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Relationship between apolipoprotein(a) size polymorphism and coronary heart disease in overweight subjects

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Abstract

Background: Overweight is associated with an increased cardiovascular risk which is only partially explained by conventional risk factors. The objective of this study was to evaluate lipoprotein(a) [Lp(a)] plasma levels and apolipoprotein(a) [apo(a)] phenotypes in relation to coronary heart disease (CHD) in overweight subjects.

Methods: A total of 275 overweight (BMI \geq 27 kg/m²) subjects, of which 155 had experienced a CHD event, 337 normal weight subjects with prior CHD and 103 CHD-free normal weight subjects were enrolled in the study. Lp(a) levels were determined by an ELISA technique and apo(a) isoforms were detected by a high-resolution immunoblotting method.

Results: Lp(a) levels were similar in the three study groups. Overweight subjects with CHD had Lp(a) concentrations significantly higher than those without [median (interquartile range): 20 (5–50.3) versus 12.6 (2.6–38.6) mg/dl, $P < 0.05$]. Furthermore, overweight subjects with CHD showed a higher prevalence of low molecular weight apo(a) isoforms than those without (55.5% versus 40.8%, $P < 0.05$) and with respect to the control group (55.5% versus 39.8%, $P < 0.05$). Stepwise regression analysis showed that apo(a) phenotypes, but not Lp(a) levels, entered the model as significant independent predictors of CHD in overweight subjects.

Conclusions: Our data indicate that small-sized apo(a) isoforms are associated with CHD in overweight subjects. The characterization of apo(a) phenotypes might serve as a reliable biomarker to better assess the overall CHD risk of each subject with elevated BMI, leading to more intensive treatment of modifiable cardiovascular risk factors.

Background

Several studies revealed a strong relationship between elevated body mass index (BMI) and coronary heart disease (CHD) [1-3]. In fact, overweight has been recognized as a modifiable cardiovascular risk factor [4]. The association between body weight and cardiovascular events, however, is influenced by several other cardiovascular risk factors, including age, gender, hypertension, smoking, diabetes, and hypercholesterolemia [2,5]. It implicates that in identifying overweight subjects with a particularly high CHD risk, it is useful to analyse various cardiovascular risk factors in order to better assess the overall CHD predisposition of each subject with increased BMI. In addition to the conventional cardiovascular risk factors, lipoprotein(a) [Lp(a)] has been found to be a strong and independent predictor of CHD [6-8]. Lp(a) consists of a low density lipoprotein (LDL) particle linked with a single disulfide bond to apolipoprotein(a) [apo(a)] [9].

Apo(a), the specific apolipoprotein of Lp(a), shows a structural similarity with plasminogen [10] and is thought to interfere with plasma fibrinolysis by inhibiting the generation of the thrombolytic enzyme plasmin [11]. It has been therefore suggested that Lp(a) may display both atherogenic and thrombogenic capacities [12]. Apo(a) shows a high degree of genetic polymorphism, resulting from differences in the number of kringle-IV (K-IV) type 2 repeats in the LPA gene located in 6q26-27 [13]. There are several apo(a) isoforms of molecular weight varying from 280 to 820 kDa, encoded by an autosomal co-dominant allele system [14]. A 'null' allele, which does not manifest any type of isoform, has also been described [15]. Although the LPA gene, or sequences closely linked to it, was believed to account for >90% of the variability in the plasma Lp(a) level [16], it has been recently demonstrated that the apo(a) length polymorphism explains only 38% of the variability in Lp(a) concentration [17].

A number of investigations have demonstrated that small apo(a) isoforms are associated to clinical CHD independent of the corresponding Lp(a) concentrations [18-20], although these findings were not replicated by other authors [21].

Furthermore, the evidence of an association between apo(a) phenotypes and CHD in a large case-control study of overweight (BMI ≥ 27 kg/m²) subjects is still lacking. To evaluate the Lp(a)-related predisposition in the development of CHD in overweight subjects, we determined Lp(a) plasma levels and apo(a) phenotypes in a group of 275 overweight subjects and compared the overweight subjects who had a prior CHD with those who did not.

Methods

Study population

A total of 275 overweight (BMI ≥ 27 kg/m²) Caucasian Italian subjects (211 males and 64 females; mean age 61.36 ± 10.39 years; BMI 29.67 ± 2.56 kg/m²) were recruited among the patients attending the Cardiology Division, IRCCS San Matteo Hospital, Pavia. For each enrolled subject, body weight was measured to the nearest kg and height to the nearest centimeter. BMI was calculated as body weight divided by height² (kg/m²). According to previous methodology [22-24], we used a BMI ≥ 27 kg/m² as the criterion for inclusion in the overweight group. Furthermore, this cut-off was chosen since it has been previously adopted as the criterion to indicate overweight in a Western Europe population [25].

We then assembled an age- and gender-matched normal weight control group, consisting of 103 subjects (74 males and 29 females; mean age 61.95 ± 14.79 years; BMI 23.92 ± 1.88 kg/m²). Controls were, mainly, individuals who visited the outpatients' internal medicine department of the same hospital and at the same period with the coronary patients, for routine clinical or laboratory examinations. Controls were subjects without any clinical symptoms, signs or any suspicion of cardiovascular disease in their medical history, as determined by a physician [26].

Among the overweight subjects, 107 (38.9%) subjects suffered from diabetes mellitus, which was diagnosed according to the ADA criteria [27], or the use of insulin or oral hypoglycemic drugs. 173 (62.9%) overweight subjects were hypertensive. Diagnosis of hypertension was performed as either the blood pressure measured was higher than 140/90 mmHg or by the need of antihypertensive medications. Furthermore, 121 (44%) subjects showed hypercholesterolemia, defined as a serum total cholesterol major than 200 mg/dl, or the use of lipid lowering drugs [28]. Smoking was considered to be present if the subject had smoked more than three cigarettes a day for at least one year.

The overweight subjects were further divided into two subgroups according to whether they had CHD or not. The retrospective diagnosis of CHD was performed on the basis of a documented history of myocardial infarction (clinical history of retrosternal pain that lasted for at least 30 min without response to nitroglycerine, ST segment elevation on a standard twelve-lead electrocardiogram and an increase in serum creatine kinase to at least twice the normal upper limit); coronary artery disease documented by angiography (stenosis > 75% in at least one coronary artery); coronary artery bypass grafting or a positive history of anginous chest pain together with a positive exercise test. Among the overweight subjects, 155

(56.3%) had CHD; for the other patients the possibility of CHD was excluded on the grounds of their medical history and an exercise stress test, as described previously [29].

In order to assess possible differences in the Lp(a)-associated risk between overweight and normal weight subjects, 337 normal weight subjects with CHD (251 males and 86 females; mean age 62.01 ± 11.18 years; BMI 23.85 ± 2.01 kg/m²), were recruited from the cardiology section.

The study protocol followed the guidelines of our local ethics committee and all subjects gave full informed consent to participate in the study.

Lp(a) quantification and apo(a) isoforms phenotyping

Venous blood was collected from subjects after an overnight fast of about 12 hours. Quantification of Lp(a) and phenotyping of apo(a) protein were done using plasma obtained from blood collected in EDTA tubes, which were centrifuged at 4°C, at low-speed, for 12 minutes. After centrifugation, the plasma aliquots were frozen and stored at -80°C.

Lp(a) plasma concentrations were determined by a sandwich-ELISA technique, using the commercially available kit Macra-Lp(a) (SDI, Delaware, USA). The intra-assay and inter-assay coefficients of variation of this method is 5 and 9%, without cross-reaction with plasminogen.

Apo(a) isoforms phenotyping was performed by an immunoblotting method, as previously described in detail [30], with slight modifications.

Briefly, 15 µL of EDTA-plasma samples were pretreated with 30 µL of a reducing solution. The submarine electrophoretic run was performed on 1% sodium dodecyl sulfate-agarose gel and electrophoresis was carried out in tank buffer for 14 h at 80 V and 0.04 A. Reduced samples (20 µL) were applied in wells, at 3 cm from the cathode of the gel. The separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Segrate, Italy) by a capillary blotting technique and tested with a polyclonal antihuman Lp(a) antiserum from rabbit (DAKO, Glostrup, Denmark). A peroxidase-conjugated goat antirabbit immunoglobulin (DAKO, Glostrup, Denmark) was used as the second antibody. Relative band mobility was determined as referred to apo(a) standard isoform mobility included in each blot (values: 35, 27, 23, 19, and 14 K-IV repeats; Immuno AG, Wien, Austria). Thus the estimated number of K-IV repeats in each sample was calculated.

Statistical analysis

Normally distributed data are presented as means \pm SD. For Lp(a) levels, which showed a skewed distribution, we

used medians and interquartile ranges. Student's t test was exploited for comparison in normally distributed data between 2 groups and the Mann-Whitney *U* test was performed for comparison of Lp(a) concentrations between 2 groups. Categorical variables were compared by means of the chi-square test. The significance of clinical and biochemical parameters as independent predictors of CHD in overweight subjects was tested in stepwise regression analysis. Predictors or independent variables were: gender, age, BMI, smoking, diabetes, hypertension, hypercholesterolemia, Lp(a) levels and apo(a) phenotypes. The criterion for inclusion into the model was a *P* value less than 0.05, as determined with the *F* test. A probability value below 0.05 was considered to indicate statistical significance. All statistical analyses were performed using the SPSS statistical package, version 11.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Baseline characteristics and Lp(a) levels of the study patients

Clinical characteristics of overweight subjects, normal weight subjects with CHD and controls are summarized in Table 1. Among the overweight subjects, the prevalence of diabetes mellitus, hypertension and hypercholesterolemia was significantly higher than in controls. Among the normal weight CHD subjects, the prevalence of smoking and diabetes mellitus was significantly higher than in controls, whereas no significant differences in the prevalence of hypertension and hypercholesterolemia were found. Furthermore, no difference in median Lp(a) level was found between the three study groups.

The features of overweight subjects with and without CHD are shown in Table 2. The two groups show no statistically significant differences in age, gender, hypertension, smoking status and BMI. The CHD group included significantly more subjects with diabetes mellitus and hypercholesterolemia. Overweight subjects with CHD had Lp(a) plasma significantly higher than those without [median (interquartile range): 20 (5–50.3) versus 12.6 (2.6–38.6) mg/dl, *P* < 0.05].

Analysis of apo(a) polymorphism

Out of the 715 subjects recruited, we identified twenty-three different apo(a) isoforms with molecular weight varying from 400 and 835 kDa. The detection method showed good sensitivity, specificity and reproducibility, as described [30].

Because of the high degree of apo(a) polymorphism, we divided apo(a) isoforms in two subgroups according to a previously identified cut-off between 640 and 655 kDa. This cut-off seems to discriminate well apo(a) isoforms associated with higher atherothrombotic predisposition

Table 1: Baseline characteristics and conventional atherothrombotic risk factors of the study groups

	Overweight subjects	Normal weight subjects with CHD	Controls
No. of subjects	275	337	103
BMI, kg/m ²	29.67 ± 2.56***	23.85 ± 2.01	23.92 ± 1.88
Age, yr	61.36 ± 10.39	62.01 ± 11.18	61.95 ± 14.79
Male gender, n (%)	211 (76.7%)	251 (74.4%)	74 (71.8%)
Diabetes mellitus, n (%)	107 (38.9%)*	168 (49.8%)*	6 (5.8%)
Hypertension, n (%)	173 (62.9%)*	184 (54.6%)	49 (47.5%)
Smokers, n (%)	32 (11.6%)	68 (20.2%)*	7 (6.8%)
Hypercholesterolemia, n (%)	121 (44%)*	115 (34.1%)	30 (29.1%)
Lp(a) (mg/dl)	16.2 (4–43)	19.6 (5.2–49)	14.9 (4.3–45.3)

BMI, body mass index; CHD, coronary heart disease; Lp(a), lipoprotein(a). Lp(a) levels are expressed as medians (interquartile range within brackets). *P < 0.01 ***P < 0.001, versus controls.

Table 2: Features and conventional atherothrombotic risk factors of overweight subjects with and without coronary heart disease (CHD)

	CHD	No CHD
n	155	120
BMI, kg/m ²	29.39 ± 2.13	30.03 ± 3.00
Age, yr	61.70 ± 9.56	60.90 ± 11.40
Male gender, n (%)	120 (77.4%)	91 (75.8%)
Diabetes mellitus, n (%)	83 (53.4%)	24 (20%)*
Hypertension, n (%)	93 (60%)	85 (70.8%)
Smokers, n (%)	33 (21.3%)	20 (16.7%)
Hypercholesterolemia, n (%)	80 (51.6%)	41 (34.1%)*
Lp(a) (mg/dl)	20 (5–50.3)	12.6 (2.6–38.6)*

BMI, body mass index; Lp(a), lipoprotein(a). Lp(a) levels are expressed as medians (interquartile ranges within brackets). *P < 0.05 **P < 0.01 ***P < 0.001, versus CHD

Table 3: Apo(a) phenotypes distribution in the study groups.

	n	Subjects with at least one apo(a) isoform of low molecular weight	Subjects with only apo(a) isoforms of high molecular weight
Overweight subjects with CHD	155	86 (55.5%)*	69 (44.5%)
Overweight subjects without CHD	120	49 (40.8%)	71 (59.2%)
Normal weight subjects with CHD	337	192 (57%)*	145 (43%)
Controls	103	41 (39.8%)	62 (60.2%)

*P < 0.05 **P < 0.01, versus controls.

[31]. According to previous methodology [19,32,33], we decided to use only the smaller apo(a) isoform detected for categorization.

Table 3 shows the apo(a) phenotype distribution in the study groups. In the group of normal weight subjects with CHD, low molecular weight apo(a) isoforms prevailed

(57%) and the difference in comparison with controls was significant (P < 0.01).

In the subgroup of overweight patients with CHD low molecular weight apo(a) isoforms were more prevalent (55.5%), whereas among subjects without CHD apo(a) isoforms of high molecular weight prevailed (59.2%). The

difference between the two groups of overweight subjects was statistically significant ($P < 0.05$).

The frequencies of apo(a) isoforms for the overweight subjects with CHD and the CHD normal weight subjects were similar. Furthermore, there was no significant difference in the distribution of apo(a) isoforms between overweight subjects without CHD and controls.

Multivariate analysis

The common cardiovascular risk factors, Lp(a) concentrations and apo(a) isoforms of low molecular weight were tested as predictors of CHD in overweight and normal weight subjects in a stepwise regression analysis (Table 4). The presence of diabetes mellitus, small-sized apo(a) isoforms, hypercholesterolemia, and BMI were found to be significant predictors of CHD in overweight subjects. In normal weight subjects the presence of diabetes mellitus, smoking, hypercholesterolemia, hypertension and at least one small-sized apo(a) isoform were found, in the order they entered into the model, to be independent predictors of CHD.

Table 4: Results of stepwise regression analysis with the presence of coronary heart disease (CHD) as the dependent variable in overweight and normal weight subjects.

Overweight subjects			
Predictor	step	t	P value
Diabetes mellitus	1	5.892	<0.0001
Apo(a) isoforms of low molecular weight	2	2.665	0.008
Hypercholesterolemia	3	2.466	0.014
BMI	4	2.120	0.035
Normal weight subjects			
Diabetes mellitus	1	8.521	<0.0001
Smoking	2	4.135	<0.0001
Hypercholesterolemia	3	3.296	0.001
Hypertension	4	3.097	0.002
Apo(a) isoforms of low molecular weight	5	2.500	0.013

Discussion

In this report we investigated both Lp(a) concentrations and apo(a) isoforms in a sample of overweight Caucasian Italian subjects. The few studies available in the literature that examined Lp(a) levels in overweight/obese subjects gave rise to conflicting results. Indeed, Wassef *et al* [34] found that obese subjects had higher Lp(a) levels than did the control subjects, whereas Donatelli *et al* [35] reported similar Lp(a) concentrations in normal glucose tolerant obese subjects compared with controls. Although the

reasons for these contrasting findings remain unclear, different experimental designs, different number and characteristics of subjects recruited, ethnical differences, medical treatments (aspirin, beta carotene, lipid-lowering drugs), lack of standardization of methods for the determination of Lp(a) levels and different ways in which plasma samples were handled might at least partially explain the differences among the studies. In any case, it has been shown by Akanji *et al* [36] a significant correlation between BMI and blood Lp(a) levels. Therefore, it seems to be worthy of investigation whether Lp(a) levels and apo(a) isoforms are independent risk factors for CHD in overweight subjects.

In our study we found that Lp(a) plasma levels do not seem to show significant differences between the whole group of overweight and normal weight controls. However, we showed that Lp(a) plasma levels were significantly higher in CHD overweight subjects with respect to the CHD-free. This finding may be interpreted in that it is possible to discriminate through Lp(a) levels among CHD and CHD-free overweight subjects. However, this result should be considered with caution, since stepwise regression analysis did not confirm an independent association between Lp(a) levels and CHD in our sample of overweight subjects. Furthermore, a potential methodological limitation of our study should be discussed. Indeed, Lp(a) quantification was performed with a commercial method which may be potentially sensitive to the size of apo(a) protein. Therefore, our results on Lp(a) plasma levels should be at least in part confounded by the influence of variation in apo(a) isoform size [37].

After these considerations, we decided to extend our analysis to the distribution of apo(a) isoforms in our sample of overweight subjects. To date, no data concerning the analysis of apo(a) phenotypes in relation to CHD in overweight subjects have been published. The analysis of apo(a) size polymorphism in the subgroups of overweight subjects with or without CHD has indicated that overweight subjects with CHD have a higher prevalence of low molecular weight apo(a) isoforms, whereas overweight subjects without CHD showed a higher prevalence of high molecular weight apo(a) phenotypes. The regression analysis confirmed that, among the cardiovascular risk factors considered, the presence of at least one low molecular weight apo(a) isoform is a reliable discriminant between overweight subjects with CHD and those without.

Therefore, the main finding of this study is that low molecular weight apo(a) isoforms seem to be associated with CHD not only in normal weight but also in overweight subjects. In overweight subjects, the CHD predisposition related to the genetically determined apo(a)

isoforms should be added to the CHD risk due to increased BMI and other risk factors associated with overweight. Furthermore, it seems to be reasonable to hypothesize that overweight subjects with at least one apo(a) isoform could be prone to an earlier development of atherothrombotic complications. Such a risk could be related to both the proatherogenic and thrombogenic effect of small-sized apo(a) isoforms, since it has been recently demonstrated that low molecular weight apo(a) isoforms display a high antifibrinolytic activity and could be involved in fibrinolysis impairment [38].

Interestingly, it should be noted that the higher prevalence of low molecular weight apo(a) isoforms in CHD overweight subjects was associated with higher Lp(a) plasma levels, whereas this was not the case of normal weight CHD subjects. In any case, it has been recently shown that the contribution of the apo(a) isoform size to the control of plasma Lp(a) level is considerably lower than previously calculated, because the variability in plasma Lp(a) concentration is not uniform across the apo(a) size spectrum [17].

Conclusions

Our results indicate that apo(a) isoforms are reliable markers for CHD predisposition in subjects with elevated BMI, and should be used together with other risk factors to assess the overall risk status for CHD in overweight subjects.

Finally, it should be emphasized the importance of apo(a) phenotyping among overweight patients, since the identification of at least one apo(a) isoform of low molecular weight may be useful in guiding the physician toward a better control of modifiable cardiovascular risk factors among overweight subjects, and could therefore reduce the negative impact of coronary heart disease in subjects with elevated BMI.

Competing interest

None declared.

Authors' contributions

EE and EP contributed equally to this work. EE: data analysis and writing the paper. EP: data collection and writing the paper. PM: data collection and revising the paper. CF: conception and design. AD and LM: laboratory analyses. DG: conception and design. All authors read and approved the final manuscript.

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