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# Influence of APOH protein polymorphism on apoH levels in normal and diabetic subjects

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Apolipoprotein (apo)H (also known as  $\beta$ 2 glycoprotein-I) is a glycoprotein synthesized by liver cells and it is present in the blood associated with plasma lipoproteins. APOH displays a genetically determined structural polymorphism: three alleles (APOH\*1, APOH\*2, APOH\*3) at a single locus on chromosome 17 code for different isoforms, and population studies have shown that APOH\*2 is the most frequent allele. This paper assesses the relation between APOH phenotypes and plasma apoH levels in a population composed of 278 healthy subjects (243 H2/2, 32 H3/2, 2 H3/3, 1 H2/1; allele frequencies APOH\*1 0.002, APOH\*2 0.934, APOH\*3 0.064) and 245 diabetics (212 H2/2, 30 H3/2, 3 H3/3; allele frequencies APOH\*2 0.927 and APOH\*3 0.073).

Determination of apoH levels by competitive ELISA gave a mean value of  $26.3 \pm 9.8$  mg/dl for all subjects,  $22.6 \pm 7.7$  in normals vs  $30.6 \pm 10.3$  in diabetics ( $p=0.0001$ ), and  $23.0 \pm 7.9$ ,  $19.3 \pm 5.4$  and  $18.5 \pm 3.5$  mg/dl for H2/2, H3/2 and H3/3 in normals and  $31.1 \pm 10.1$ ,  $28.2 \pm 10.8$  and  $15.7 \pm 9.0$  mg/dl in diabetics, respectively. ANCOVA of the adjusted data revealed a significant difference in apoH levels for the three phenotypes in both the normal subjects ( $p=0.01$ ) and the diabetics ( $p=0.02$ ). ANCOVA of the whole samples of subjects, controlling for diabetes as well as age, sex and total cholesterol, indicated a substantial effect of phenotype, independent of the other variables ( $p=0.0007$ ).

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ApoH, also known as  $\beta$ 2 glycoprotein-I, is a single, approximately 50 kDa chain glycoprotein (Lozier et al. 1984) present in the blood in a mature 326 amino acid form and associated with plasma lipoproteins to the extent of 35% (Polz & Kostner 1979). Its chemical and physical characteristics, particularly its affinity for negatively charged molecules, suggest that apoH is a coagulation inhibitor. It does, in fact, inhibit ADP-induced platelet aggregation (Nimpf et al. 1987), platelet prothrombinase activity (Nimpf et al. 1986), and contact activation of the coagulation pathway (Schousboe 1985). Its role in lipid metabolism has been examined by several workers (Nakaya et al. 1980, Wurm et al. 1982, Eichner et al. 1989a, Kamboh & Ferrell 1991). The *in vivo* and *in vitro* data indicate that it is involved in triglyceride (Tg) metabolism (Nakaya et al. 1980, Wurm et al. 1982). Many studies have shown that apoH is an obligate cofactor for binding

some groups of antibodies to anionic phospholipids (McNeil et al. 1990, Jones et al. 1992, Gharavi et al. 1992, Hunt et al. 1993, Roubey 1994, Kamboh et al. 1995). When present in patients with autoimmune diseases, these antibodies are associated with thrombosis episodes (Love & Santoro 1990). It has recently been shown that apoH itself may be the antigenic determinant of autoantibodies thought to be responsible for occlusion (Viard et al. 1992), but the relation between apoH level and thrombosis (Bancsi et al. 1992) is uncertain, whereas a relation between apoH level and cholesterol (Chol) level has recently been observed in dyslipidaemia (McNally et al. 1994). Plasma apoH levels are about 20 mg/dl (Propert 1978).

APOH displays a genetically determined structural polymorphism. Three alleles (APOH\*1, APOH\*2, APOH\*3) in Caucasians (plus APOH\*4 in blacks only) at a single locus on chromosome 17

code for isoforms identified by isoelectric focusing electrophoresis (IEF) and immunoblotting (Kamboh et al. 1988). Population studies have shown that APOH\*2 is the most frequent allele (Kamboh et al. 1988, Kamboh et al. 1991, Saha et al. 1992, Cassader et al. 1994), and that allele distribution shows ethnic variability (Crews et al. 1991, Kamboh et al. 1991, Cleve et al. 1992, Crews et al. 1993). The relation between APOH phenotype and plasma Tg levels is still a matter of discussion (Sepehrnia et al. 1989, Eichner et al. 1989b, Kaprio et al. 1991, Saha et al. 1993, Cassader et al. 1994).

This paper assesses the relation between APOH alleles and plasma apoH levels in a Piedmont population consisting of 278 healthy and 245 diabetic subjects, in view of our personal observation of a link between apoH and this disease (Cassader et al. 1997).

## Materials and methods

### Materials

All the materials were analytical grade. Nitrocellulose sheets were purchased from Bio-Rad (Bio-Rad Laboratories, Milan, Italy). Goat anti-rabbit IgG (alkaline phosphatase conjugated) was from Sigma-Chemical Co. (St. Louis, MI, USA), rabbit anti-Apo H antiserum was purchased from Istituto Behring (Scoppito, Italy), and the alkaline phosphatase conjugate substrate kit from Bio-Rad.

### Subjects

A total of 278 unrelated subjects was enrolled from the University of Turin's Department of Internal Medicine staff and its blood bank donors. The inclusion criteria were: absence of diabetes, liver and kidney diseases, altered thyroid function, and taking drugs affecting lipid metabolism. The only subject carrying the APOH\*1 allele was not considered for statistical analysis. The 245 diabetics were outpatients at the department's diabetological section. Diabetes was diagnosed in accordance with the WHO criteria. It is treated free in Italy and the patients can thus be regarded as representative of the Piedmont Region. They consisted of 129 non-insulin-dependent (NIDDM) subjects receiving oral hypoglycaemising agents (98 patients) or diet management alone (31 patients), and 116 insulin-dependent (IDDM) subjects usually treated with prompt insulin plus one slow insulin in the evening. Glycosylated haemoglobin (HbA<sub>1c</sub>) was determined by high-pressure liquid chromatography (HPLC) (Bio-Rad, Diamat Model 723 Analyzer). Body mass index (BMI) was evaluated as body weight (kg) divided by height squared (m<sup>2</sup>).

### Plasma lipid determinations

Blood samples (1 mg/ml EDTA-Na<sub>2</sub>) were centrifuged for 30 min at 2500 rev/min and 4°C in a Beckman J6B centrifuge (Beckman, Palo Alto, CA, USA) and stored at -20°C until processed. Chol and Tg were measured enzymatically (Poli Diagnostici, Milan, Italy), HDL-cholesterol was determined after precipitation of Apo B-containing lipoproteins with heparin and manganese chloride by means of automated enzymatic methods with a Shimadzu CL-7000 (Shimadzu Instruments, Kyoto, Japan). Plasma samples if not immediately used were stored at -70°C for at least 6 months.

### Determination of apoH phenotype

This was done with a previously described modification (Cassader et al. 1994) of Kamboh et al.'s method (Kamboh et al. 1988). Briefly, 10 µl plasma were delipidated in 5 ml acetone-ethanol 1:1 (v/v) at -20°C for 2 h, resuspended in acetone-ethanol, washed with cold ether and dried under nitrogen. Pellets were incubated in 100 µl 0.01 M TRIS-HCl, 3M urea and isoelectrofocussed on polyacrylamide gel (T=5%, C=3.33%, urea 3M, pH=4-8) (BioPhoresis [TM] horizontal electrophoresis cell, Bio-Rad), using a Model 3000/300 Power Supply. Focusing was carried out under 10 W constant power for 3 h. After the run, the proteins were transferred overnight onto 0.45-µm pore size nitrocellulose membrane by simple diffusion. The nitrocellulose was then incubated with rabbit anti-apoH antiserum (Istituto Behring) (1:2000, v/v, as first antibody) and goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) (1:6000, v/v, secondary antibody). The nitrocellulose was developed at room temperature according to the kit manufacturer's instructions (Bio-Rad).

### Determination of apoH levels

Plasma apoH was determined with a competitive ELISA. Pure apoH was obtained from pooled human plasma by affinity chromatography and elution electro-phoresis as described elsewhere (Gambino et al. 1996). Rabbit anti-apoH antibodies (Istituto Behring) were diluted 1:8000 with 0.05 M carbonate/bicarbonate buffer pH 9.5, and 100 µl were pipetted into each well of a 96-well microtitre plate. This was then sealed and left at 37°C for 1 h. Washing with 0.02 M sodium phosphate pH 7.8, 0.15 M NaCl (PBS) to remove unbound antibodies was followed by blockade of the remaining sites by incubation with 350 µl 4% bovine serum albumin (BSA) in PBS for 1 h at 37°C. Unknown plasma samples and standards (obtained with serial dilution

from the stock solution of pure apoH) were diluted in assay buffer solution before assay. The optimum ELISA conditions were: 50 µl standards of apoH or plasma diluted 400-fold and 50 µl purified apoH labelled with horseradish peroxidase diluted 1000-fold. Samples were pipetted into the wells of a pre-coated microtitre plate, which was then covered and incubated for 2.5 h at 37°C. The plate was washed six times and developed with 1 mg/ml p-nitrophenol phosphate in 0.05 M phosphate-citrated buffer pH 5.0. Well absorbance was measured with a Bio-Rad 3550 reader at 490 nm and serum concentrations were expressed in mg/dl. The working range was from 1.9 to 120 mg/dl. The intra-assay coefficient of variation (CV) of our competitive ELISA was calculated from the results of 10 pairs of wells in a single assay. The inter-assay CV was calculated from the results of pairs of wells in five assays. In the absence of appropriate control plasma for apoH, accuracy was determined from the recovery obtained after addition of our isolated apoH in known concentrations to normal plasma.

Statistics

Fisher's test (Kendall & Stuart 1979) was used to compare the observed vs. expected phenotype frequencies, according to Hardy-Weinberg equilibrium. The null hypothesis that APOH locus does not affect apoH plasma levels was tested by analysis of variance (ANOVA) performed on the three phenotypes (H2/2, H3/2, H3/3). In order to adjust for the effect of potentially confounding variables, which could be unevenly distributed among the three phenotypes, and therefore explain the observed differences in apoH levels, we performed analysis of covariance (ANCOVA), using age, gender, body mass index and total cholesterol as covariates. When simultaneous analysis was made on both groups of subjects, the means were adjusted also for presence of diabetes.

To test the hypothesis that a trend in apoH levels exists when the number of copies of allele APOH\*3 in the phenotype increases from zero to two, we performed linear regression analysis on adjusted data.

To test whether this effect is different in normal and diabetic subjects (i.e. if the regression coefficient differs in the two groups), we checked the significance of the interaction term between number of APOH\*3 allele and presence of diabetes. The average effects of APOH\*2 and APOH\*3 on these levels were calculated in accordance with Boerwinkle et al.'s formula (Boerwinkle et al. 1988).

Results

APOH phenotypes were determined in 278 normal subjects (243 H2/2, 32 H3/2, 2 H3/3, 1 H2/1) and 245 diabetics (212 H2/2, 30 H3/2, 3 H3/3). Allele frequencies were APOH\*1 0.002, APOH\*2 0.934 and APOH\*3 0.064 in normals; APOH\*2 0.927 and APOH\*3 0.073 in diabetic subjects. Fisher's test did not reveal any significant differences in these frequencies from those expected assuming Hardy-Weinberg equilibrium.

Determination of apoH levels by competitive ELISA gave a mean value of 26.3 ± 9.8 mg/dl for all subjects, 22.6 ± 7.7 in normals vs 30.6 ± 10.3 in diabetics (p=0.0001). The clinical and metabolic data for the two groups are set out in Table 1.

Table 2 reports apoH raw means divided by group and APOH phenotype. ANCOVA of data adjusted for age, sex, BMI and total cholesterol is reported in Table 3, and shows a significant effect of phenotype on apoH levels in both groups of subjects. The agreement between raw and adjusted means shows that the interference of covariates is not very important. No significant association was found between apoH levels and gender, age or Tg levels, in either normal or diabetic subjects.

An apparently decreasing trend of apoH levels,

Table 1. General particulars and plasma lipoprotein values (in mg/dl) in the normal and diabetic subjects (mean ± SD)

	Normals	Diabetics
n	277	245
M/F	139/138	136/109
Age	48.7 ± 20.1	49.9 ± 19.5
BMI	24.3 ± 3.1	26.0 ± 4.7
HbA <sub>1c</sub> (%)	-	8.7 ± 1.8
Tg	131 ± 101	118 ± 79
Chol	222 ± 43	198 ± 42
HDL-Chol	57.6 ± 12.8	50.6 ± 15.5
ApoH	22.6 ± 7.7	30.6 ± 10.3

BMI = body mass index.  
HbA<sub>1c</sub> = glycosylating haemoglobin.  
Tg = triglyceride.  
Chol = cholesterol.  
HDL = high density lipoprotein.  
ApoH = apolipoprotein H.

Table 2. Apolipoprotein H (apoH) levels according to APOH phenotype in normal and diabetic subjects. (SEM = standard error of the mean)

Group	H2/2		H3/2		H3/3	
	n (%)	Mean (SEM)	n (%)	Mean (SEM)	n (%)	Mean (SEM)
Normal	243 (87.7)	23.0 (0.5)	32 (11.6)	19.3 (1.0)	2 (0.7)	18.5 (2.5)
Diabetics	245 (86.5)	31.1 (0.7)	30 (12.2)	28.2 (2.0)	3 (1.2)	15.7 (5.1)

Table 3. Analysis of covariance (ANCOVA) adjusting for age, sex, body mass index and total cholesterol. Apolipoprotein H levels are reported as adjusted means and (SEM)

Group	ANCOVA			F-value	P-value
	H2/2	H3/2	H3/3		
Normal	23.0 (0.5)	19.1 (1.4)	18.1 (5.4)	4.50	0.01
Diabetic	31.1 (0.7)	28.2 (1.9)	16.4 (5.9)	3.90	0.02

when the number of APOH\*3 alleles in the phenotype increased, was observed in both normal and diabetic subjects (Table 3). The significance of this trend was checked by linear regression in the two groups separately, and in the whole sample after including also the presence of diabetes as a covariate. The results are shown in Table 4 and are consistent with a cumulative effect of the dose of APOH\*3 alleles.

To test whether the regression coefficients in normal and diabetic subjects are significantly different, as suggested by the means shown in Table 2, we computed the interaction term between the number of APOH\*3 alleles and the presence of diabetes. The resulting coefficient ( $0.66 \pm 2.08$ ) is not significantly different from zero ( $p=0.75$ ), thus indicating that our data do not support the hypothesis of a different effect of APOH\*3 allele in the two groups.

The average effect of APOH\*3 allele was to reduce apoH levels by 3.59 (normals) and 3.38 (diabetics) mg/dl, whereas that of APOH\*2 was to increase them by 0.23 and 0.33 mg/dl respectively. It can also be estimated that 2.5% of the between-individual variability of apoH levels was attributable to differences in phenotype.

## Discussion

Genetic and environmental factors control plasma lipoprotein levels (Lusis et al. 1988). As components of the lipoproteins, apolipoproteins can increase the risk of the appearance and development of atherosclerosis (Sedlis et al. 1986). Our data show that

Table 4. Regression analysis of apolipoprotein H plasma levels versus number of H\*3 alleles. Data adjusted for age, gender, body mass index (BMI) and total cholesterol

Group	Regression coefficient	Standard error	P-value
Normals	-3.51	1.25	0.005
Diabetics	-4.20	1.69	0.013
All*	-3.85	1.04	0.0002

\* Values adjusted for age, sex, BMI, total cholesterol and diabetes.

apoH levels are under the control of at least two alleles in a population composed of 278 normal and 245 diabetic subjects. Their levels are higher in those homozygous for APOH\*2 and lower in those homozygous for APOH\*3. This effect is independent of age, sex, diabetes, BMI and total Chol. Diabetes, in fact, seems to increase plasma apoH levels significantly, and they are also correlated with those of total Chol (McNally et al. 1994). The decreasing trend of plasma apoH levels, according to the number of APOH\*3 alleles in the phenotype, is statistically significant in both groups of subjects, although there are some dissimilarities: in normals the main difference among the three phenotypes is between H2/2 and H3/2 subjects, while in diabetics the main reduction is between H3/2 and H3/3 subjects. However, there are by far too few APOH\*3 homozygous individuals to allow pairwise comparison between phenotypes, and in this condition it is not possible to reject the hypothesis of a codominant effect of allele APOH\*3.

ApoH seems to be involved in lipid metabolism and coagulation (Nakaya et al. 1980, Wurm et al. 1982, Eichner et al. 1989a, Roubey 1994, Kamboh & Ferrell 1991), and also as a cofactor for the formation of antiphospholipid antibodies in some autoimmune diseases (Roubey 1994). A correlation has equally been sought between its plasma levels and various disorders, especially those related to lipid metabolism and coagulation (Roubey 1994). Increased levels have been reported in dyslipidaemia (McNally et al. 1994), while no association between apoH levels and other metabolic disease has so far been observed (Bancsi et al. 1992).

Recent studies have evaluated the relation between APOH polymorphism and plasma lipid levels (Sephehrnia et al. 1989, Eichner et al. 1989b, Kaprio et al. 1991, Saha et al. 1993, Cassader et al. 1994). No association has so far been established, however, between phenotype and apoH levels.

No reason can readily be found for the observation that apoH levels are markedly increased in diabetics, even when the data are corrected for confounding variables. In this connection, it will be recalled that variations in plasma Lp(a) in diabetes have been reported by some workers (Ramirez et al. 1992), though not by others (Scherthaner et al. 1983), with differing results in IDDM and NIDDM types, and a tendency towards higher values in poorly controlled IDDM patients (Haffner 1993). The conclusion reached in a recent review on the non-enzymatic glycation of Lp(a) was that this process increases the negative charge of the molecule (Makino et al. 1995), and hence its chemical and physical properties, but not its atherogenic potential. It is clear that studies of similar glycation of

apoH *in vitro* would be of great assistance in explaining our results in diabetes.

ApoH is synthesised in the liver and probably catabolised in the kidney. It is a highly glycosylated protein (Lee et al. 1983): glycosylation may thus increase in relation to blood glucose levels in diabetic patients, prolonging apoH catabolism and hence influencing its circulating levels. We have demonstrated a relation between degree of glycosylation and apoH levels, as indicated by the correlation between these levels and HbA<sub>1c</sub> in diabetics (Cassader et al. 1997).

These findings require corroboration in other populations, since the allele frequency observed was similar to that reported by some workers, though not all (Saha et al. 1993). It would thus be of interest to determine whether there is a direct allele effect on apoH levels. Further studies are also needed to assess the ratio between apolipoprotein levels and the various alleles in disorders such as autoimmune diseases, in which apoH itself appears to be involved.

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