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STUDIES ON \( \text{Lp(a)} \) DISTRIBUTION IN ULCRACTRIFUGALLY SEPARATED LIPOPROTEIN FRACTIONS

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SUMMARY
In this study we have determined how lipoprotein(a) (\( \text{Lp(a)} \)) influences the HDL-cholesterol level when present in human plasma. Six hundred and eighty one individuals were chosen for the study disregarding age, sex or illness criteria. Their plasma was processed in order to assess the cholesterol and triglyceride concentrations in HDL particles isolated both by MgCl\(_2\)-Phosphotungstic acid precipitation and differential ultracentrifugation in the density range of 1.063-1.21 kg/L. The presence of \( \text{Lp(a)} \) was identified noting the presence or not of a band with a pre-\( \beta \) electrophoretic mobility (“sinking pre-\( \beta \)” lipoprotein) in agarose gel electrophoresis of VLDL-free plasma (plasma infranatant at \( d > 1.006 \) kg/L). On this basis, the samples were classified as sinking pre-\( \beta (+) \) and sinking pre-\( \beta (-) \), respectively. \( \text{Lp(a)} \) was also quantified in plasma by means of an enzyme-linked immunoassay and a close correspondence between the existence of “sinking pre-\( \beta \)” lipoprotein, and a plasmatic concentration of \( \text{Lp(a)} \) higher than 300 mg/L was observed. The HDL-cholesterol level obtained by ultracentrifugation was higher than the one assessed by precipitation in the sinking pre-\( \beta (+) \) samples, but not in the sinking pre-\( \beta (-) \). There was a positive linear correlation between the difference of such HDL-cholesterol levels and plasma \( \text{Lp(a)} \) concentration. A \( \text{Lp(a)} \) concentration of 300 mg/L (value found in more than 27% of the population study) settles an ultracentrifugation HDL-cholesterol value that was 0.097 mmol/L higher than the precipitation HDL-cholesterol level.

The presence of \( \text{Lp(a)} \) in the lipoprotein fractions prepared by ultracentrifugation was determined. \( \text{Lp(a)} \) was mostly detected in the HDL fraction. This latter result together with the observation that \( \text{Lp(a)} \) precipitates along with LDL under the action of the MgCl\(_2\)-Phosphotungstic acid reagent, explains the discrepancy between HDL-cholesterol values commented above.

The proportion of \( \text{Lp(a)} \) present in the LDL fraction (density range: 1.006-1.063 kg/L) was in average one thire of that in HDL. \( \text{Lp(a)} \) in the VLDL fraction was detectable in plasma with a high concentration of \( \text{Lp(a)} \); in these samples, \( \text{Lp(a)} \) in VLDL was positively correlated with VLDL-triglycerides. In conclusion, \( \text{Lp(a)} \) is mostly separated along with HDL, but also LDL and even VLDL particles from ultracentrifuged plasma. This means a \( \text{Lp(a)} \) interference in the quantification of HDL-cholesterol by ultracentrifugation that is proportional to plasma \( \text{Lp(a)} \) concentration.

INTRODUCTION
In recent years, an increasing interest about \( \text{Lp(a)} \) has raised because its double atherogenic and antifibrinolytic character (1), could be an independent risk factor for coronary artery disease (2-5). In normolipemic individuals, a plasma \( \text{Lp(a)} \) concentration of 300 mg/L results in a two-fold risk for coronary artery disease when compared to the general population (2, 3). On the other hand, \( \text{Lp(a)} \) is the most powerful di-
Lipoprotein(a) consists of the association of one apolipoprotein(a) molecule to a LDL particle linked by a disulfide bond to Apo B-100 (8). Apolipoprotein(a) is a glycoprotein with a high content in syalic acid, which confers the characteristic electrophoretic pre-β mobility. Lipoprotein(a) ranges in density from 1.04 to 1.11 kg/L (9). Due to the aforementioned features, Lp(a) was early identified as “sinking pre-β” lipoprotein, which means a band with pre-β mobility that was present in d> 1.006 kg/L plasma infranatant of some subjects (10).

Apolipoprotein(a) shows a high degree of homology with plasminogen (11, 12). It has a serine-protease domain (94% identity to plasminogen homologous domain) and two kinds of homologous kringle-plasminogen domains: one copy of kringle-5 and 15-37 copies of kringle-4 (12, 13).

Lipoprotein(a) competes with plasminogen for its binding-site to fibrin (14, 15), fibrinogen (15, 16), and different cellular types of receptors (17, 19), with equivalent capacities and affinities (10), through the kringle-4 domain.

It has been estimated that at plasma concentration of 300 mg/L, Lp(a) reduces cellular plasminogen binding by 20%, thereby lowering fibrinolytic capacity (18). On the other hand, there is an accumulation of Lp(a) in atherosclerotic arteries but not in normal blood vessels, while the opposite situation is found with plasminogen (17). These characteristics can explain the association between high Lp(a) concentration and coronary artery disease.

To assess atherogenic risk, in routine analysis we measured HDL concentration by two methods separately: plasma precipitation with MgCl₂-Phosphotungstic acid and by differential ultracentrifugation of plasma in density equilibrium (1.063-1.21 kg/L). In the first method, polyanions interact with plasma lipoprotein precipitating VLDL, LDL, and Lp(a) fractions, selectively (20), while Lp(a) overlaps with HDL particles at their density range (9); as a matter of fact, in the present study we have analysed the presumed influence of Lp(a), when present in plasma, on HDL-cholesterol values obtained by ultracentrifugation versus precipitation.

SUBJECTS, MATERIALS AND METHODS
Subjects
The study was carried out with plasma from 681 individuals, 354 females and 327 males. Considering the methodologic finality of this work, we did not apply any exclusion criterium, so the population studied consisted of presumed healthy, hyperlipemic or endocrinologically altered subjects. Plasma cholesterol and triglyceride levels were 6.32 ± 0.08 (1.96-17.11) mmol/L and 1.88 ± 0.12 (0.39-50.17) mmol/L, respectively.

Lipoprotein analysis
Blood was drained from subjects after a 10-12 hours fasting period, in tubes containing Na₂-EDTA as anticoagulant (final concentration 1 g/L). Plasma total cholesterol and triglyceride levels were enzymatically determined (Menarini and Boehringer Mannheim, respectively) using an HITACHI 705 autoanalyzer. A plasma aliquot was precipitated with MgCl₂-Phosphotungstic acid (Boehringer-Mannheim) and cholesterol and triglyceride concentration in supernatant fractions (ppHDL) were determined. Another plasma aliquot was placed in Beckman 50Ti rotor tubes, the volume was completed with 0.189 mol/L MaCl, 1 mmol/L EDTA (d= 1.006 kg/L) and was centrifuged for 18 hours at 45.000 rpm 10°C, in a Beckman L5-50 ultracentrifuge, in order to isolate VLDL particles in the supernatant. The infranatant fraction was adjusted to a density of 1.063 kg/L with solid KBr (82.1 g/L) and was again centrifuged for 20 hours at 47.000 rpm, 10°C. LDL particles were recovered in the supernatant and the infranatant was transferred to other tubes, the density was again adjusted to 1.21 kg/L with solid KBr (221 g/L) and was finally centrifuged for 44 hours at 47.000 rpm, 10°C. This latter supernatant (ucHDL) was used for cholesterol and triglyceride determinations as before.

Supernatant (VLDL fraction) and infranatant (VLDL-free plasma) aliquots from the centrifugation at d= 1.006 kg/L, as well as total plasma were subjected to zonal electrophoresis. This electrophoresis was carried out on 0.5% agarose gel plates (Beckman Paragon-Lipo) with barbital buffer (pH 8.6) and lipid staining with Sudan-black. Samples that showed a pre-β mobility band on visual examination as the VLDL-free plasma were identified as sinking pre-β(+).
Lipoprotein(a) was quantified by enzyme-linked immunoassay (Biopool, TintElize Lp(a), Sweden) with polyclonal antibodies against purified Lp(a) and lyophilized human plasma as control and the results were expressed as total Lp(a) (mg/L).

Calculation and statistical treatment
The results showed in the text correspond to mean ± standard error (range). Visual identification of Lp(a) in electrophoresis was compared with Lp(a) values assessed by enzyme-linked immunoassay, and a value of 300 mg/L was taken as reference limit (3). Specificity was calculated as the percentage of true negative cases related to false positive plus true negative cases. Sensibility was obtained as the percentage of true positive cases related to true positive plus false negative cases. Finally, efficiency was calculated as the percentage of true positive plus true negative cases related to all cases.
Groups were statistically compared by means of the t-Student test. Mathematical equations and plots were performed by means of the STATGRAPHICS statistical package (Statistical Graphics Corporation) in an IBM PS-2, 55SX computer.

RESULTS
Individuals with Lp(a) were firstly identified regarding the existence of a band with a pre-β electrophoretic mobility in the VLDL-free plasma. Such a band was present in 23.1% of the studied subjects who were classified as sinking pre-β(+). Lipoprotein(a) was assessed in the whole set of samples: 524 sinking pre-β(−) and 157 sinking pre-β(+); their respective frequency distributions are shown in figure 1. As can be seen, both populations are definitely different since, in the sinking pre-β(−) less than 7% showed Lp(a) values higher than 300 mg/L, while in the sinking pre-β(+) more than 96% of the individuals had Lp(a) values higher than 300 mg/L.
The assessment of the results shows that the appearance of the “sinking pre-β” band related to Lp(a) quantification has a specificity of 99%, a

Figure 1: Frequency distribution of plasma Lp(a) concentration values in sinking pre-β(−) (upper panel) and sinking pre-β(+) (lower panel) samples.
Plasma Lp(a) concentration was determined in 681 samples, 524 sinking pre-β(−) and 157 sinking pre-β(+). The classification into sinking pre-β(+) or sinking pre-β(−) was established by the presence of a pre-β band on agarose electrophoresis of VLDL-free plasma (“sinking pre-β”).

Figure 2: Frequency distribution of (ucHDL-ppHDL) cholesterol values in sinking pre-β(−) (upper panel) and sinking pre-β(+) (lower panel) samples.
Plasma from 681 subject was processed separately to determine HDL-cholesterol by plasma ultracentrifugation at density range of 1.063-1.21 kg/L (ucHDL) and plasma precipitation with MgCl₂-Phosphotungstic acid (ppHDL). Samples were subdivided into sinking pre-β(−) or sinking pre-β(+) regarding to their electrophoretic pattern (see foot to figure 1).
sensibility of 81% and an efficiency of 96%. Therefore, the classification of individuals into sinking pre-β(+) or sinking pre-β(−) groups, was suitable for our purposes.

A highly significant correlation was found between HDL-cholesterol levels from precipitated (ppHDL) and ultracentrifuged (ucHDL) samples. In sinking pre-β(−) individuals the regression equation between ucHDL and ppHDL was y = 0.939x + 0.100 mmol/L (r = 0.931, n = 456, p < 0.001) while in the sinking pre-β(+) group the equation was y = 0.954x + 0.253 mmol/L (r = 0.887, n = 149, p < 0.001). The higher independent value for the latter regression line suggested an additive effect of Lp(a) on HDL-cholesterol values obtained by ultracentrifugation. In order to analyze such a possibility, differences between ucHDL-cholesterol and ppHDL-cholesterol values for the same sample, were calculated. These differences were 0.020 ± 0.008 mmol/L and 0.190 ± 0.017 mmol/L in the sinking pre-β(−) and sinking pre-β(+) groups, respectively (p < 0.001). The frequency histogram delineates a normal distribution for sinking pre-β(−) cases (Figure 2, upper panel), with a mode close to zero, while shift to the right can be seen in the sinking pre-β(+) group (Figure 2, lower panel), in more than 90% of the cases the difference being greater than zero. These results reveal a suitable agreement between HDL-cholesterol values obtained by means of ultracentrifugation and those from precipitation in the sinking pre-β(−) group, but there exists significant differences in the sinking pre-β(+) group, in which the values obtained from ultracentrifugation were higher than those from precipitation.

Triglyceride concentration was determined in the same samples (ucHDL and ppHDL). Not significant differences between ucHDL-triglyceride and ppHDL-triglyceride were observed in any group (data not shown). Differences between ucHDL and pp HDL - triglyceride were calculated and the results are shown as a frequency histogram (Figure 3). As can be seen, values approach a normal distribution in both groups, there being no significant differences between them (sinking pre-β(−)): -0.037 ± 0.004 mmol/L; sinking pre-β(+) : -0.046 ± 0.002 mmol/L; p > 0.07). Therefore, the presence the Lp(a) in a sample does not affect the ultracentrifugation HDL-triglyceride values as compared to precipitation ones.

The fact that HDL-triglyceride levels measured in precipitated plasmas are higher than in ultracentrifuged ones (as observed from the negative difference obtained) must be attributed to the presence.

Figure 4: Linear correlation between (ucHDL-ppHDL) cholesterol values and plasma Lp(a) concentration. Regression line was adjusted to y = 0.00039 - 0.023 (r = 0.443, p < 0.001, n = 592).
ce of free glycerol, that is obviously assessed in the sample submitted to precipitation but in the ucHDL sample it is diluted three times due to methodologic requirements. In fact, a mean plasma glycerol concentration of 0.1 mmol/L would result in a −0.066 mmol/L difference between ucHDL-triglyceride and ppHDL-triglyceride values, which is close to the one found. Regression lines between (ucHDL-ppHDL) differences on one side and plasma Lp(a) concentration on the other, were analyzed. For this, both sinking pre-β(−) and sinking pre-β(+) samples were included. In figure 4 can be observed the positive lineal correlation between (ucHDL-ppHDL) cholesterol and Lp(a) concentration (y = 0.00039x - 0.023, r = 0.443, n = 592, p < 0.001). On the contrary, (ucHDL-ppHDL) triglyceride values were not significantly correlated to plasma Lp(a) concentration (y = 0.000023x - 0.049, r = 0.113, n = 577, p > 0.006) (Figure 5).

Lipoprotein(a) distribution among the different lipoprotein fractions was assessed by directly determining Lp(a) concentration in ultracentrifuged HDL and LDL (ucHDL and ucLDL) samples from 42 patients which were selected to sample a wide variety of Lp(a) concentrations. Correlations between Lp(a) in ucHDL and ucLDL subfractions and plasma Lp(a) concentration were analyzed separately. A positive lineal correlation between Lp(a) values in ucHDL and plasma Lp(a) concentration (y = 0.595x + 16.7, r = 0.957, n = 42, p < 0.001) was observed (Figure 6). Lipoprotein(a) values in ucLDL and plasma Lp(a) concentration were also significantly correlated (y = 0.195x - 0.31, r = 0.776, n = 42, p < 0.001) (figure 7) but the slope was 3 times lower than in the previous case. Finally, the Lp(a) in ucHDL/Lp(a) in ucLDL ratio was observed to be negatively correlated plasma Lp(a) concentration (y = -0.011x + 11.8, r = -0.569, n = 29, p < 0.002) (Figure 8).
To further confirm that Lp(a) is the agent that causes overestimation of HDL-cholesterol when assessed by ultracentrifugation, regression between the difference ucHDL-cholesterol minus ppHDL-cholesterol (ucHDL-ppHDL) and the value of cholesterol corresponding to Lp(a) in ucHDL (as theoretically estimated considering that one third of Lp(a) mass is cholesterol (21)), was established and a positive correlation was obtained (y = 1.244x - 0.0067, r = 0.790, n = 29, p < 0.001) (Figure 9).

To check whether Lp(a) would interfere with HDL-cholesterol evaluation after plasma precipitation, we determined Lp(a) concentration in MgCl₂-foosfotungstic acid supernatants from plasmas with high Lp(a) concentration, and found non detectable Lp(a) in any cases.

Lipoprotein(a) was analyzed in VLDL, fractions from the aforementioned 42 individuals to further study the Lp(a) distribution in lipoprotein fractions obtained by ultracentrifugation. In only 8 of 42 cases Lp(a) was detectable and their VLDL-Lp(a) accounted for 7% of whole plasma. When a value of 5 mg/L (half the sensibility limit) was assigned to those samples with undetectable Lp(a), a significant and positive correlation between Lp(a) values in VLDL and plasma triglyceride concentration was seen, but a more dramatic correlation was found when the group of individuals with plasma Lp(a) higher than 300 mg/L (data not shown) was separately considered. To confirm such an interaction, regression between the percentage of Lp(a) recovered in VLDL fraction and the VLDL-triglyceride concentration was analyzed, and a close correlation was found in patients with plasma Lp(a) higher than 300 mg/L (y = 1.40 + 0.48, r = 0.752, n = 27, p < 0.001) (Figure 10).

Finally, once demostrated that Lp(a) could be separated even within VLDL fractions in some individuals the possibility that the double pre-β phenotype (22) could correspond to the presence of Lp(a) in the d< 1.006 kg/L fraction, was raised. On such a basis, Lp(a) concentration from 16 VLDL samples that showed the double band in agarose gel electrophoresis was determined. Contrary to the hypothesis, lipoprotein(a) was undetectable in all of them.

As the population under study was a heterogenous group comprising of individuals suffering from different diseases, we were interested in comparing the Lp(a) values observed to those from a normal population. For this, a group of 321 individuals, 229 females and 92 males, from the Hospital Ramon y Cajal working population with a range of age from 22 to 65 years, were randomly chosen and studied. Results corresponding to both

![Figure 8: Lineal correlation between Lp(a) in ucHDL / Lp(a) in ucLDL mass ratio, and plasma Lp(a) concentration. Regression line was adjusted to y = 0.011x + 11.86 (r = 0.569, p < 0.002, n = 29). Samples with undetectable Lp(a) in either ucHDL or ucLDL were discarded.](image1)

![Figure 9: Lineal correlation between (ucHDL-ppHDL) cholesterol values, and Lp(a) cholesterol in ucHDL. Lp(a) cholesterol in ucHDL was estimated by considering Lp(a) cholesterol content as one third of Lp(a) total mass. Regression line was adjusted to y = 1.244x - 0.0067 (r = 0.790, p < 0.001, n = 29).](image2)
population are depicted in Figure 11. Distribution of Lp(a) concentration in patients (Figure 11, upper panel) was similar to that in the working population (Figure 11, lower panel), with values of 147±8.8 mg/L (median ± standard error) and 130 ±10.3 mg/L, respectively. Nevertheless, the percentage of individuals with Lp(a) higher than 300 mg/L was 27.5% in patients and 25.5% in the working population.

DISCUSSION

In the present work, the interference of Lp(a) on HDL isolated by ultracentrifugation has been evaluated. This study was justified by the fact that Lp(a) has a density range (d= 1.04 - 1.11 kg/L, approximately) which overlaps the separation limit of LDL and HDL particles (9). Thus a fraction of Lp(a), if present, is obtained along with HDL particles by ultracentrifugation. On the contrary, as we have also demonstrated in this work, MgCl2-Losphotungstic acid reagent precipitates Lp(a) and so it is quantified with LDL particles with these kind of precipitation methods.

Early in this work, Lp(a) presence in samples was identified noting the appearance of a band with pre-β mobility in VLDL-free plasma. Identification was carried out by visual examination and then samples were classified into sinking pre-β(+) and sinking pre-β(−), respectively. Using this method, lipoproteins separated in this way turned out to be Lp(a), as others authors have reported (10). When comparing this method with enzyme-linked immunoassay Lp(a), more than 96% sinking pre-β(+) samples have a Lp(a) higher than 300 mg/L (specificity = 99%). As efficiency was also very high (>96%), this result allowed us to keep the aforementioned two groups for the methodological aim we were searching for.

In sinking pre-β(+) samples, ucHDL-cholesterol values were significantly higher than the ones from precipitated plasma. Which indicates that, differential ultracentrifugation partially renders Lp(a) along with HDL particles, as could be predicted from Lp(a) physical features. Therefore, these HDL-cholesterol values are partially artificial. Regress on analysis (Figure 4) showed that a Lp(a) concentration of 300 mg/L determines a value 0.097 mmol/L higher for ucHDL-cholesterol than ppHDL-cholesterol.

This observation was earlier reported by Bachorik et al. (25) in some, but not all, of their samples series studied and in fact, this finding had limited repercussion. Nevertheless, the artifact caused by Lp(a) in ucHDL-cholesterol value is nor irrelevant nor anecdotal since in the population under study, mean Lp(a) concentration was 226 mg/L and more than 27% showed plasma levels higher than reference value of 300 mg/L. Therefore, these results are in contrast to the general assumption that ultracentrifugation is the reference method for separation and quantification of HDL particles. The perfect correlation between ucHDL-cholesterol values and ppHDL levels is only achieved in Lp(a) free samples.

Lipoprotein(a) presence does not remarkably interfere with ucHDL-triglyceride values. Firstly, the difference ucHDL-ppHDL did not correlate with plasma Lp(a) concentration (Figure 5) and secondly, this difference is similar in sinking pre-β(+) and sinking pre-β(−) samples (Figure 3). The explanation must be sought in the scant content of triglyceride in Lp(a) (<5%) (21) and so Lp(a)-tri
glyceride contribution to HDL fraction has little significance.

As is demonstrated here, Lp(a) is mostly isolated with HDL particles by ultracentrifugation. However, it is also separated with LDL particles,
as can be seen from the correlations obtained between Lp(a) amount in those fractions and total plasma Lp(a) concentration (Figures 6 & 7). We have even observed that the HDL-Lp(a)/LDL-
Lp(a) ratio decreases as plasma Lp(a) concentration increases (Figure 8). On the basis of the negative correlation described between apo(a) size and plasma Lp(a) concentration (26), it can be suggested that Lp(a) shifts to LDL range in a subject with a low molecular weight apo(a). Interestingly, even in the VLDL density range Lp(a) was detected.

This finding is in agreement with the observations by Selinger et al. (27) and Borsot et al. (28) who detected Lp(a) in d < 1.006 Kg/L fraction from hypertriglyceridemic subjects. We have observed that Lp(a) concentration in VLDL fraction is positively correlated with VLDL-triglyceride concentration in patients with Lp(a) higher than 300 mg/L. These results clearly demonstrate that, although to a minor extent, apo(a) is also associated to triglyceride-rich lipoproteins. On this basis, we were interested in ascertaining the possible correspondence between double pre-β phenotype (22) and the presence of Lp(a) in VLDL fraction. None of the 16 cases with such a phenotype had a detectable Lp(a) in the d<1.006 Kg/L fraction. Therefore, the additional pre-β band, which is characteristic of this double pre-β phenotype, is not Lp(a).

Finally, in this work plasma Lp(a) concentration was measured in a significant sample population from the Hospital Ramón y Cajal employees, that could be representative of the general adult population. Approximately, 25% of the studied subjects had Lp(a) concentration higher than 300 mg/L, thus having at high risk for coronary artery disease. Comparison of our results with those by others, is difficult due to different methods used yet there does not exist a control nor standardization for all of them and there are even disagreements when depicting results (total Lp(a) mass versus apo-Lp(a) mass or apo(a) mass). On the other hand, since plasma Lp(a) concentration does not fix a gaussian distribution, mean values have little significance.

In spite of this, our median values are similar (6,29) or slightly higher (4,9) than the ones reported in other European populations.

In conclusion, Lp(a) is mostly separated along with HDL, but also with LDL and even VLDL particles from ultracentrifuged plasma. This means a Lp(a) interference in ucHDL-cholesterol levels proportional to plasma Lp(a) concentration. Consequently, ucHDL-cholesterol values are clearly higher than ppHDL-cholesterol levels in Lp(a) containing samples. This interference is not an anecdote since 25% of the general population shows a plasma Lp(a) concentration higher than 300 mg/L.

Figure 11: Frequency distribution of plasma Lp(a) concentration in patients (upper panel) and the Hospital Ramón y Cajal working population (lower panel).
The study populations consisted in 681 patients suffering from different diseases who were submitted to lipoprotein profile study for diagnostic reasons, and 321 presumed healthy, workers from Hospital Ramon y Cajal who were randomly chosen, respectively.
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