Homocysteine or Renal Impairment
Which Is the Real Cardiovascular Risk Factor?

Kathleen Potter, Graeme J. Hankey, Daniel J. Green, John W. Eikelboom, Leonard F. Arnolda

Objective—The purpose of this study was to determine whether adjustment for renal function eliminates the relationship between total plasma homocysteine (tHcy) and vascular risk, assessed by carotid intima medial thickness (CIMT) and flow-mediated dilation (FMD) of the brachial artery.

Methods and Results—We used cross-sectional data from 173 stroke patients treated with B-vitamins (folic acid 2 mg, vitamin B₆ 25 mg, and vitamin B₁₂ 0.5 mg) or placebo in a randomized double-blinded trial to test the relationships between posttreatment tHcy, cystatin C (a marker of glomerular filtration rate), estimated glomerular filtration rate (eGFR, Modification of Diet in Renal Disease equation) creatinine, CIMT, and FMD in stepwise and multivariable regression models. The strong linear relationship between tHcy and cystatin C was not altered by long-term B-vitamin treatment. tHcy lost significance as a predictor of the vascular measurements after adjustment for any single marker of renal function. Cystatin C, but not tHcy, was a significant independent predictor of FMD after adjustment for age, sex, smoking, systolic blood pressure, high-density lipoprotein cholesterol, and treatment group.

Conclusions—Adjusting for renal function eliminates the relationship between tHcy and CIMT and FMD, supporting the hypothesis that elevated tHcy is a marker for renal impairment rather than an independent cardiovascular risk factor. (Arterioscler Thromb Vasc Biol. 2008;28:000-000)

Key Words: homocysteine □ cystatin c □ stroke □ flow-mediated dilation □ carotid intima-medial thickness

Elevated plasma homocysteine concentrations (tHcy) are associated with an increased risk of stroke and myocardial infarction, but it remains unclear whether elevated tHcy causes atherosclerosis or is a marker of risk burden. Glomerular filtration rate (GFR) is the strongest determinant of tHcy in individuals with an adequate B-vitamin intake, and it has been suggested that elevated tHcy may account for some of the excess cardiovascular risk associated with renal failure.1 Evidence that homocysteine-lowering treatment does not reduce vascular event rates or mortality in people with renal insufficiency has undermined this hypothesis.2–4 Recent data from 2 large prospective trials indicate that small reductions in GFR increase cardiovascular risk more significantly than previously appreciated, supporting the alternative hypothesis that renal impairment is an independent causal risk factor for atherosclerosis and mildly elevated tHcy a marker for reduced GFR.5–7 Observational studies reporting an association between tHcy and surrogate markers of vascular risk, such as carotid intima medial thickness (CIMT) and flow-mediated dilation (FMD), frequently fail to adjust for renal function or correct only for creatinine.8,9 Serum creatinine is influenced by many factors and is thus a relatively insensitive marker for renal insufficiency. Cystatin C is a more accurate indicator of GFR than creatinine, particularly in the elderly, as its concentration is not substantially affected by age, muscle mass or nutritional status.10 Our hypothesis is that adequate adjustment for renal function with cystatin C will eliminate the association between tHcy and vascular risk, assessed by CIMT and FMD. Our aim is to test this hypothesis with a posthoc analysis of data from a randomized, double-blind, placebo-controlled homocysteine-lowering trial.

Methods
The study was conducted in accordance with the Declaration of Helsinki. The Royal Perth Hospital (RPH) Ethics Committee approved the study protocol, and each subject gave written informed consent before taking part.

Study Design and Intervention
This study was a posthoc analysis of cross-sectional data collected from a subgroup of participants in the VITAMINS TO Prevent Stroke (VITATOPS) trial. VITATOPS is a large, randomized, double blind, placebo-controlled trial designed to examine the efficacy and safety of homocysteine lowering with B-vitamin treatment in the prevention of vascular events in patients with a recent stroke.11
subjects were randomly assigned to treatment with a single daily tablet containing folic acid 2 mg, vitamin B₆ 25 mg, and vitamin B₁₂ 500 μg or an identical placebo tablet. Figure 1 shows the recruitment flow-chart for this substudy. All VITATOPS participants enrolled in Perth, Australia who had been taking the study medication for a minimum of 2 years were eligible for inclusion (n=532). Subjects who had died, withdrawn from VITATOPS, ceased taking the study medication, moved away from Perth, or were severely disabled were excluded (n=189). Recruitment letters were sent to all eligible subjects and those who responded positively were contacted by telephone and enrolled in this study (n=173).

Data Collection at >2 years After Randomization
All subjects attended for a single appointment at which age, sex, smoking status, alcohol intake, medications and cardiovascular risk factors were recorded, blood pressure, height, weight, hip and waist girth, CIMT, and FMD were measured and a fasting blood sample was collected.

CIMT and FMD were measured using methods described previously. Subjects fasted overnight, withheld any morning medications, and refrained from smoking or consuming caffeine or alcohol for at least 6 hours before the study session. We recorded B-mode ultrasound images in the carotid and brachial arteries with a 10 MHz multi-frequency linear array probe attached to a high-resolution ultrasound (Acuson Aspen). Digital images of the right and left common carotid arteries were recorded proximal to the bifurcation from 3 angles of insonation; posterior, lateral, and anterior. CIMT was measured off-line using edge-tracking software described previously. The final CIMT values for each subject were calculated as the mean of these 6 measurements.

FMD was measured in the left arm, and B-mode images of the brachial artery were recorded for 1 minute to assess baseline arterial diameter. A rapid inflation/deflation pneumatic cuff placed around the forearm was inflated to 250 mm Hg for 5 minutes to provide the ischemic stimulus. After cuff deflation, images were recorded continuously for 2 minutes to capture peak arterial dilation. FMD was measured off-line using edge-detection software described previously. All measurements were made by a single observer blinded to treatment status.

A venous blood sample was collected after the vascular measurements. Blood for tHcy measurements was collected onto ice and centrifuged (4°C, 3000 rpm, 8 minutes) within an hour of collection. tHcy, red blood cell (RBC) folate, and serum B₁₂ were measured using a competitive immunoassay (Immulite 2000, Diagnostic Products Corporation). Serum B₆ was determined by microbiological assay using Lactobacillus casei as the test organism. Cholesterol, triglycerides, glucose, and glycohemoglobin (HBA1c) were also measured.

Renal function was primarily assessed using cystatin C. Baseline and posttreatment cystatin C concentrations were measured in a single batch by nephelometry (Dade Behring BNII) on serum samples stored at −80°C. We also measured serum creatinine and estimated GFR (eGFR) using the 4 variable formula from the Modification of Diet in Renal Disease (MDRD) study. Serum creatinine was measured by a kinetic assay (Jaffe method) on a Roche Hitachi 917 analyser (Roche Diagnostics GmbH).

Statistical Analysis
Variables with a log-normal distribution, including tHcy, cystatin C, and creatinine, were log-transformed and results reported as geometric mean (95% CI). Continuous variables were compared by t test or 1-way ANOVA and proportions were tested using a normal approximation to the binomial. We used linear regression to determine whether adjustment for cystatin C, eGFR, or creatinine eliminated the relationship between tHcy and FMD or CIMT. We tested the relationships in univariate models, a model containing both tHcy and a marker of renal function and a model adjusted for variables strongly associated with the vascular measurements on univariate analysis (age, sex, smoking, systolic blood pressure, high density lipoprotein (HDL) cholesterol, arterial lumen diameter (brachial or carotid), ambient laboratory temperature (FMD model only), and waist circumference and HBA1c (CIMT model only). All models...
Results

Subject Characteristics

Table 1 shows subject characteristics at follow-up. There were no significant differences between the groups in post-treatment CIMT or FMD.

Blood Results at Follow-Up

Table 2 shows the blood results at follow-up. After a mean treatment period of 3.9 ± 0.9 years (range 1.8 to 6.0 years), tHcy was significantly reduced from baseline among subjects randomized to vitamins (−2.7 ± 2.9 μmol/L, P < 0.001) compared with a nonsignificant increase from baseline in the placebo group (0.1 ± 2.8 μmol/L, P = 0.99). Serum folic acid, B12, and B6 concentrations were significantly higher in vitamin-treated subjects.

Univariate Correlations between tHcy, Renal Function, FMD, and CIMT

The renal function markers were all significantly correlated with tHcy (r = 0.5, P < 0.001) and appeared to have a stronger relationship with the vascular measurements than tHcy (Table 3). Cystatin C and eGFR were more strongly correlated with FMD than tHcy (r = −2.96, df = 169, P < 0.005 and t = 5.17, df = 169, P < 0.001, respectively) and the strength of the relationship was graded according to the accuracy of the renal function marker. Cystatin C had a stronger correlation with FMD than eGFR (r = 7.34, df = 169, P < 0.001) and eGFR had a stronger correlation with FMD than creatinine (r = −10.8, df = 169, P < 0.001). Cystatin C was no better as a predictor of CIMT than tHcy, eGFR, or creatinine. RBC folate, serum B12, and serum B6 concentrations were not correlated with FMD, CIMT, or any marker of renal function.

Table 3. Correlations Between FMD, CIMT, tHcy, and Renal Function

<table>
<thead>
<tr>
<th></th>
<th>FMD</th>
<th>CIMT</th>
<th>tHcy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIMT</td>
<td>−0.20 (&lt;0.01)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>tHcy*</td>
<td>−0.15 (0.05)</td>
<td>0.14 (0.07)</td>
<td>...</td>
</tr>
<tr>
<td>Cystatin C*</td>
<td>−0.37 (&lt;0.001)</td>
<td>0.29 (&lt;0.001)</td>
<td>0.50 (&lt;0.001)</td>
</tr>
<tr>
<td>eGFR</td>
<td>0.26 (&lt;0.01)</td>
<td>−0.18 (&lt;0.05)</td>
<td>−0.46 (&lt;0.001)</td>
</tr>
<tr>
<td>Creatinine*</td>
<td>−0.17 (&lt;0.05)</td>
<td>0.22 (&lt;0.01)</td>
<td>0.46 (&lt;0.001)</td>
</tr>
</tbody>
</table>

Values are Pearson correlation coefficient (P value).
*Values log-transformed before analysis.
Adjustment for Renal Function in the Relationship Between tHcy, FMD, and CIMT

Table 4 shows results from regression analyses with FMD and CIMT as response variables. In univariate models adjusted for actual treatment group (Model 1), cystatin C appeared to be a stronger predictor of FMD and CIMT than tHcy, eGFR, or creatinine. In Model 2, adjusted for cystatin C, the negative association between tHcy and FMD was reversed, confirming multicollinearity between the predictors. In data not shown, the association between tHcy and FMD lost significance if the model was adjusted for eGFR (β = 0.05 ± 0.05, P = 0.31, adjusted R² = 2.1%), or creatinine (β = 0.03 ± 0.05, P = 0.61, adjusted R² = 3.2%). In Model 3, adjusted for multiple potential confounders, cystatin C was a significant independent predictor of FMD but tHcy was not. In backwards stepwise-regression, cystatin C was a significant independent predictor of FMD but tHcy, eGFR, and creatinine were not. Neither tHcy nor any of the renal function markers independently predicted CIMT in Model 3 or in a backwards stepwise-regression model.

Effect of B-Vitamins on the Relationship Between tHcy and Cystatin C

Figure 2 shows the relationship between tHcy and cystatin C at baseline and follow-up in 132 subjects with serum stored before randomization. The regression lines separated significantly at follow-up (regression constant β = 0.12 ± 0.24 placebo versus 0.10 ± 0.33 vitamins; P < 0.001) but the gradient of the regression line in the vitamin group after treatment (0.17 ± 0.37) was not significantly different from the gradient...
at baseline (0.78 ± 1.05; \( P = 0.49 \)) or in the placebo group after treatment (0.78 ± 1.01; \( P = 0.55 \)).

**Discussion**

Adjustment for renal function, whether assessed by cystatin C, eGFR, or creatinine, eliminated the relationship between tHcy and vascular risk, assessed by CIMT and FMD. Renal function, as assessed by cystatin C, was an independent predictor of FMD and the univariate correlation between renal function and FMD appeared to be graded according to the accuracy of the renal marker. Long-term homocysteine-lowering treatment did not alter the strong linear relationship between tHcy and cystatin C.

Previous investigators have reported that elevated tHcy is associated with reduced FMD in healthy middle-aged and elderly subjects.16–18 These studies compared FMD in subjects with “high” or “low” tHcy levels without correcting for renal function, so it is possible that the groups also had “low” and “high” GFR, respectively. In the 2 studies that reported serum creatinine, the levels were greater in the “high” tHcy group. The difference was not significant (79 ± 11 μmol/L versus 75 ± 17 μmol/L, \( P = 0.90 \)) in 1 article16 and was not reported as significant in the other, although a 2-sample \( t \) test of the stated values (87.5 ± 8.8 μmol/L versus 72.5 ± 8.8 μmol/L) returns a probability value of less than 0.001.18 Similar studies in younger adults report that elevated tHcy is not associated with impaired FMD, perhaps because factors other than renal impairment have more influence on tHcy in this age group.18–21

A recent study investigating the relationship between FMD and asymmetrical dimethylarginine in healthy subjects aged between 24 and 39 years (\( n = 2096 \)) found that neither eGFR (estimated using the Cockcroft-Gault equation) nor tHcy were independent predictors of FMD after adjustment for multiple cardiovascular risk factors.22 It is possible that the spread of eGFR and FMD values was too narrow to detect a linear relationship between renal insufficiency and endothelial dysfunction in these young healthy subjects. However, the limitations of creatinine-based equations for estimating renal function eliminated the relationship between tHcy and FMD, particularly if the renal impairment is mild. We found that eGFR did not independently predict FMD in our older subjects, whereas cystatin C did.

The univariate relationship between tHcy and CIMT in our subjects was weak and was eliminated by correction for cystatin C, eGFR, or creatinine. Of the large studies that have reported a significant association between tHcy and CIMT after adjustment for confounding variables,9,23–25 only 1 adjusted for creatinine and reported the relationship was not substantially altered.9 Other studies have found no evidence to support an independent association between tHcy and CIMT.26–28

Our data indicate that cystatin C is not a significant independent predictor of CIMT. Two previous studies have used cystatin C to investigate the relationship between renal function and CIMT. A small cross-sectional Japanese study in 60 hypertensive patients found a strong univariate association between cystatin C and CIMT (\( r = 0.5, P < 0.001 \)).29 However, despite reporting close correlations between cystatin C, age, and systolic blood pressure, variables also strongly associated with CIMT, the authors failed to adjust the relationship for these or other confounders. The second study, conducted in a middle-aged population-based cohort from the Seychelles (\( n = 523 \)), found that cystatin C was not an independent predictor of CIMT.30

Our study had a number of strengths that make us reasonably confident that the reported relationships between tHcy, renal function, and the vascular outcomes are reliable. A single observer blinded to treatment allocation measured FMD and CIMT using edge-detection software, reducing the risk of systematic biases. We also collected complete and detailed follow-up data that allowed us adjust the regression models for important potential confounders. However, the greatest strength of our study was using cystatin C to estimate renal function, as we were able to show that the association between renal function and vascular risk was graded according to the sensitivity of the renal marker. In addition, we were able to detect renal function as an independent predictor of FMD.

Our study also had some potential limitations. Previous investigators have suggested that tHcy is a marker for a low B-vitamin intake and that an inadequate nutritional status is thus the underlying causal risk factor for atherosclerosis.31,32 Very few of our subjects had low serum vitamin levels so we were unable to explore this hypothesis. However, we found no correlations between RBC folate, serum B<sub>6</sub>, or serum B<sub>12</sub> and the vascular measurements suggesting that, in vitamin-replete individuals at least, these factors do not strongly influence FMD and CIMT. We also adjusted the regression models for any possible effect of B-vitamin supplementation by including an indicator variable for actual treatment group. Although the design was a randomized intervention trial with 2 treatment groups, we treated the cross-sectional posttreatment data from both groups as a single cohort for this regression analysis. We believe this approach was a reasonable way to address the question of whether adjustment for renal function eliminated the relationship between tHcy and vascular risk. B-vitamin treatment did not significantly alter the key variables, FMD, CIMT, creatinine, or cystatin C, and the intervention created a wide spread of tHcy values without altering the linear relationship between tHcy and GFR (as assessed by cystatin C).

As some subjects did not have serum stored at baseline, the impact of B-vitamin treatment on the relationship between tHcy and renal function was tested in a subgroup (\( n = 132 \)) rather than the whole cohort (\( n = 173 \)). There were no systematic differences between the subjects with and without stored serum, so we have no reason to believe the reported null effect would have been different in the full cohort. We had 80% power (\( \alpha = 0.05, n = 132 \)) to declare an absolute change in the regression coefficient of 0.37 statistically significant, compared with 0.31 if baseline data were available for all 173 subjects.

Our study is the first to report cystatin C as an independent predictor of FMD. Our data are consistent with recent evidence that cystatin C is a sensitive indicator for the presence and severity of coronary artery disease and a
predictor of vascular events.\textsuperscript{5,33} However, we also found that cystatin C was not an independent predictor of CIMT and the reason for the discrepancy is not clear. Given the limitations discussed above, the relatively small subject numbers and the specificity of regression results to a particular data set, it is possible that either the FMD or CIMT regression results are attributable to chance. It is also possible that renal impairment contributes to cardiovascular risk through mechanisms that cause functional rather than structural change in vasculature, but this hypothesis will need to be tested in future studies. We have demonstrated that adjusting for renal function not only eliminates the relationship between \textit{tHcy} and markers of vascular risk in subjects with proven cerebrovascular disease, but also that elevated cystatin C, a sensitive indicator of GFR, is independently associated with reduced FMD. Our data are thus consistent with the hypothesis that mild renal impairment is an independent risk factor for vascular disease and elevated \textit{tHcy} simply a marker for reduced GFR. The underlying relationship between \textit{tHcy} and renal function is not altered by long-term B-vitamin supplementation and it is possible that, by treating homocysteine, we may be shooting the messenger rather than attacking the true risk factor.

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Disclosures

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