

Post-Methionine-Load Hyperhomocysteinemia and Increased Lipoprotein(a) Are Associated With Renal Metabolic Dysfunction: A Hypothesis

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Previous studies have shown that homocysteine influences the structure of lipoprotein(a) [Lp(a)] and its affinity to fibrin, and that there is an increased risk of vascular disease when both homocysteine and Lp(a) are elevated. The aim of this study was to determine whether there is a correlation between increased total homocysteine (tHCY) and high Lp(a) concentrations, and whether increased concentrations of tHCY affect the concentration of unbound serum apolipoprotein(a) [Apo(a)]. Forty-seven male subjects recruited from a primary prevention screening program with normal serum creatinine and Lp(a) concentrations above 30 mg/dL were included and underwent a standardized oral methionine-loading test to increase the plasma tHCY concentration. This increase might lead to a modification of the Apo(a) structure, thus possibly influencing the serum concentration of unbound Apo(a). Fasting blood samples were taken before the tests and after 6 hours. The median values of tHCY increased about 4-fold after the methionine-loading test. Fasting tHCY did not show an association with Apo(a) and a post-methionine load increase of unbound Apo(a) was not observed. Backward multiple linear regression analysis, however, revealed that only post-load tHCY was independently and significantly influenced by Lp(a). Furthermore, Lp(a) correlated significantly with post-load tHCY, but not with fasting tHCY. Subdividing the subjects according to the Lp(a) concentration showed a significantly higher median concentration of tHCY after methionine load in subjects with Lp(a) over 50 mg/dL compared to subjects with Lp(a) under 50 mg/dL ($P = .009$). A similar cut-off was seen for post-load Apo(a) at 7.3 mg/dL ($P = .04$). Factors such as age, C677T-methylene-tetrahydrofolate-reductase (MTHFR) mutation, folate, vitamin B₁₂, and creatinine showed no significant influence on post-load tHCY in the different subgroups. The reasons for our findings remain partially unclear. However, considering our results and the current knowledge on the association of tHCY and Lp(a) concentration with the renal function, we hypothesize that both parameters may be linked by commencing renal metabolic dysfunction. It should be stressed that our hypothesis is speculative and that further studies will be necessary to improve the understanding of the interrelation of tHCY and Lp(a) concentration.

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A LARGE BODY of evidence has linked fasting homocysteine^{1,2} and lipoprotein(a) [Lp(a)]^{3,4} independently with coronary artery disease (CAD) and myocardial infarction. Each has been suggested to promote progression of atherosclerosis through thrombogenic and atherogenic effects, but the exact mechanisms are as yet not fully understood. Elevated Lp(a) is found in about 30% and increased homocysteine in about 40% of CAD patients.^{5,6}

Lp(a) consists of an apolipoprotein B-100 (ApoB-100) containing low-density lipoprotein (LDL) particle with apolipoprotein(a) [Apo(a)] attached to the ApoB-100 by a single disulfide bridge.⁷ The unique character of Lp(a) is based on the Apo(a), a highly glycosylated protein, structurally homologous to plasminogen.⁸ Recently published data indicate the existence of unbound (free) forms of Apo(a) in blood and urinary excretion of Apo(a) fragments.⁹⁻¹¹ Furthermore, Apo(a) has also been reported to be correlated to CAD as well as renal disease.¹²⁻¹⁵ Animal experiments indicate that Apo(a) not only serves as a distinctive marker of Lp(a), but also represents an atherogenic component of Lp(a).¹⁶ Dissociation of Apo(a) may lead to the exposure of an additional lysine-binding site, increasing the affinity of free Apo(a) for plasmin-modified fibrin, thus impeding fibrinolysis.¹⁷ Several publications have reported increased plasma concentrations of Lp(a) in patients with renal disease.^{15,18-22} The enhanced atherosclerotic risk in these patients seems to be related to the serum concentration of Lp(a) and also to the Apo(a) phenotype.^{20,23} The increased serum concentration of Lp(a) during the chronic renal failure phase and its decline after kidney transplantation is basically related to the loss of renal function,²⁴ presenting a possible role of the kidney in Lp(a) metabolism.

Homocysteine is a sulfur-containing amino acid that occurs in all cells as a consequence of the normal methylation process.

It is metabolized either by transsulfuration to cysteine via cystathionine or by remethylation to methionine. Excess homocysteine that is not metabolized in the cell is exported to the plasma compartment.²⁵ Although there is abundant evidence suggesting that the kidney likely plays an important role in homocysteine clearance and metabolism, there is considerable controversy surrounding the extent and mechanism of this role.²⁶ In recent years, numerous studies have associated moderate hyperhomocysteinemia with premature cerebrovascular, peripheral, and coronary artery disease.²⁷⁻³² Disturbed methionine metabolism with moderate hyperhomocysteinemia (15 to 30 $\mu\text{mol/L}$) has thus been recognized as an independent atherogenic risk factor. Moreover, marked hyperhomocysteinemia is frequently observed in renal patients,¹⁸ which seems to involve reduced clearance of plasma homocysteine.^{33,34} Studies by Bostom and Lathrop³ and Guttormsen et al³⁰ point at the kidney as a major site for the elimination of homocysteine from plasma, while van Guldener et al³⁶ observed no significant net renal extraction of homocysteine when measuring the arteriovenous difference. Hence, the reduced clearance of plasma homocys-

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teine in renal disease may be attributable to defective renal clearance and/or extrarenal clearance and metabolism, the latter possibly resulting from retained uremic inhibitory substances. The reverse relationship between homocysteine levels and glomerular filtration rate (GFR), which is consistent throughout a nonuremic range of kidney function, supports the premise that it is reduced renal function, not the accumulation of uremic toxins, that causes homocysteine levels to increase.²⁶

In a previous study we showed that patients after renal transplantation with fasting total homocysteine (tHCY) concentrations above 22 $\mu\text{mol/L}$ had significantly higher concentrations of unbound Apo(a).³⁷ We discussed 3 possible mechanisms that could influence the level of Apo(a) in serum: (1) homocysteine-induced structural changes in Lp(a) leading to a partial release of Apo(a) from Lp(a); (2) decreased renal excretion of Apo(a); and perhaps (3) an increase of hepatic Apo(a) synthesis.

Harpel et al³⁸ as well as Leerink et al³⁹ reported that homocysteine so alters the intact Lp(a) particle as to increase the reactivity of plasminogen-like Apo(a). An *in vitro* tHCY concentration of 8 $\mu\text{mol/L}$ induced a 20-fold increase in the affinity between Lp(a) and plasmin-treated fibrin. Enhanced binding of Lp(a)/Apo(a) to fibrin may delay fibrinolysis by plasmin. This observation suggests a biochemical relationship between homocysteine metabolism, thrombosis, and atherogenesis. Furthermore, a distinctly increased *in vitro* homocysteine concentration ($> 1 \text{ mmol/L}$) has been reported to effect a partial dissociation of Apo(a) from the Lp(a) molecule.⁴⁰

Bostom et al¹⁸ reported in a matched case-control study that combined hyperhomocysteinemia, hyperfibrinogenemia, and Lp(a) excess may contribute to the high incidence of vascular disease experienced by dialysis patients, which is poorly explained by traditional risk factors for atherothrombotic events. In a recent cross-sectional study on young CAD patients, combined elevation of tHCY and Lp(a) concentration was found to increase the risk for the presence of CAD nearly 5 times compared with a more modest risk association with each variable individually.⁴¹ Moreover, this report discussed a possible interactive effect beyond that attributable to either additive or multiplicative effects of homocysteine and Lp(a). In contrast, elevations of both parameters showed evidence of being independent risk factors. Hopkins et al⁴² reported a striking interaction between tHCY and Lp(a) concentration. They investigated risk factors in early familial coronary artery disease and found marked elevations in risk when Lp(a) over 40 mg/dL and high tHCY concentration ($>90\text{th}$ percentile) were combined.

To test our hypothesis that hyperhomocysteinemia might be associated with increased serum Lp(a)/Apo(a), we investigated the relation between tHCY and Lp(a)/Apo(a) concentrations in 47 males with Lp(a) greater than 30 mg/dL. Lp(a) concentrations over 30 mg/dL were chosen because below this concentration the level of unbound Apo(a) is expected to be very low. All subjects underwent an oral methionine loading test to determine possible disturbances in homocysteine metabolism. Furthermore, we wanted to find out whether post-load tHCY concentrations affect the *in vivo* concentration of unbound Apo(a).

MATERIALS AND METHODS

Subjects

All subjects were recruited from a primary prevention screening program and were residents of the Province of Styria (Austria). Written informed consent was obtained from each participant and the Ethics Board of the Karl-Franzens University approved the study. Forty-seven males with normal serum creatinine and plasma Lp(a) concentrations greater than 30 mg/dL underwent a standardized oral methionine loading test.⁴² Median age was 53.3 years (5th/95th percentile, 49.1/55 years). The status of C677T-methylene-tetrahydrofolate-reductase (MTHFR) mutation was known for 40 of 47 subjects. Eleven were wild-type, 27 heterozygous, and 2 homozygous genotypes. We selected male subjects only, because certain conditions, e.g., menopause, contraceptive, or hormone treatment, may affect the tHCY status in females and thus complicate the evaluation of data related to homocysteine metabolism. Other exclusion criteria were intake of vitamins, antidepressants, lipid-lowering drugs, liver dysfunction, impaired kidney function, myocardial infarction, inflammation, or invasive procedures within the past 6 months. Blood samples were taken from an antecubital vein between 7 and 8 AM after an overnight fast of at least 8 hours. L-methionine (0.1 g/kg/body weight) in 200 mL orange juice was then given to each participant and a second (post-load) blood sample was obtained 6 hours later.

Measurements

All blood samples were processed immediately, centrifuged at 4°C ($3,000 \times g$ for 10 minutes) within 15 minutes and stored at -70°C until analysis. All serological analyses were performed without prior knowledge of clinical data.

tHCY Determination

Measurements of plasma homocyst(e)ine concentrations, folate, and vitamin B₁₂ were done at the Department of Laboratory Medicine, Karl-Franzens University School of Medicine, Graz, Austria. Analyses in EDTA plasma for homocyst(e)ine were performed using high-performance liquid chromatography (HPLC) and fluorescence detection according to Araki and Sako⁴⁴ with modifications by Ubbink et al⁴⁵ and Vester and Rasmussen.⁴⁶ Briefly, after treatment with tri-*N*-butylphosphine, the free thiol groups were derivatized with 7-fluorobenzofurazane-4-sulfonic acid (SBD-F). Separation was performed under isocratic conditions on a revers-phase column at pH 2.1 with mercaptopropionyl-glycine as internal standard. The intra-assay variability of the method was between 1.3% (27 $\mu\text{mol/L}$) and 4.5% (10 $\mu\text{mol/L}$). Because the procedure involved a reducing step, the method did not distinguish between homocysteine and its oxidized analogs. The measured moiety is therefore referred to as total homocysteine (tHCY).

Vitamin Determination

Folate and vitamin B₁₂ (plasma) concentrations were determined with commercially available "Ion Capture Assay" and "Microparticle Enzyme Immunoassay," respectively (Abbott Diagnostics, Wiesbaden, Germany).

Lp(a)/Free Apo(a) Determination

Determination of Lp(a) and free Apo(a) concentrations was performed at the Central Laboratory, Saarland University School of Medicine, Homburg/Saar, Germany utilizing a PROGEN Immunozyom Lp(a) enzyme-linked immunosorbent assay (ELISA) (PROGEN, Heidelberg, Germany). In brief, this test immobilizes Lp(a)/free Apo(a) on an α -Apo(a)-coated microplate and utilizes a second peroxidase-labeled α -Apo(a) for detection. For free Apo(a) testing, the Lp(a) was precipitated prior to this measurement by an excess of α -ApoB-100.

Table 1. Medians of tHCY, Lp(a), Apo(a), Vitamin B₁₂, Folate, and Creatinine

Parameter (N = 47)	Median (5th/95th Percentile)
tHCY _{Fasting} (μmol/L)	8.5 (6.5/14.8)
tHCY _{PML} (μmol/L)	33.6 (23.5/45.5)
Lp(a) (mg/dL)	79.7 (31.6/172.6)
Apo(a) _{Fasting} (mg/L)	5.27 (2.51/13.19)
Apo(a) _{PML} (mg/L)	5.33 (2.44/11.36)
Vitamin B ₁₂ (ng/L)	427.0 (178.3/805.4)
Folate (μg/L)	8.4 (4.6/32.4)
Creatinine (mg/dL)	1.0 (0.83/1.4)

*According to Mann-Whitney test.

Statistical Analysis

Median values, 5th and 95th percentiles, Mann-Whitney test, correlation analysis by Spearman rho, and backward regression analysis were calculated with the SPSS software package (version 9.0 for Windows; SPSS Inc, Chicago, IL).

RESULTS

The median values of fasting tHCY (tHCY_{Fasting}) and post-methionine load tHCY (tHCY_{PML}), Lp(a), and unbound Apo(a), as well as folate, vitamin B₁₂, and creatinine, are listed in Table 1. Under fasting conditions tHCY was not associated with Apo(a) or Lp(a). Both folate and vitamin B₁₂ were within normal range. The median of tHCY_{PML} was about 4 times higher than the median of tHCY_{Fasting}. Free Apo(a) concentration, however, was not significantly changed by the methionine-load test. Subdividing the subjects according to their Lp(a) concentration, we found a significantly higher median of tHCY_{PML} in subjects with Lp(a) above 50 mg/dL than in subjects with Lp(a) below 50 mg/dL (Table 2 and Fig 1). Age, creatinine, folate, and vitamin B₁₂ did not differ significantly in these subgroups (Table 2). Similar results were obtained for free Apo(a) after the methionine load test [Apo(a)_{PML}]. The cut-off value leading to a significant difference between the 2 Apo(a)_{PML} subgroups was determined at 7.3 mg/L (Fig 2). The group of subjects with Apo(a)_{PML} above 7.3 mg/L was smaller and all of these subjects had Lp(a) concentrations exceeding 100 mg/dL. Utilizing backward multiple linear regression analysis we could show that tHCY_{PML} as a dependent variable was only independently and significantly influenced by the tHCY_{Fasting} (P = .006) and by the Lp(a) concentration (P =

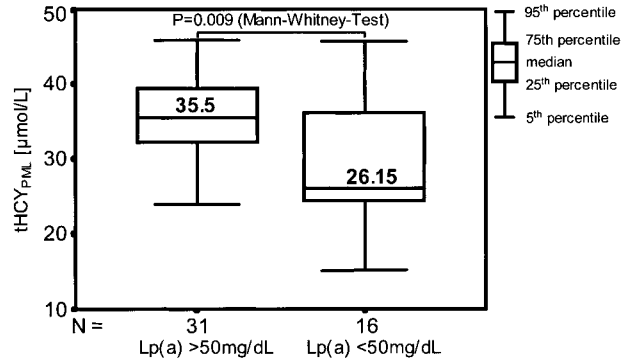


Fig 1. Post-methionine load total homocysteine concentration (tHCY_{PML}) in subjects based on Lp(a) concentrations above and below 50 mg/dL (P = .009).

.016). Additionally, Spearman rho correlation analysis showed that the Lp(a) concentration and the Apo(a)_{PML} concentration correlated significantly with tHCY_{PML} (Table 3).

DISCUSSION

Several publications have found an increased risk for vascular disease in the case of combined elevation of tHCY and Lp(a) concentration.^{18,41,42} Other studies reported that homocysteine influences the structure of Lp(a) as well as its affinity to fibrin, and that in-vitro hyperhomocysteinemia can effect a release of Apo(a) from Lp(a).³⁸⁻⁴⁰

In the present study we used a methionine loading test to induce experimental hyperhomocysteinemia, thus testing if increased tHCY concentration induces structural changes in Lp(a) leading to a partial release of Apo(a) from Lp(a). Statistical analysis nonetheless showed no significant difference between fasting and post-load Apo(a) concentrations. Still, this mechanism cannot be ruled out completely, because the post-load hyperhomocysteinemia was only temporary. Possibly, only a higher and/or chronic increase in tHCY concentration can mediate an elevation in Apo(a) concentration in vivo.

Further statistical analysis of our data revealed a significant correlation of post-load hyperhomocysteinemia with fasting Lp(a) concentrations, whereby subjects with strongly elevated Lp(a) concentrations (>50 mg/dL) showed a significantly higher tHCY elevation after methionine load compared to those

Table 2. Medians of tHCY, Vitamin B₁₂, Folate, Creatinine, and Age Subdivided According to Lp(a) Concentrations Above and Below 50 mg/dL

Parameter	Lp(a) Subgroups [median (5th/95th percentile)]		Significant*
	Lp(a) > 50 mg/dL (n = 31)	Lp(a) < 50 mg/dL (n = 16)	
tHCY _{Fasting} (μmol/L)	8.7 (6.5/15.1)	7.7 (4.2/14.2)	No
tHCY _{PML} (μmol/L)	35.5 (24.4/45.7)	26.15 (15.1/45.0)	Yes (P = .009)
Vitamin B ₁₂ (ng/L)	414.2 (170.3/783.2)	463.6 (188.3/822.1)	No
Folate (μg/L)	10.2 (3.7/55.5)	9.4 (5.4/16.1)	No
Creatinine (mg/dL)	1.00 (0.7/1.5)	1.05 (0.9/1.2)	No
Age (yr)	55.5 (46.4/59.7)	54.0 (32.1/58.1)	No

*According to Mann-Whitney test.

with moderately elevated Lp(a) concentrations (30 to 50 mg/dL). Age, creatinine, folate, and vitamin B₁₂ did not differ significantly in the 2 subgroups. It is commonly accepted that mutations in enzymes such as cystathionine- β -synthase (CBS) and C677T-MTHFR can lead to elevated tHCY_{PML} concentrations.^{25,47,48} We did not determine CBS mutation. However, we did find that the C677T-MTHFR mutation did not increase homocysteine in our subjects. Furthermore, the significant increase of tHCY_{PML} in subjects with Lp(a) greater than 50 mg/dL was also independent of the C677T-MTHFR genotype. This observation may be explained by the normal folate concentrations, and by the fact that there were only 2 subjects with a homozygous C677T-MTHFR mutation in the investigated group.

The reasons for our findings remain partially unclear. It seems likely, however, that pathological post-load tHCY concentrations and increased Lp(a) concentrations are not just concurrent, but somehow physiologically linked. This is not supposed to mean that both parameters are dependent on one another, but that there might be a common origin for the pathological increase in both concentrations. Considering this brings to mind, that both tHCY^{18,33,34} and Lp(a)^{15,18-22} are elevated in patients with chronic renal disease. The role of the kidney in plasma homocysteine handling and the cause of hyperhomocysteinemia in renal disease are still under investigation. One approach discusses the involvement of unidentified uremic inhibitors that block normal extrarenal homocysteine metabolism. Other data nonetheless suggest, but do not prove, that hyperhomocysteinemia is primarily attributable to decreases in homocysteine plasma clearance and metabolism by decreased functioning renal mass.²⁶ The impaired homocysteine metabolism in the kidney of patients with renal dysfunction could be in part due to vitamin deficiency and is also a consequence of other factors, such as oxidative stress^{49,50}

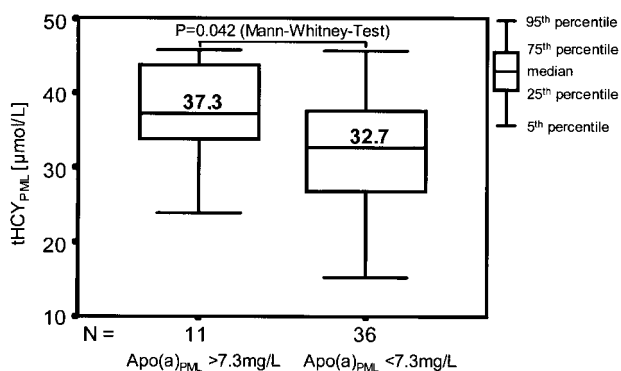


Fig 2. Post-methionine load total homocysteine concentration (tHCY_{PML}) in subjects based on Apo(a)_{PML} concentrations above and below 7.3 mg/L ($P = .042$).

Table 3. Correlations of tHCY, Lp(a), and Apo(a) according to Spearman Rho Test

Parameter (N = 47)	tHCY _{Fasting}	tHCY _{PML}	Lp(a)	Apo(a) _{Fasting}	Apo(a) _{PML}
tHCY _{Fasting}	1.000				
tHCY _{PML}	0.393†	1.000			
Lp(a)	0.104	0.360*	1.000		
Apo(a) _{Fasting}	0.054	0.283	0.795**	1.000	
Apo(a) _{PML}	0.190	0.336*	0.793**	0.806**	1.000

* $P < .05$.

† $P < .01$.

and/or elevated nitrous oxide exposure^{51,52} of enzymes (eg, CBS and C677T-MTHFR) involved in homocysteine metabolism.⁵³⁻⁵⁶ In our study, creatinine was within normal range and showed no association with tHCY_{PML}. However, it is well established that substantial reductions in renal function may occur before plasma creatinine becomes abnormal.⁵⁷ Hence, it is not a suitable diagnostic parameter for early renal metabolic dysfunction. Since the kidney is discussed to play a major role in the daily tHCY elimination from plasma,^{35,58} it cannot be ruled out that our observations may be in some way associated with a slight renal metabolic dysfunction. In this respect, our finding of an association of high Lp(a) and increased tHCY_{PML} could be explained by different approaches. On the one hand, high Lp(a) concentrations could somehow impair transsulfuration. However, a recent publication on patients with chronic renal failure (CRF) refutes this approach, demonstrating a significant reduction of tHCY, Lp(a), and fibrinogen after 4 weeks of homocysteine-lowering treatment (folic acid 15 mg/d, pyridoxine 150 mg/d, and cyanocobalamin 1 mg/wk).⁵⁹ This indicates that, on the other hand, hyperhomocysteinemia could have an increasing effect on Lp(a). Possibly the condition of disturbed renal homocysteine metabolism with frequent hyperhomocysteinemic episodes after food intake contributes to increased oxidative stress, which may inhibit enzymes involved in homocysteine metabolism and may support vascular inflammation leading to increased Lp(a).⁵⁹ The release of Apo(a) from Lp(a) by cleaving of the disulfide bond of Apo(a) to ApoB-100 and its tubular excretion are crucial steps in renal Lp(a) catabolism, which are possibly sensitive to oxidative stress. The reduced urinary excretion of Apo(a) and elevated serum concentrations of free Apo(a) and Lp(a) were proven in patients with CRF.^{14,15,37}

In conclusion, we hypothesize that pathologically elevated tHCY_{PML} concentrations and increased Lp(a) concentrations may be physiologically linked. Both renal homocysteine metabolism and Lp(a) catabolism could be impaired due to a slight renal metabolic dysfunction. This may also account for the increased risk for CAD, beyond that attributable to additive or multiplicative effects of homocysteine and Lp(a), observed in case of concomitant elevation of tHCY and Lp(a) concentrations.^{41,42}

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