0.398	<0.001	<0.001
Plasma-Apo E (mg/dl)[a]	0.260	0.001	0.001
VLDL-cholesterol (mmol/l)[a]	0.323	<0.001	<0.001
VLDL-triglycerides (mmol/l)[a]	0.359	<0.001	<0.001
VLDL-Apo B (mg/dl)[a]	0.298	<0.001	<0.001
Adiponectin (μg/l)[a]	−0.039	ns	–
FABP4 (μg/l)[a]	0.190	0.014	0.019
RBP4 (mg/l)[a]	0.346	<0.001	<0.001
hsCRP (mg/l)[a]	0.152	0.051	ns
Vitamin E (μmol/l)	0.208	0.007	0.006
Lipoperoxides (μmol/l)	−0.097	ns	–
Oxidized LDL (U/l)	−0.084	ns	–
Oxidized LDL antibodies (kU/l)[a]	−0.012	ns	–
LDL-lag phase (min)	−0.162	0.046	0.049
LDL-maximal rate (mol diene/mol LDL/min)	0.070	ns	–
LDL-maximal diene production (mol diene/mol LDL)	−0.203	0.013	0.012

Adjusted p: p-value adjusted for age, gender and body mass index.
[a] Log transformed before analysis.

Finally, multiple linear regression analysis, including age, gender, BMI, creatinine, triglycerides, FABP4, RBP4, showed that APOH levels were significantly and independently determined by RBP4 and triglyceride levels. These 2 variables accounted for the 22% of variations in APOH plasma values in this type 2 diabetic population. These independent associations between RBP4 and triglycerides with APOH, remained significant when adjusted for age, gender and BMI ($\beta = 0.06$, $p < 0.001$ and $\beta = 1.28$, $p < 0.001$, respectively).

4. Discussion

In this study we have analyzed the plasma levels of APOH in type 2 diabetic patients, and investigated the relationship to additional metabolic factors. In our hands APOH is strongly correlated with MS components. We have observed a clear relationship between APOH and total triglycerides, and also components of TRL such as VLDL-cholesterol, VLDL-triglycerides, VLDL-APOB and APOC3 and APOE plasma concentrations. Those subjects with low HDL-cholesterol had also higher APOH. The available data about the relationship between APOH and lipoproteins is rather controversial. It has been reported that APOH is linked to triglycerides, and also to cholesterol and HDL. Although there is a body of evidence suggesting that APOH is transported in part in TRL, some authors have observed that only a small percentage (3–14 to 35%) is transported associated with lipoprotein, while the rest is recovered from lipoprotein-free plasma [2]. Moreover, APOH levels are not modified in the postprandial state. Our results suggest a metabolic association between APOH and TRL, although we cannot conclude that APOH is structurally associated with this lipoprotein fraction. In these subjects, there is a derangement of TRL metabolism that is classically explained by a hyperlipolytic status in adipose tissue, associated with insulin resistance [22]. In this metabolic situation the liver is overloaded by free fatty acids that stimulate TRL synthesis [14]. Perhaps this could also influence APOH liver production. APOH was associated with RBP4 levels, which have been previously correlated with insulin resistance and adipose tissue dysfunction status. Because of these results, it could be argued that adipose tissue could participate in APOH synthesis in the presence of obesity and MS. However, we have shown a lack of APOH expression in adipose from six morbidly obese patients undergoing bariatric surgery. By contrast, APOH was expressed in the liver and was significantly higher in obese subjects with type 2 diabetes and MS than in obese subjects without diabetes and MS. Furthermore, APOH plasma concentrations were higher in those diabetic patients that met the MS criteria. These results suggest that the same metabolic factors stimulating TRL production rate determine the increase in APOH at hepatic level, thus at plasma levels.

Patients who had suffered a previous major cardiovascular event had higher APOH levels. It has been suggested that APOH can play a role in atheroma development. This molecule has been associated to LDL oxidation and inflammation, which is in accordance with our results, and it has been detected in atheroma plaques and myocardial infarction areas associated with lymphocyte infiltration [10]. Most likely APOH can induce immunoreactivity at the artery wall contributing to arteriosclerosis production. APOH could be one of the many molecules that mediate the vascular risk in this group of individuals.

The determinants of APOH synthesis are relatively unknown and genetic and environmental factors probably play a role. Although in other clinical situations it has been reported that some genetic variants of the *APOH* gene could influence its synthesis rate [3,19], in this study the APOH plasma values were independent of *APOH* gene polymorphisms. Therefore, at least in diabetic patients, *APOH* gene polymorphisms do not seem to influence its plasma levels.

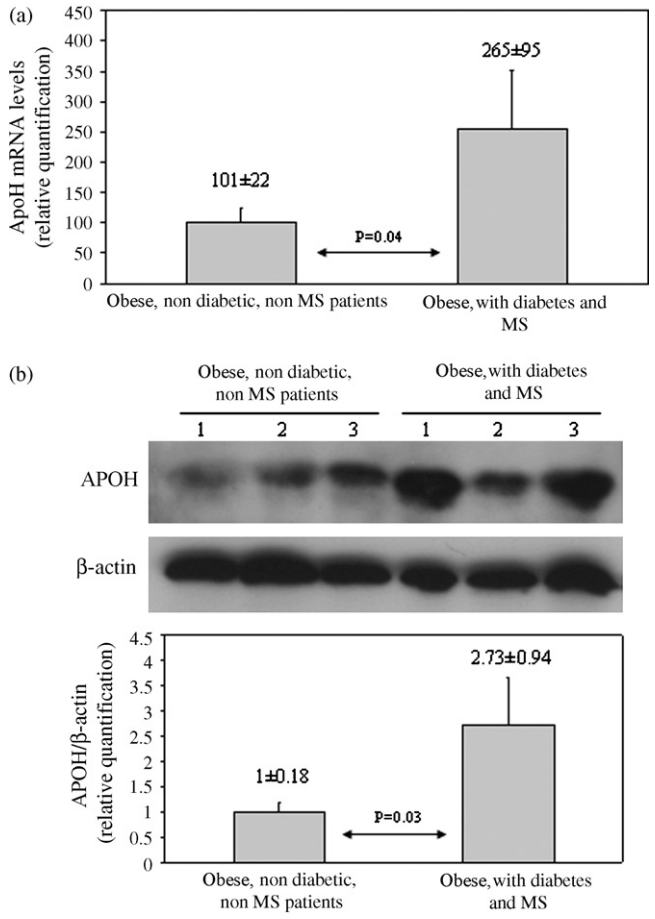

Fig. 1. Increased expression of ApoH mRNA and protein levels in livers of obese patients with type 2 diabetes and metabolic syndrome (MS). (a) Real-time PCR analysis of ApoH mRNA levels in obese patients with ($n = 3$) and without ($n = 3$) type 2 diabetes and MS. Values of ApoH mRNA are relative to β-actin mRNA levels that was used as gene control. (b) Western blot of APOH and β-actin in total protein levels of livers from obese patients with ($n = 3$) and without ($n = 3$) type 2 diabetes and MS.

In conclusion, we communicate for the first time that APOH plasma concentrations are elevated in type 2 diabetic patients according to the presence of MS components, and that the liver from obese patients with type 2 diabetes and MS shows an increased APOH mRNA and protein expression. RBP4 and triglycerides were independently related to APOH plasma levels accounting for 22% of its variablility. APOH was also associated to the presence of clinically evident arteriosclerosis. Because of its role in immunity, inflammation thrombosis and lipid oxidation, we suggest that APOH plays a role as a link between the abnormalities of these processes and MS in type 2 diabetic patients, ultimately increasing the cardiovascular risk of these patients.

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