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APO(a) PHENOTYPING AND LONG-TERM PROGNOSIS FOR CORONARY
ARTERY DISEASE.

Baños-González MA^{1,3}, Anglés-Cano E⁶, Peña-Duque MA¹, Bahena A⁵,
Valente-Acosta B², Cardoso-Saldaña G¹, Escalante-Acosta B³, Angulo-Ortíz J¹,
De la Peña-Díaz A^{1,2}.

1 Instituto Nacional de Cardiología “Ignacio Chávez”, Departamentos de Biología Molecular, Cardiología Intervencionista, Endocrinología. México, D.F.

2 Facultad de Medicina, Departamento de Farmacología, Universidad Nacional Autónoma de México, México, D.F.

3 Centro de Investigación y Estudios Avanzados de IPN. Departamento de Biomedicina Molecular. México, DF.

4 Institut National de la Sante et de la Recherche Medicale, U919 Serine proteases in neurovascular pathology GIP Cyceron/Bd Henri Becquerel 14074-cdx, Caen, France.

⁵ Instituto Nacional de Neurología, México, D.F.

Corresponding author: Aurora de la Peña, Departamento de Farmacología, Edificio D, 1er piso, Facultad de Medicina, Universidad Nacional Autónoma de México. CP04510. México, D.F. Tel. 52 55 23 21 64 e mail: aurorade2002@yahoo.com

Running head: Apo(a) phenotype and coronary artery disease.

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Background: The aim of this study is to identify whether the plasma concentration of Lp(a) helps predict the likelihood of cardiac death, non-fatal myocardial infarction, unstable angina, the need for additional revascularization procedures, and stroke.

Materials and Methods: We analyzed the clinical prognosis of 68 patients with coronary artery disease included in a case-controlled study which evaluated Lp(a) concentration, apo(a) size, and the ability of Lp(a) to bind to fibrin (de la Peña et al. Eur J of Clin Invest 2003; 33: 99-105). Cohort analysis of prognosis data was conducted over a median follow-up of 8 years in Mexico City at Instituto Nacional de Cardiología "Ignacio Chavez". We used Kaplan-Meier survival tables to evaluate cardiovascular and cerebrovascular events in the follow-up period.

Results: Based on a Kaplan-Meier analysis, apo-(a) isoforms of small size (<22 kringle domain repeats) are predictors of coronary risk. We also find an association between Lp(a) plasma concentration and apo(a) fibrin- binding with major adverse cardiovascular and cerebrovascular events, although without statistically significant results.

Conclusions: This study identifies small-sized apo(a) isoforms as an independent risk factor for cardiovascular and cerebrovascular events in patients with coronary artery disease in follow-up. We also found an association, although not significant, with Lp(a) plasma concentration and apo(a) fibrin-binding using multivariate statistic analysis.

Key words: apo(a) phenotyping, Lp(a) and long term prognosis

Introduction

Lipoprotein (a) [Lp(a)] is a complex lipoprotein particle in human plasma. It is composed of apolipoprotein B (apoB100) and apolipoprotein (a) [apo(a)] which are linked by a disulfide bond. Apo(a) consists of a serine proteinase region and several kringle domains, derived from those of plasminogen but presenting some distinctive features.¹ Only plasminogen's kringle 5 (one copy) and 4 (multiple copies) are present in apo(a). The copies of apo(a) kringle 4 are not alike: 10 different types have been recognized.² Each type is present only once, except for kringle 4, type 2, which is present in multiple copies. This variation in copy number of kringle 4, type 2 domains results in structural heterogeneity of Lp(a) and isoforms whose molecular weights range from 280 to 800 kDa. Smaller isoforms of apo(a) with correspondingly fewer kringle 4, type 2 domains correlate inversely with Lp(a) plasma concentration.³

Plasma levels of Lp(a) vary greatly among individuals. Elevated plasma levels of Lp(a) have been shown to be an independent risk factor for coronary artery diseases (CAD). The level of Lp(a) is controlled by a single gene with multiple alleles; each allele affects the concentration of Lp(a)⁴ differently. The interindividual variation in plasma concentrations is almost entirely controlled by the apo(a) locus on chromosome 6q26-q27 and can be explained by a variable number of transcribed kringle4 repeats (KIV-VNTR).

External environmental factors have little impact on the pathogenicity of plasma Lp(a); however internal environmental characteristics such as the ability to undergo oxidation or the action of proteolytic or lipolytic enzymes can modify Lp(a)'s pathogenicity. Transient circumstances such as inflammation can also cause increases in plasma concentration and result in the apo-(a) being partially

divided by polymorphonuclear neutrophils (PMNs) elastase, forming lower molecular weight structures⁵. It is important to stress this fact because when low molecular weight isoforms of apo(a) are present in the Lp(a) molecule, Lp(a) exhibits a greater affinity for fibrin and competitively inhibits plasminogen binding, thus generating a fibrinolytic shortfall⁶. Surprisingly, isolated apo(a) (10 to 34 krings) has been shown to display a high affinity for fibrin⁷, independent of its size.

The pathological routes by which Lp(a) can lead to an atherosclerotic-prone condition or by which a procoagulant mechanism is created by Lp(a) in the atherosclerotic plaque have been explored previously. Proposed mechanisms include the inhibition of the conversion of Glu-plasminogen to Lys-plasminogen⁸, the alteration of the secretion of tissue-type plasminogen activator, the prolongation of fibrinolysis time due to the thinning of fibrin fibers⁹, an increase in binding to tissue factor pathway inhibitor (TFPI)¹⁰, and an increase in platelet aggregation through the interaction of apo(a) with a specific receptor at the platelet surface¹¹ which induces endothelial dysfunction¹²⁻¹³. In transgenic mice, Lp(a) has been shown to increase the concentration of cholesterol-rich remnant lipoproteins and the atherosclerosis lesion area in the aortic root¹⁴. Moreover, Lp(a) shows a synergistic effect with homocysteine¹⁵ another atherogenic particle.

It is also important to note that apo(a) has properties independent of Lp(a). For example, oxidized proinflammatory phospholipids in apoB-100 particles are preferentially sequestered on Lp(a),¹⁶ which induces avid uptake by monocyte-macrophages recruits¹⁷. Another very interesting study shows evidence that the origin of oxidized phospholipids in Lp(a) is apo(a) rather than

LDL, independent of plasma concentration or the isoform of apo(a)¹⁸. It has been shown that vascular endothelial cell growth, migration, contraction and cell permeability^{19, 20} are all induced by apo(a).

The evidence suggests that there is no single pathophysiological mechanism leading to the development of CAD and induced by Lp(a). Several mechanisms may be involved, and different populations may display different sensitivities. The association between Lp(a) and cardiovascular disease has been the subject of numerous studies, in vitro and in vivo. Population retrospective studies consistently support a pathophysiologic role for Lp(a), but prospective studies have been inconsistent. Two meta-analyses of prospective studies^{21, 22} with at least 1 year of follow-up showed a risk ratio of 1.40 (95% CI 1.22–1.57) and 1.6 (95% CI 1.4 to 1.8), respectively. These features demonstrate a clear association between Lp(a) and CAD in a Caucasian population, although this conclusion is not necessarily generalizable to a different population with distinct genetic profiles. Mexicans living in the United States have decreased Lp(a) plasma concentrations relative to non-Hispanic whites²³. Native Mexicans and the Mestizo group living in Mexico²⁴ have significant differences in Lp(a) concentration and apo(a) phenotype distribution between them, and there is evidence that atherothrombotic disease is associated with apo(a) fibrin-binding and small size apo(a) in this population²⁵.

In the present work we analyze the impact of apo(a) on the course of cardiovascular and cerebrovascular diseases in a population using a functional approach (the binding of apo(a) and fibrin), Lp(a) plasma concentration, and apo(a) phenotyping determined about eight years previously. Death, non-fatal myocardial infarction, unstable angina, need for myocardial revascularization

(percutaneous or surgical), and ischemic stroke were monitored over 8 years of follow-up.

Methods

We included patients born in Mexico who had a history of CAD defined as myocardial infarction and/or unstable angina (> 3 months) or stable angina and who had been followed up at the outpatient clinic of the National Institute of Cardiology "Ignacio Chavez". From January 1997 to December 1999, we conducted a case controlled study with the aim of evaluating the impact of Lp(a) in CAD and cerebrovascular disease. Sixty-eight patients with CAD were monitored and included in this analysis.

Patients were considered positive for type 2 diabetes mellitus if they were receiving hypoglycemic and/or insulin treatment or if fasting glucose levels were 126 mg/dL on 2 or more occasions. Patients were classified as having systemic hypertension with a prior diagnosis or with established antihypertensive treatment. Dyslipidemic patients had hypercholesterolemia (as determined by prior diagnosis, total cholesterol levels equal to or greater than 240 mg/dL, LDL cholesterol levels equal to or greater than 160 mg/dL or HDL cholesterol levels lower than 40 mg/dL) and/or hypertriglyceridemia (as determined by prior diagnosis, treatment with fibrates or serum triglycerides equal to or greater than 150 mg/dL). Patients were classified as prior smokers if they had a history of smoking 5 or more cigarettes a day and had abstained for longer than a year. Patients were considered active smokers if they smoked 5 or more cigarettes a day at the beginning of the study or had abstained for less than a year. All patients were treated with aspirin, statins, beta blockers and angiotensin-converting enzyme inhibitors (ACE-i).

The extent of CAD was estimated by determining the percentage of internal luminal narrowing. Involvement of one vessel was considered hemodynamically significant with more than a 50% reduction in the diameter of an important vessel (left anterior descending, circumflex or right coronary). Involvement of 2 vessels meant significant stenosis in two coronary vessels or a narrowing of 50% or more in the left main trunk, and involvement of 3 vessels meant significant stenosis in three coronary vessels.

As previously reported³³ Lp(a) plasma concentration was measured employing an immunonephelometric method (reagents and equipment from Beckman Co, Palo Alto CA, USA).

Apo(a) isoforms were detected by SDS-PAGE of plasma under reducing conditions. Apo(a) isoforms were identified using a standard composed of recombinant apo(a) containing 10, 14, 18, 26 and 34 kringles²⁶.

The binding assay is based on the ability of Lp(a) to compete with plasminogen for fibrin binding^{27, 28}. The amount of Lp(a) bound is expressed in nanomoles of apo(a) by referring to a calibration curve. The standard curve was obtained from a reference standard composed of equimolar amounts of five recombinant apo(a) isoforms of different lengths (10 to 34 kringles).

Data were collected from medical records or by telephone interviews. Major adverse cardiovascular and cerebrovascular events (MACCE) were recorded during follow-up. A comprehensive catalog of all causes of death, myocardial infarction, unstable angina and/or percutaneous revascularization or bypass surgery, and ischemic stroke was compiled. Myocardial infarction was defined as angina symptoms with ST-segment elevation and/or elevated necrosis cardiac markers at least 3 times above the normal value. Unstable

angina was defined as characteristic symptoms of angina *in crescendo* or angina at rest. Deaths were classified as either cardiac or not cardiac. Deaths that could not be classified were considered cardiac.

Statistics

We used descriptive statistics with median $\pm$ SD and median with minimum and maximum values in accordance with their distribution. The Kolmogorov-Smirnov test was used as evidence of normality. The student t test or the Mann-Whitney U test was used to compare continuous variables between groups. Categorical variables were reported as absolute values or percentages and compared with a chi-square test or Fisher exact test for frequencies lower than 5. Lp(a) concentration, binding and apo(a) size, as well as age, gender, traditional risk factors, three-vessel disease, and left ventricle systolic dysfunction were included in a logistic regression model. Apo(a) size was dichotomized for analysis at the 22K isoform. Low molecular weight apo(a) isoforms were designated by convention as $\leq 22\text{K-IV}$ repeats²⁹.

The primary end point of the study was the incidence of MACCE at follow-up. The primary end point was defined to be the event which occurred first. Events were counted only once,. We used tables of Kaplan-Meier survival to evaluate the primary endpoint as either death, non-fatal myocardial infarction, need for revascularization (percutaneous or surgical) or ischemic stroke. We censored cases that were losses in monitoring (patients lost to follow-up were considered at risk until the date of last contact, at which point they were removed from consideration). Comparison between groups was carried out using the logrank test (Log Rank). All tests were 2-tails. Differences were

considered significant if $p < 0.05$. All analyses were performed with SPSS (version 13.0).

Results

Clinical and demographic characteristics of patients are presented in Table 1. Prevalent risk factors in both groups were hypertension, dyslipidemia and smoking. At baseline, no differences were observed for age, sex, cardiovascular risk factors, profile of lipids, and medical treatment between both groups. The majority of patients were undergoing pharmacological treatment including aspirin, betablockers and ACEi. A few patients were taking statins and calcium channels blockers at baseline. There was a statistically significant difference in Lp(a) concentration in patients with small apo(a) isoforms compared with large apo(a) isoforms ($p=0.02$). Interestingly, there were no differences in apo(a) fibrin binding in the groups.

In the multivariate analysis (Table 2), apo(a) KIV $\leq$ 22 was an independent risk factor for cardiovascular and cerebrovascular events in patients with CAD (odds ratio (OR) of 4.4, CI of 1.24-15.62, $p=0.021$). Apo(a) binding and Lp(a) >30 mg/dL did show a tendency for an increased risk of MACCE, although without the statistical significance seen for apo(a) K-IV $\leq$ 22.

MACCE in the follow up.

Clinical follow-up was achieved in patients after an average of 6.9 years ± 3.04 . There were 3 deaths in the small kringle group (10%) and no deaths in the large kringle group ($p=0.081$). MACCE-free survival at follow-up was 27% in patients with apo(a) KIV $\leq$ 22 and 48% in patients with the larger apo(a) KIV $>$ 22 isoform ($P=0.051$). Incidence of MACCE at follow-up was significantly

lower in patients who had the large isoform compared to patients with the small isoform (Table 3).

Discussion

The evidence presented here shows that the best approach to evaluate the cardiovascular risk associated with Lp(a) is to determine the isoforms of circulating apo(a).³⁰ When just measuring plasma concentration of Lp(a), it is not always possible to find such an association³¹. Nevertheless, Kamstrup et al, in a recent meta-analysis of 36 prospective studies, found a modest association of elevated Lp(a) concentration with coronary heart disease and stroke³².

We previously reported³³ experimental evidence to support the hypothesis that smaller-sized isoforms of apo(a) increased the potential antifibrinolytic effect in ischemic cardiopathy and cerebrovascular disease. This effect was more evident in the cerebrovascular group, suggesting the existence of peculiar and poorly understood differences in antithrombotic mechanisms in the cerebral and coronary arteries of these patients.

In this study, we found an increase in MACCE even when follow-up was only two years after initial contact in the apo(a) KIV $\leq$ 22 group, demonstrating that, in a hospital-based population, small apo(a) isoforms are associated with the development of major adverse cardiovascular and cerebrovascular events. These data agree with other studies that found an association between Lp(a) levels and stroke and death from vascular disease in the elderly.³⁴

When we examined the relationship between apo(a) fibrin binding and MACCE, we did find an association, although it was not significant according to the multivariate statistical analysis. It is possible that mechanisms other than apo(a) fibrin binding could influence the thrombogenicity of apo(a) and the risk

of adverse cardiovascular events. Apo(a) has the ability to preferentially bind proinflammatory oxidized phospholipid (OxPL)³⁵, and it has been shown that there is an association between OxPL and small apo(a) isoforms despite differences in Lp(a) levels.

The interaction of Lp(a) with other particles likely enhances its atherogenic and thrombogenic properties. The combination of high Lp(a) plasma concentrations and LDL cholesterol levels increased the risk and advance of CAD³⁶. Also homocysteine can increase the antifibrinolytic effect of Lp(a) by dissociating apo(a) from the Lp(a) particle³⁷³⁸

This study does have limitations, including the fact that the analysis was retrospective and the fact that we did not include in the analysis the response of patients to different treatments. However, we believe that when the results presented here are carefully considered, we can conclude that there is a mild mortality risk associated with apo(a) K-IV $\leq$ 22 in Mexican patients with coronary artery disease. This association may prove clinically useful in risk stratification, helping identify patients with an increased risk of major adverse cardiovascular and cerebrovascular events in the long term.

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Table 1. Demographic and clinical characteristics of patients according to apo(a) size, (n= 68).

Variables	Apo(a) K-IV $\leq$ 22 n=30	Apo(a) K-IV >22 n=38	p
Age, median $\pm$ SD	51.8 $\pm$ 10.3	53.2 $\pm$ 10.7	0.557
Male, n(%)	24 (80)	34 (89)	0.318
Systemic hypertension, n(%)	15 (50)	18 (47)	0.829
Diabetes mellitus, n(%)	7 (23)	3 (7)	0.094
Previous smoking habits, n(%)	9 (30)	15 (39)	0.417
Present smoking habits, n(%)	16 (53)	17 (44)	0.481
Dyslipidemia, n(%)	14 (46)	21 (55)	0.481
Previous MI, n(%)	29 (96)	32 (84)	0.124
Body-mass index, median $\pm$ SD	26.7 $\pm$ 4.1	25.9 $\pm$ 2.9	0.341
Cholesterol (mg/dl) median $\pm$ SD	192 $\pm$ 42.8	196 $\pm$ 39.1	0.687
Triglycerides (mg/dl) median $\pm$ SD	215 $\pm$ 95.6	195 $\pm$ 102.4	0.417
LVEF (%)	51.2 $\pm$ 8.2	53.2 $\pm$ 10.7	0.389
LVEF low (<50%), n (%)	11 (36)	10 (26)	0.359
Multivessel disease, n (%)	5 (16)	5 (13)	0.740
Apo(a) fibrin binding, (median, minimum-maximum value)	0.14 (0.01-0.58)	0.13 (0-0.60)	0.546
Apo(a) high-affinity binding to fibrin, n (%)	7 (23.3)	5 (13.2)	0.274

Lp (a) lipoprotein (mg/dL), median (minimum-maximum value)	26 (0-128)	10 (0-128)	0.021
Medical treatment, n(%)			
- Aspirin	26 (86)	36 (94)	0.394
- Estatin	7 (23)	14 (36)	0.231
- Beta-blocker	16 (53)	19 (50)	0.785
- Calcium channel blockers	11 (36)	14 (36)	0.988
- ACE-i	11 (36)	19 (50)	0.272

LVEF= left ventricular ejection fraction, apo(a)K-IV $\leq$ 22= low molecular weight apolipoprotein-(a) isoforms, Lp(a)= lipoprotein-(a), ACE-i= angiotensin converting-enzyme inhibitors, SD= standard deviation, MI= myocardial infarction.

Table 2. Variables included in a logistic regression model

	Sig.	OR	95.0% CI	
			Lower	Upper
Male Gender	.611	.667	.14	3.17
Hypertension	.524	.704	.239	2.07
Diabetes Mellitus	.299	2.359	.467	11.916
Smoking	.536	1.443	.452	4.612
Dyslipidemia	.379	0.618	.211	1.805
Low LVEF	.931	1.052	.329	3.369
Multivessel disease	.366	0.472	0.093	2.398
Apo(a) KIV < 22K	.021	4.418	1.249	15.625
Apoa) binding	.276	2.354	.505	10.968
Lp (a) > 30 mg/dL	.563	1.547	.353	6.787

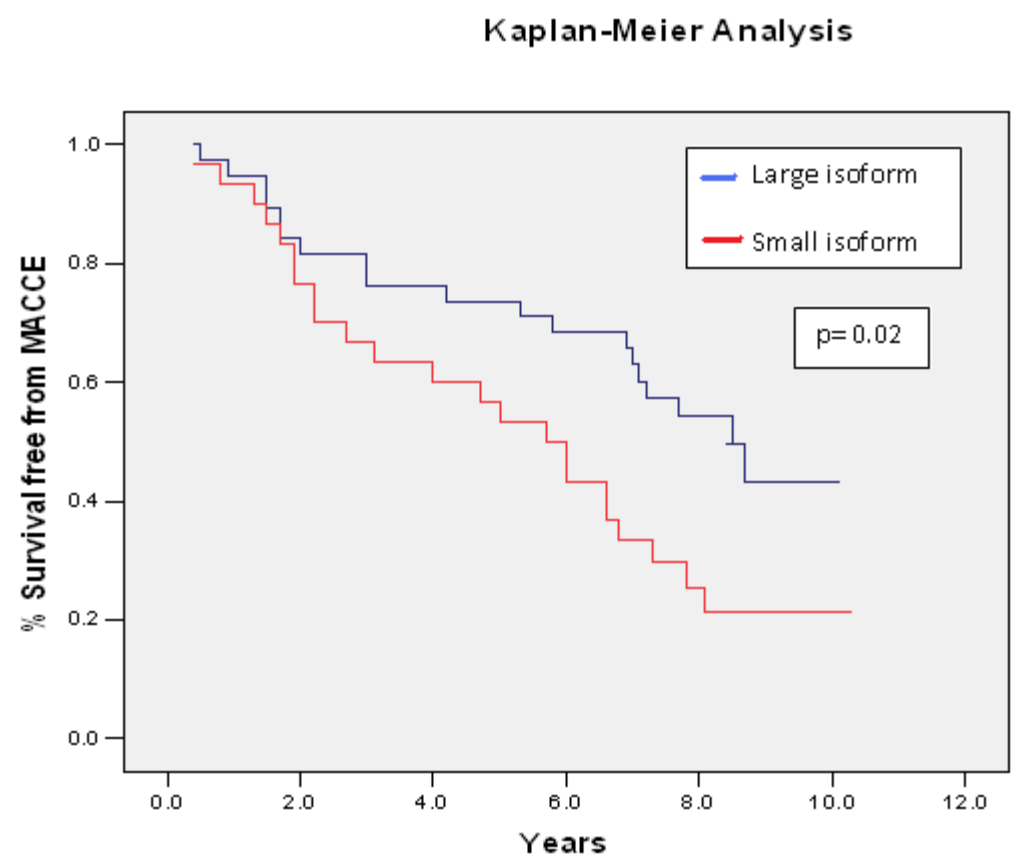
Lp(a)= lipoprotein(a); apo(a)= apolipoprotein(a); MI= myocardial infarction;
 LVEF= left ventricular ejection fraction; OR = odds ratio; CI= confidence
 intervals

Table 3. Incidence of major adverse cardiovascular and cerebrovascular events.

	Apo(a) KIV< 22, n=30	Apo(a) KIV >22, n=38	OR(95% CI)
Death	3 (10%)	0	2.4 (1.8-3.2)
- Cardiac	2 (6.7%)		
- Not cardiac	1 (3.3%)		
Myocardial infarction	4 (13.3)	5 (13.2)	1.0 (0.4-2.2)
Unstable angina	5 (16.7)	6 (15.8)	1.0 (0.5-2.1)
Ischemic stroke	4 (13.3)	1 (2.6)	1.9(1.1-3.2)
Coronary revascularization	3 (10)	2 (5.3)	1.4 (0.6-3.0)
MACCE	19 (63%)	15 (39%)	1.7(0.9-3.0)

OR=odds ratio; CI= confidence intervals

Figure 1.



Patients at risk

Apo(a) KIV<22	30	28	23	20	19	17	15	10	6	5
Apo(a) KIV>22	38	36	32	31	29	28	26	24	18	6
Total	68	64	55	51	48	45	41	34	24	11

Freedom from major adverse cardiovascular and cerebrovascular events (death, myocardial infarction, unstable angina, myocardial revascularization , and stroke) at 10 years.