Cardiovascular Topics

Hyperhomocyst(e)inemia is an important risk factor for vascular disease in subjects with high-molecular weight apo(a) isoforms

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Summary

Background: **Homocyst(e)ine is reported to increase the binding of lipoprotein(a) [Lp(a)] to fibrin, which may increase the thrombogenic effects of Lp(a) *in vivo*. The aim of this study was to investigate whether there is a relationship between homocyst(e)ine and Lp(a) levels and vascular disease risk, and if the relationship depends on the apo(a) isoforms.**

Methods: **A case-control study was performed in 91 Caucasian male subjects with vascular disease due to atherosclerosis, and in 100 healthy age- and sex-matched control subjects.**

Results: **Both hyperhomocyst(e)inemia and elevated Lp(a) were significantly more prevalent in patients. Concordant elevated Lp(a) and hyperhomocyst(e)inemia were not associated with increased vascular disease risk (relative odds 2.96; 95% CI: 0.90-9.80), while hyperhomocyst(e)inemia in the absence of elevated Lp(a) was associated with increased vascular disease risk (relative odds 7.20; 95% CI: 2.37–21.91). Hyperhomocyst(e)inemia in individuals with high-molecular weight apo(a) isoforms [smaller apo(a) isoform > S3] was observed to be associated with increased vascular disease risk (relative odds 11.02; 95% CI: 3.54–34.30), while vascular disease risk in subjects with low-molecular weight apo(a) isoforms [smaller apo(a) isoform < S3] was not significantly increased, the relative odds being 1.92; 95% CI: 0.51–7.24.**

Conclusions: **We conclude that hyperhomocyst(e)inemia is an important risk factor in individuals with high-molecular weight apo(a) isoforms.**

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Cardiovasc J South Afr 2004; **15**: 207–213. www.cvjsa.co.za

The association between elevated plasma Lp(a) levels and increased risk for vascular disease is well established,[1-10] although negative findings have also been reported.[11-16] A variation at the apo(a) gene locus and the apo(a) size polymorphism, which determines Lp(a) levels to a great extent, are also determinants of coronary artery disease (CAD) risk.[17] Small apo(a) isoforms are associated with increased risk for CAD.[18-26]

Although *in vitro* and population studies suggest that elevated plasma Lp(a) levels are causally related to atherosclerosis, the process of atherogenesis is not fully understood. Increased Lp(a) levels are associated with ischaemic heart disease in particular when LDL levels are also increased.[27,28] Howard and Pizzo speculated that Lp(a) is not atherogenic on its own but needs to be modified structurally or needs the presence of evolving atherosclerotic vascular disease to exhibit its pro-thrombotic effects.[29] Intact Lp(a) may cross the vascular endothelial layer, and in the intima it may undergo structural changes due to interaction with fibrin, free radicals, proteoglycans or glycosaminoglycans.[30] After modification, Lp(a), like LDL, may be taken up by macrophage receptors, resulting in the formation of foam cells,[31] although *in vitro* studies of Lp(a) uptake by macrophages have been less than convincing.[32-34] Modified Lp(a) is also reported to increase monocyte chemo-attractant activity of vascular endothelial cells,[35] stimulate smooth muscle cell proliferation,[36] and impair endothelium-dependent vasodilation.[37]

Due to the structural homology with plasminogen, Lp(a) may affect fibrinolysis by competing with plasminogen for binding to endothelial cells,[38,39] fibrin,[40,41] fibrinogen and fibrin fragments,[42,43] mononuclear cells and platelets.[44] The binding of plasminogen to fibrin is mediated by lysine–fibrin-binding sub-sites localised to Kringles I and IV of plasminogen.[45,46] The accessibility of these binding sites as well as the individual's Lp(a) phenotype may

determine the ability of Lp(a) to compete with plasminogen for fibrin binding.[47,48] As a result, Lp(a) acts as a competitive antagonist for plasminogen, and thrombogenesis may be promoted *in vivo* if Lp(a) levels are increased. Even at low plasma concentrations, homocysteine increases the binding of Lp(a) to fibrin, especially when Lp(a) levels are increased, and this may contribute to the atherogenicity and thrombogenicity of Lp(a).[49] Furthermore, free apo(a) can more readily inhibit the fibrinolytic system than complete Lp(a), and homocysteine is reported to release apolipoprotein(a) from Lp(a) when total homocyst(e)ine (sum of free and protein-bound homocysteine and homocystine) exceeded 22 µmol/l.[50]

The risk for vascular disease associated with both hyperhomocyst(e)inemia and elevated plasma Lp(a) levels has been investigated in several studies,[8,9,24,51-56] but in none of these studies has the possible interaction between the apo(a) size polymorphism and circulating total homocyst(e)ine concentrations been analysed. The various apo(a) isoforms that are distinguished by a different number of Kringle IV repeats differ also with respect to their affinity for fibrin.[47,48] We hypothesised that the interactive effect between Lp(a) and homocysteine, which is postulated to enhance thrombogenesis *in vivo*, may depend on the size of the individual apo(a) isoform. To investigate our hypothesis, we determined apo(a) phenotypes together with serum Lp(a) and plasma total homocyst(e)ine (tHcy) concentrations in patients with coronary or peripheral vascular disease (VD) due to atherosclerosis, and apparently healthy age- and sex-matched controls from the same geographic region.

Aim

The aim of the study was to assess whether there is a relationship between circulating Lp(a) concentrations, apo(a) isoform size, plasma tHcy concentrations and risk for vascular disease.

Methods

Patients and controls

Ethical approval was granted by the Human Ethics Committee of the University of Pretoria to perform this serial study on male Caucasian patients with clinical symptoms of vascular disease who were admitted for angiography to the Pretoria Heart and Unitas Hospitals. Apparently healthy male controls with no acute or chronic disease, or history of vascular disease were recruited from governmental and semi-private business institutions in such a way that the control group represented the same socio-economic subclasses that constituted the patient group.

From these two groups, 100 controls [mean (SD) age 49.1 (6.6)] and 91 patients with vascular disease due to atherosclerosis [mean (SD) age 49.5 (6.3)] were selected according to age, to perform a case-control study in which the possible interaction between plasma tHcy, Lp(a) and apo(a) isoform was assessed. Eighty-four patients had angiographically documented coronary artery disease (CAD) (> 50% stenosis of a major epicardial coronary artery), while four had angiographically documented peripheral vascular disease (PVD) of the iliac or femoral vessels, and three had both CAD and PVD. Of the 84 patients with CAD, 44 had survived a myocardial infarction, coronary artery bypass grafting or percutaneous transluminal angioplasty, but not within the three months preceding angiography. The rest of the patients had newly diagnosed VD. A questionnaire was completed by all subjects and provided information concerning age, general health status, current medication used, and current smoking practice.

Laboratory analyses

Blood samples with EDTA as anticoagulant, and clotted blood samples were collected from the patients after an overnight fast prior to angiography. Fasting blood samples were also collected from the apparently healthy controls. The EDTA samples were kept on ice until the plasma was separated within 30 minutes by low-speed centrifugation. The plasma was aliquoted and transported on dry ice to the laboratory where it was stored at –80°C until the analyses were performed. The serum samples were left to clot and were separated within four hours at the laboratory. A biochemical profile was determined on all subjects with an automated analyser (results not shown) to identify individuals with renal or hepatic dysfunctions.

Plasma Lp(a) concentrations were measured in batches using a radio-immunoassay method [Mercodia Apo(a) RIA, Mercodia AB, Uppsala, Sweden] within six months of collection and were recorded in mg/dl. The day-to-day analytical coefficient of variation of the method was 4.6%. The lipid profiles were determined either the same or the following day. Serum total cholesterol (TC), determined with an automated analyser (Technicon DAX® System; Miles Inc, Tarrytown, New York 10591-5097, USA), and high-density lipoprotein cholesterol (HDL-C), determined with the CHOD-Iodide method (14350 Merckotest® Merck, Darmstadt, Germany), were used to calculate serum low-density lipoprotein cholesterol (LDL-C) concentrations. Serum triglycerides were determined with an automated analyser (Technicon DAX® System; Miles Inc, Tarrytown, New York 10591-5097, USA) and serum apolipoprotein A_1 and B (Apo A and Apo B) were determined with nephelometry (Behringwerke AG, Marburg, Germany).

Apo(a) phenotyping was performed by SDS agarose gel electrophoresis. The method was based on the one described by Kraft *et al*.[57] Plasma samples (2–10 µl, depending on the plasma Lp(a) concentrations) were diluted with a reducing buffer to a volume of 100 µl and boiled for 10 minutes in a water bath. The buffer contained 3.8% (w/v) sodium dodecyl sulfate (SDS), 7.7% (v/v) β-mercaptoethanol, 0.077% (w/v) bromophenol blue and 0.77% (v/v) glycerol. The final Lp(a) concentration was approximately 20 ng/µl. The gel contained 1.5% (w/v) ultrapure agarose (Agarose, L, Cat. No. 17-04240-02, Pharmacia Biotech, Uppsala, Sweden), 90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA and 0.1% (w/v) SDS. The size of the gel was $15 \times 25 \times 0.4$ cm ($L \times W \times H$) with 30 slots of 2-mm depth and 1.5-mm width. One microlitre of the standard or sample was loaded under buffer into the slots.

Electrophoresis was performed at a constant 15 W for seven hours with a Pharmacia gel electrophoresis apparatus (GNA-200, Pharmacia Biotech, Uppsala, Sweden) and a

Pharmacia power supply (EPS 600 Pharmacia Biotech, Uppsala, Sweden). The gel was cooled down to 25°C in a buffer containing 45 mM Tris-HCl, 45 mM boric acid, 2 mM EDTA and 0.1% (w/v) SDS. Semi-dry electroblotting was performed to nitrocellulose filters (PolyScreen® PVDF Transfer membrane, Cat. No. NEF-1002, Du Pont Biotechnology Systems, Boston, Massachusetts, USA) with an LKB transfer system over 120 minutes at 2.5 mA/cm^2. A transfer buffer system was used with two anode buffers (0.3 M Tris, pH 10.4 and 25 mM Tris, pH 10.4) and one cathode buffer (25 mM Tris, 40 mM glycine and 40 mM 6-amino-*n*-hexanoic acid, pH 9.4), all containing 10% methanol. The gel was equilibrated for 5 min in the cathodic buffer and the transfer membrane was immersed in methanol for a few seconds, left in water for five minutes to elute the methanol, and equilibrated for five minutes in the second anode buffer.

The nitrocellulose filter was blocked overnight at 4°C in a solution of 1% (w/v) bovine serum albumin, 10 mM Tris-HCl, 85 mM NaCl and 0.2% Triton X-100. The membrane was incubated for two hours at 37°C, in blocking solution to which the first antibody, apo(a)-specific 1A^2 monoclonal (mouse-anti human) antibody was added. After extensive washing the blot was exposed for one hour to the second antibody, which was a horseradish peroxidase conjugated polyclonal (sheep anti-mouse) antibody (Cat. No. NA 9310, Amersham, Life Sciences, UK). After further washing, an ECL-detection system (ECL Western blotting detection reagents, Cat. No. RPN 2106, Amersham, Life Sciences, UK) was used for detection on a Fuji RX-V 18 × 24 PT 361515 film.

The various apo(a) isoforms were designated according to the number of Kringle IV repeats[56] by comparing the electrophoretic mobility of the isoforms with that of a standard in which the number of K-IV repeats had been determined by genomic southern blotting. To allow an adequate statistical treatment and also to enable comparability with previous studies with lower resolution, the following grouping was performed: apo(a) isoforms containing 11–13 Kringle IV repeats were summarised as F, 14–16 Kringle IV repeats as B, 17–19 Kringle IV repeats as S1, 20–22 Kringle IV repeats as S2, 23–26 Kringle IV repeats as S3 and those > 26 Kringle IV repeats as S4, in accordance with the original classification.[56] In addition, the following phenotypic groups were constructed as described:[21,58] phenotype group I: S4, 0; phenotype group II: S3, S3S4; phenotype group III: S2, S2S3, S2S4; phenotype group IV: phenotype combinations containing the isoforms F, B or S1. Phenotype groups II and I were regarded as high-molecular weight isoforms, and phenotype group III and IV as low-molecular weight isoforms.

Plasma tHcy concentrations were determined with HPLC as described,[59,60] and total (free plus protein-bound) homocyst(e)ine was measured. The day-to-day analytical coefficient of variation of the method was 5.2%.

Statistical analyses

Analyses were performed with SPSS software. Between-group comparisons were performed with the student's *t*-test or the Mann Whitney U-test for continuous variables depending on the distribution of the variable. The χ^2 test was used for contingency tables, and logistic regression was used to determine relative risk. A value of 12.0 µmol/l was used as cut-off level to establish vascular disease risk related to hyperhomocyst(e)inemia.[61] Plasma Lp(a) concentrations above 30.0 mg/dl, the commonly accepted threshold, were regarded as elevated, which was less than the 75th percentile of control plasma Lp(a) concentrations of 32.7 mg/dl. Only one VD subject had increased serum urea and creatinine concentrations. As exclusion of this subject did not alter the statistical findings, and as none of the other variables measured could be regarded as a possible statistical outlier, the subject was included in the study.

Results

Risk profile of subjects and controls

No significant difference was observed in the prevalence of hypertension (diastolic blood pressure > 90 mm Hg and systolic blood pressure > 160 mm Hg / treated with anti-hypertensive drugs) between the two groups. The prevalence of tobacco smoking appeared to be much higher in the VD subjects, the observed prevalence of pipe or cigarette smoking being 61% for patients and 18% for control subjects, which is suspiciously low. Of the patient group, 37.4% were treated with cholesterol-reducing drugs, while 1% of controls were treated with these drugs. The serum TC concentrations of patients were significantly increased compared with controls ($p = 0.035$) despite more frequent cholesterol-reducing drug use, while the LDL-C, HDL-C and triglyceride levels did not differ significantly (Table I). Apo A and Apo B levels of patients were also less favorable in patients compared with controls ($p < 0.001$) (Table I).

Plasma Lp(a) concentrations as risk factor for VD

The median (IQD interquartile distance) plasma Lp(a) con-

TABLE I. VASCULAR DISEASE RISK FACTORS IN VASCULAR DISEASE PATIENTS AND CONTROLS

	Controls (n = *100)*	*Patients* (n = *91)*
Age (y)	49.1 (6.6)	49.5 (6.3)
Hypertension[a]	17.0%	28.6%
Current tobacco smoking[b]	16/89 (18%)	35/57 (61%)
Serum TC (mmol/l)	5.63 (1.06)	5.98 (1.22)*
Serum LDL-C (mmol/l)	4.06 (1.14)	4.37 (1.29)
Serum HDL-C (mmol/l)	1.05 (0.25)	1.02 (0.47)
Serum Apo A (g/l)	1.44 (0.22)	1.20 (0.24)**
Serum Apo B (g/l)	1.20 (0.34)	1.48 (0.38)**
Serum triglycerides (mmol/l)	2.33 (1.33)	2.34 (1.19)
Plasma tHcy (mmol/l)	8.34 (4.95–18.23)	10.15 (5.24–47.06)**
Lp(a) (mg/dl)	15.3 (27.7)	26.3 (46.1)*

[a]Diastolic blood pressure > 90 mm Hg and systolic blood pressure > 160 mm Hg / treated with anti-hypertensive drugs.
[b]Smoking refers to percentage of subjects who currently either smoked a pipe or cigarettes.
All variables are expressed as mean (SD), except plasma tHcy concentrations which are expressed as median (range), and plasma Lp(a) concentrations which are expressed as median (IQD).
Significance of differences: *$p < 0.05$, **$p < 0.001$, ***overall chi-square 28.83, p-value < 0.001.

centration of the patients was 26.3 (46.1) mg/dl, which was significantly elevated compared with control concentrations of 15.3 (27.7) mg/dl (Table I). Elevated Lp(a) concentrations (> 30 mg/dl) were observed in 30.7% of controls and 48.0% of patients. Also this difference was significant (χ^2 = 7.02; p = 0.008), showing that VD was significantly associated with elevated Lp(a) levels, and the relative risk associated with VD due to increased Lp(a) concentrations was 2.02 (95% CI: 1.14–3.59).

Relation between VD risk and plasma total homocyst(e)ine

Median (range) plasma tHcy concentrations of 10.15 µmol/l (5.24–47.06) for VD patients were significantly elevated compared with 8.34 µmol/l (4.95–18.23) for controls (Table I). Hyperhomocyst(e)inemia (> 12.0 µmol/l) was prevalent in 34.0% of the patients and was significantly associated with VD (relative risk 5.94; 95% CI: 2.56–13.79).

Interaction between Lp(a) and homocysteine

Potential interaction between Lp(a) and homocyst(e)ine was tested by assessing risk relating to excess of a risk factor in the presence of excess or absence of the other risk factor (Table II), as previously reported.[56] Concordant excess of Lp(a) and hyperhomocyst(e)inemia did not result in significantly increased VD risk (relative odds 2.96; 95% CI: 0.90–9.80), whereas hyperhomocyst(e)inemia in the absence of excess Lp(a) resulted in a 7.20-fold increase in VD risk (95% CI: 2.37–21.91).

Table III shows the risk of VD associated with elevated plasma tHcy concentrations after stratification of the data into high- and low-molecular weight apo(a) isoform groups. Hyperhomocyst(e)inemia was not associated with increased VD risk in subjects with one low-molecular weight isoform (relative odds 1.92; 95% CI: 0.51–7.24), whereas in subjects with only high-molecular weight apo(a) isoforms, the relative risk of VD was 11.02 (95% CI: 3.54–34.30).

TABLE II. RISK OF VASCULAR DISEASE ASSOCIATED WITH HOMOCYST(E)INE EXCESS IN THE PRESENCE OR ABSENCE OF LP(A) EXCESS

Risk factors	*Relative odds*	*95% confidence interval*
Concordant excess of plasma total homocyst(e)ine[a] and Lp(a)[b]	2.96	0.90–9.80
Excess plasma homocyst(e)ine in the absence of excess Lp(a)	7.20	2.37–21.91

[a]Plasma total homocyst(e)ine concentrations > 12.0 µmol/l
[b]Plasma Lp(a) concentrations > 30.0 mg/dl

TABLE III. RISK OF VASCULAR DISEASE ASSOCIATED WITH HYPERHOMOCYST(E)INEMIA AFTER STRATIFICATION OF THE DATA ACCORDING TO APO(A) ISOFORM SIZE

Group	*Odds ratio*	*95% confidence interval*
Subjects with one or two low-molecular weight apo(a) isoforms	1.92	0.51–7.24
Subjects with only high-molecular weight apo(a) isoforms	11.02	3.54–34.30

Hyperhomocyst(e)inaemia is defined as > 12.0 µmol/l.

Discussion

Both elevated plasma Lp(a) concentrations and hyperhomocyst(e)inemia appear to be associated with increased risk for VD in Caucasian South African males. Concordant Lp(a) excess and hyperhomocyst(e)inemia are expected to increase VD risk significantly. The possible interaction between Lp(a) and homocyst(e)ine was investigated by Hopkins *et al*.[56] These researchers reported a significant interaction between Lp(a) and homocysteine, based on calculated relative odds of VD of 31.7 (95% CI: 6.5–155), if both risk factors were elevated.[56] In our study we found that concordant excess of plasma total homocyst(e)ine and Lp(a) was not significantly associated with VD risk (Table II) (relative odds 2.96; 95% CI: 0.90–9.80).

The study by Hopkins *et al*.[56] was performed in patients with familial CAD who had survived a myocardial infarction, percutaneous transluminal angioplasty, or coronary artery bypass grafting. Patients with such a profile constituted only 48% of our patient population. These differences may explain the different relative risks between our study and that of Hopkins *et al*. Application of Hopkins *et al*.'s higher threshold values for elevated Lp(a) (40 mg/dl) and hyperhomocyst(e)inemia (15 µmol/l) to our data did not alter our findings. Concomitant hyperhomocyst(e)inemia and Lp(a) excess may however be ascribed to renal impairment, which was not taken into account in Hopkins *et al*.'s study. In our study we found that neither hyperhomocyst(e)inemia nor Lp(a) excess could be explained by renal impairment.

Parsons *et al*.[51] reported that 20% of patients with mild renal impairment had both risk factors present, whereas 58% of patients with a GFR less than 10 ml/min had both hyperhomocyst(e)inemia and Lp(a) excess. In another study, mean plasma tHcy, fibrinogen and Lp(a) levels were reported to be substantially increased in end-stage renal disease patients.[54] Prevalent CAD was however not associated with levels of plasma tHcy, fibrinogen or Lp(a) in the maintenance dialysis population.[54] Kronenberg *et al*. found that the prevalence of peripheral arterial calcifications in patients with ESRD was related to Lp(a) and homocyst(e)ine levels and suggested that the progression of arterial calcifications is related to hyperhomocyst(e)inemia.[55]

A prospective study would probably be the ideal study design to investigate the cumulative or additive effect of concomitant hyperhomocyst(e)inemia and Lp(a) excess, although long-time storage of samples may affect the measurement of Lp(a).[62] This may have been the case in a prospective study performed in Finland,[63] which evaluated vascular risk relating to plasma concentrations of Lp(a) and homocyst(e)ine, and found that neither of these two risk factors contributed to VD prevalence.

Our results suggest that hyperhomocyst(e)inemia is an important risk factor for VD, especially in individuals with Lp(a) levels of < 30.0 mg/dl (Table II) (relative odds 7.20; 95% CI: 2.37–21.91), which are by default individuals with the larger apo(a) isoforms. Logistic regression analyses on the data, stratified according to high- or low-molecular weight apo(a) isoforms, showed that the risk of VD relating to elevated plasma tHcy concentrations was highly significant in subjects with high-molecular weight apo(a) isoforms (relative odds 11.02; 95% CI: 3.54–34.30) (Table III).

Kronenberg *et al*. recently demonstrated that the decrease of Lp(a) concentrations following kidney transplantation was caused by changes in the expression of HMW apo(a) isoforms.[64] They concluded from this study that an interaction of apo(a) genetic variability and kidney function on Lp(a) concentrations exists.[64] Renal function is one of the main determinants of plasma tHcy concentrations.[51-55,65] Whether the association between hyperhomocyst(e)ine and high-molecular weight apo(a) isoforms is causally related to VD risk and whether this relation to VD risk can be explained by renal function needs to be clarified.

Findings by Huby *et al*.[46] show that 'mini-Lp(a)' containing the C-terminal domain of apo(a) (comprising the region from Kringle IV–V to the protease domain) linked to apo B exhibits binding affinity to fibrin, whereas the repeated Kringle IV-2 domain does not appear to possess lysine-binding sites. Steric changes within the Kringle IV-2 repeat region would therefore not affect binding to fibrin within this region, but could facilitate binding within the C-terminal domain to fibrin. Conformational changes of apo(a) induced by homocysteine may therefore have a greater impact on VD progression in the high-molecular weight apo(a) isoforms compared with the low-molecular weight apo(a) isoforms.

Eritsland *et al*.[52] performed a study in which the influence of serum Lp(a) and homocyst(e)ine on vein graft patency after coronary artery bypass grafting was assessed. Neither Lp(a) nor homocyst(e)ine could explain occlusion of the grafts, but the number of vein graft occlusions was significantly higher in the lowest quartile of Lp(a) concentrations [therefore in the individuals who probably had high-molecular weight apo(a) isoforms] than in the higher three quartiles of Lp(a) concentrations. This study may support the notion that high-molecular weight apo(a) isoforms may also play a significant role in VD progression.

Conclusion

The present data show that homocyst(e)ine is an independent risk factor for VD in individuals with high-molecular weight apo(a) isoforms, who up to now have been considered to be at low risk because of their low Lp(a) levels. Determination of plasma tHcy concentrations seems imperative in these subjects.

The cardiologists practicing at the Pretoria Heart and the Unitas Hospitals and the management of these hospitals are thanked for their kind co-operation and support. Lea Goddard and Annetjie van der Merwe and other laboratory staff of the Institute for Pathology, University of Pretoria, are thanked for their contribution to the study.

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