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Glutathione Peroxidase-1 and Homocysteine for Cardiovascular Risk Prediction

Results from the Athero*Gene* Study

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OBJECTIVES This prospective study was designed to evaluate the effect of joint determination of two important contrary biomarkers—homocysteine and glutathione peroxidase (GPx)-1—on cardiovascular risk stratification.

BACKGROUND Homocysteine plasma levels have been associated with cardiovascular risk. Experimental data suggest that antioxidative GPx-1 activity modulates cardiovascular risk associated with homocysteine.

METHODS In 643 patients with coronary artery disease, we performed a prospective study to assess the risk of homocysteine plasma levels and GPx-1 activity on long-term cardiovascular risk with a median follow-up of 7.1 years.

RESULTS Both homocysteine and GPx-1 were among the strongest univariate predictors of future cardiovascular risk, even after adjustment for cardiovascular confounders. Homocysteine levels were significantly elevated in individuals with future cardiovascular events (15.4 vs. 13.4 μmol/l; $p < 0.0001$); GPx-1 activity was lower (45.3 ± 13.1 vs. 50.2 ± 11.0 U/g hemoglobin; $p < 0.0001$). In patients with GPx-1 activity below the median value, homocysteine plasma levels above the median were associated with a 3.2-fold (95% confidence interval 1.8 to 5.6; $p < 0.0001$) increase in cardiovascular risk, whereas it lost its independent risk prediction in individuals with increased antioxidative capacity, as reflected by high GPx-1 activity. In contrast to single determination, combined assessment revealed a significant increase in the area under the curve of cardiovascular risk predictive models from 0.72, including traditional risk factors to 0.75 and also including homocysteine levels and GPx-1 activity.

CONCLUSIONS Plasma homocysteine levels and GPx-1 activity are complementary in identifying individuals at high cardiovascular risk. Joint determination of both biomarkers provides substantial information on top of classic risk factors in cardiovascular risk assessment. (J Am Coll Cardiol 2005;45:1631–7) © 2005 by the American College of Cardiology Foundation

Mild to moderate elevation of total plasma homocysteine has been postulated to be causally involved in the development of atherosclerotic disease (1–3). However, there are still conflicting findings whether or not homocysteine independently adds to the predictive value over and above that obtained from traditional risk factors (4,5).

Mild to moderate elevation of homocysteine plasma levels is frequent (6). The adverse effects of homocysteine on endothelial function and atherosclerotic lesion progression may be triggered, among other potential pathophysiologic mechanisms (7), by increased oxidative stress, leading to a decrease in vascular nitric oxide bioactivity (8). In mammalian cells, glutathione is involved in oxidant defense, mainly by the most abundant and ubiquitous of the glutathione peroxidase enzymes—glutathione peroxidase-1 (GPx-1) (9). Homocysteine not only leads to the formation of reactive oxygen species (ROS) but also, as recently shown, inhibits the expression of this antioxidant enzyme (10). On the other hand, in vitro models could demonstrate that the overexpression of GPx-1 can restore the normal endothelial phenotype in hyperhomocysteinemic states (11). Thus, baseline erythrocyte levels of the antioxidant enzyme GPx-1 emerges as a defense marker rather than as an indicator of oxidative stress. Recently, baseline levels of the erythrocyte GPx-1 activity have been shown to be inversely related to future cardiovascular risk in patients with coronary artery disease (CAD) during a five-year follow-up (12).

So far, the predictive value of homocysteine concentration has not been evaluated while taking into account GPx-1 activity, which may enlighten the ongoing controversy. Hence, the present study was carried out to evaluate whether homocysteine levels and GPx-1 activity add independent information for cardiovascular risk stratification on top of that obtained from traditional risk factors and whether the activity of GPx-1 as an oxidative

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Abbreviations and Acronyms

AUC = area under the curve
CAD = coronary artery disease
CI = confidence interval
GPx-1 = glutathione peroxidase-1
HR = hazard ratio
hs-CRP = high-sensitivity C-reactive protein
MI = myocardial infarction
ROS = reactive oxygen species

defense marker influences the predictive value of homocysteine.

METHODS

Study population. Between November 1996 and December 1997, 732 patients referred to the Department of Medicine II of the Johannes Gutenberg-University Mainz for suspected CAD were enrolled in the Athero*Gene* registry. Fourteen patients with acute myocardial infarction (MI) and 75 patients in whom GPx-1 activity could not be determined immediately were excluded from the present analysis. Therefore, the final study cohort consisted of 643 patients: 133 of them with symptoms of unstable and 510 with symptoms of stable angina. In all patients, coronary angiography was performed. In 558 patients, relevant CAD (i.e., >30% stenosis in at least one major coronary artery) was detected. The study has been described in detail elsewhere (12). Briefly, exclusion criteria were evidence of hemodynamically significant valvular heart disease, surgery, or trauma within the previous month, known cardiomyopathy, known malignant diseases, febrile conditions, or oral anticoagulant therapy within the previous four weeks. Diabetes mellitus was diagnosed in patients with dietary treatment or antidiabetic medication or current fasting blood sugar >125 mg/dl, hypertension in patients who had received antihypertensive treatment or had been diagnosed as hypertensive (blood pressure >160/90 mm Hg). Smoking was classified as current, past (stopped between >4 weeks and <40 years), or never smoking.

Among the 643 patients, 639 (99.4%) were followed for a median of 7.1 years (maximum 7.6; Athero*Gene* follow-up of 2 years). There were 84 deaths from cardiovascular causes, 33 deaths from causes not related to heart disease, and 28 nonfatal MIs. Information on the cause of death or clinical events was obtained from hospital or general practitioner charts.

The study was approved by the ethics committee of the University of Mainz. Participation was voluntary, and each study subject gave written, informed consent.

Laboratory methods. Blood was drawn under standardized conditions before coronary angiography. The GPx-1 activity was determined in washed red blood cells obtained immediately from whole blood anticoagulated with EDTA. Hemolyzed cells were stored frozen for up to one week, which does not lead to changes in enzyme activity. Then, GPx-1 was measured as described (13), with minor modifications (Randox, United Kingdom). Intra- and interassay coefficients of variation were 6.7% and 9.9%, respectively. Lipid serum levels were measured immediately by routine methods (total cholesterol and triglycerides, Roche Diagnostics, Mannheim, Germany; high-density lipoprotein cholesterol, Rolf Greiner Biochemica, Flacht, Germany; and low-density lipoprotein cholesterol calculated by the Friedewald formula). Fibrinogen was determined immediately by the derived method.

For all other biomarkers described here, plasma and serum were stored at −80°C until analysis, which was performed after a mean of 1.5 years storage time. Homocysteine was determined by high-pressure liquid chromatography (interassay coefficient of variation 7.1%), selenium by carbon furnace atomic absorption spectrometry with Zeeman compensation, as previously described (14), and C-reactive protein (CRP) by a highly sensitive, latex particle-enhanced immunoassay (detection range of 0.1 to 20 mg/l, Roche Diagnostics, Mannheim, Germany; interassay coefficient of variation 1.0% at 15 mg/l and 6.5% for values <4 mg/l).

Statistical methods. Mean levels and proportions of baseline cardiovascular risk factors were calculated for study participants, according to GPx-1 activity below or above the median value. The significance of mean differences between the two groups was assessed by the Student *t* test, and the significance of proportion differences was tested by the chi-square statistic. Variables with a skewed distribution—absolute value of skewness >1—were presented as median values, and the Wilcoxon rank-sum test was applied. In all survival analyses, the combined end point was death from cardiovascular causes and nonfatal MI. Patients who died from other causes were censored at the time of death. Hazard ratios (HRs) for future coronary events, according to quartiles of homocysteine and GPx-1 activity, were estimated by Cox regression analysis, adjusting for potential confounders. Three adjusted models were constructed. The HRs were determined for quartiles 2 to 4 in comparison with the lowest category, and the p value across the quartiles was calculated by use of the Wald test (1 degree of freedom). First, we adjusted for age and gender, and second, for other cardiovascular predictors. In the final model, all potential clinical and therapeutic variables, as well as confounders, were included. We further evaluated the combined role of GPx-1 activity and homocysteine on cardiovascular risk, and therefore classified the study participants into four groups according to Gpx-1 activity and homocysteine plasma levels below and above their respective median values. The homogeneity of homocysteine effects by GPx-1 activity levels on cardiovascular risk was formally tested by including a term of interaction between homocysteine and GPx-1 levels considered as dichotomous variables in the Cox regression model. The four cumulative event plots obtained by combining high/low Gpx-1 activity and high/low homocysteine concentration were estimated by the

Table 1. Baseline Characteristics of the Overall Study Population According to the Median Value (48.3 U/g Hb) of Glutathione Peroxidase-1 Baseline Levels

Variable	≤48.3 U/g Hb (n = 321)	>48.3 U/g Hb (n = 322)	p Value
Age (yrs)	61.7 ± 9.8	61.7 ± 10.3	0.97
Male gender (%)	76.3	68.4	0.03
Classic risk factors			
BMI (kg/m^2)	26.5 ± 3.8	27.0 ± 3.3	0.28
History of diabetes (%)	17.8	13.5	0.15
History of hypertension (%)	66.1	71.4	0.16
Smokers (%)	67.8	51.0	<0.0001
Hyperlipidemia	54.9	63.5	0.03
Homocysteine metabolism			
Homocysteine (μmol/l)	14.4 (11.5/17.9)	13.5 (11.3/16.0)	0.06
Vitamin B_{12} (pg/ml)	390 (313/499)	366 (300/476)	0.08
Folic acid (ng/ml)	7.0 (5.6/9.2)	7.5 (5.6/9.4)	0.39
GPx-1 metabolism			
Selenium (ng/ml)	73.2 ± 33.2	77.5 ± 33.1	0.14
Clinical variables			
Multivessel disease (>2) (%)	67.4	61.8	0.15
Left ventricular ejection fraction (%) (n = 539)	61.9 ± 15.6	62.3 ± 15.6	0.78
Medication			
Statin medication (%)	21.1	24.3	0.33
Beta-blocker (%)	49.7	51.0	0.75
Renal function			
Creatinine (mg/dl)	1.1 ± 0.9	1.1 ± 0.3	0.47
Inflammation			
hs-CRP (mg/l)	3.9 (1.9/10.2)	3.7 (1.9/9.2)	0.79
Fibrinogen (mg/dl)	331.0 ± 101.0	352.6 ± 121.6	0.75

Data are presented as the percentage of patients or mean value ± SD or median value and 25th/75th interquartile range for skewed variables. Smoking status comprises current and former smokers.

BMI = body mass index; GPx-1 = glutathione peroxidase-1; Hb = hemoglobin; hs-CRP = high-sensitivity C-reactive protein.

Kaplan-Meier method and compared using the log-rank test. The discriminative value of different predictive models was estimated by the area under the (receiver operating) curve (AUC) obtained from the *C* statistic in a logistic regression model, including all traditional risk factors and also the plasma levels of homocysteine, serum creatinine, and GPx-1 activity. The HRs and their 95% confidence intervals (CIs) are reported. The p values are two-sided; $p < 0.05$ was considered to be significant. All computations were carried out with SPSS Version 10.07 (SPSS Inc., Chicago, Illinois).

RESULTS

The mean age of the study population was 61.7 ± 10.1 years; 72.2% were male patients. The GPx-1 activity showed a mean (±SD) level of 49.3 ± 11.5 and a median (25th/75th percentile) of 48.3 (42.2/56.3) U/l hemoglobin (Hb). The respective data for homocysteine concentrations in the overall study population showed a median (25th/75th percentile) of 13.7 (11.3/16.8) μmol/l. Baseline characteristics of the study population according to median GPx-1 activity are provided in Table 1. The major determinants of GPx-1 activity were, before all, smoking and, to a lesser extent, gender and hyperlipidemia of the classic risk factors, as well as homocysteine and selenium levels of the humoral markers.

The GPx-1 activity was lower in the subgroup of patients with future cardiovascular events (45.3 ± 13.1 vs. 50.2 ± 11.0 U/g Hb; $p < 0.0001$). Likewise, the median homocysteine plasma concentration was elevated in the event group in comparison with event-free individuals (15.4 [13.1/21.1] vs. 13.4 (11.0/16.2) μmol/l; $p < 0.0001$). In a Cox proportional hazards model, the upper quartile of homocysteine was associated with a 3.0 (95% CI 1.35 to 6.66) increase in risk after adjustment for most potential clinical and therapeutic variables, including creatinine and GPx-1 (Table 2). The upper quartile of creatinine (>1.19 mg/dl) itself was also independently associated with future cardiovascular risk in a fully adjusted model (Table 2). The inverse relationship between GPx-1 activity and future cardiovascular risk also remained highly significant after a long-term follow-up period of 7.1 years.

The interaction between these two variables on cardiovascular risk was investigated by estimating the HR associated with high homocysteine, according to GPx-1 activity. In patients with GPx-1 activity above the median value (>48.3 U/g Hb), the association between homocysteine above the median (>13.7 μmol/l) and future cardiovascular events did not reach a significant level (HR 1.7, 95% CI 0.9 to 3.3; $p = 0.10$). By contrast, in patients with GPx-1 activity below the median value, elevated homocysteine plasma levels were highly significantly associated with a

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Table 2. Hazards Ratio of Future Cardiovascular Events (n = 112) According to Prognostic Variables in 639 Patients

Variable	No. of Subjects	No. of Events (%)	Age- and Gender-Adjusted		Model 2 Prognostic Factors		Model 3 Fully Adjusted	
			HR (95% CI)	p Value	HR (95% CI)	p Value	HR (95% CI)	p Value
Glutathione peroxidase-1 (U/g Hb)								
<42.0	159	44 (27.7)	1.00	<0.001	1.00	0.001	1.00	0.001
42.0–48.3	158	28 (17.7)	0.59 (0.36–0.94)		0.67 (0.41–1.10)		0.71 (0.43–1.18)	
>48.3–56.3	161	20 (12.4)	0.39 (0.23–0.66)		0.39 (0.21–0.70)		0.39 (0.21–0.74)	
>56.3	161	20 (12.4)	0.40 (0.23–0.67)		0.40 (0.22–0.72)		0.41 (0.22–0.74)	
Homocysteine (μmol/l)								
<11.3	159	11 (7.4)	1.00	<0.001	1.00	0.012	1.00	0.006
11.3–13.7	152	21 (13.8)	1.71 (0.82–3.55)		1.80 (0.83–3.90)		2.04 (0.88–4.73)	
>13.7–16.8	153	27 (17.6)	2.18 (1.28–4.41)		1.95 (0.92–4.14)		2.22 (0.98–5.03)	
16.8	151	48 (31.8)	3.76 (1.93–7.33)		2.55 (1.23–5.30)		3.00 (1.35–6.66)	
Creatinine (mg/dl)								
<0.94	157	19 (12.1)	1.00	<0.001	1.00	0.001	1.00	0.001
0.94–1.04	166	20 (12.0)	0.91 (0.47–1.75)		0.83 (0.40–1.72)		0.89 (0.42–1.87)	
>1.04–1.19	156	23 (14.7)	1.13 (0.59–2.15)		1.19 (0.59–2.42)		1.23 (0.59–2.59)	
>1.19	160	50 (31.3)	2.41 (1.32–4.38)		2.40 (1.23–4.67)		2.48 (1.22–5.02)	
High-density lipoprotein (mg/dl)								
<44	330	71 (21.5)	1.00	0.002	1.00	0.14	1.00	0.15
≥44	309	41 (13.3)	0.54 (0.37–0.80)		0.73 (0.48–1.11)		0.72 (0.46–1.13)	
Diabetes								
No	541	48 (15.3)	1.00	0.01	1.00	0.22	1.00	0.15
Yes	98	35 (29.6)	1.71 (1.12–2.62)		1.34 (0.84–2.12)		1.42 (0.88–2.30)	
Smoking								
Never	259	35 (13.5)	1.00	0.004	1.00	0.26	1.00	0.31
Former/current	380	77 (20.3)	1.86 (1.23–2.83)		1.30 (0.82–2.07)		1.28 (0.79–2.07)	
C-reactive protein (mg/l)								
<10.1	459	73 (15.9)	1.00	0.10	1.00	0.34	1.00	0.31
≥10.1	154	35 (22.7)	1.41 (0.94–2.11)		1.24 (0.80–1.91)		1.27 (0.80–2.01)	
Statin therapy								
No	496	98 (19.8)	1.00	0.04	1.00	0.09	1.00	0.12
Yes	143	14 (9.8)	0.55 (0.31–0.96)		0.58 (0.31–1.09)		0.61 (0.32–1.14)	
Extent of vessel disease								
0–1	221	26 (12.2)	1.00		1.00		1.00	
2	153	27 (17.0)	1.31 (0.76–2.25)	0.04	1.33 (0.74–2.40)	0.076	1.22 (0.65–2.29)	0.20
≥3	265	59 (22.3)	1.65 (1.03–2.64)		1.57 (0.92–2.66)		1.43 (0.81–2.51)	
Ejection fraction (%)								
≥40	506	63 (12.5)	1.00	<0.001	1.00	<0.001	1.00	<0.001
<40	57	26 (45.6)	4.41 (2.79–6.98)		3.15 (1.87–5.29)		3.46 (1.91–6.29)	

Model 2 adjusted for age, gender, and all prognostic variables presented in the table. Model 3 further adjusted for body mass index, triglycerides (continuous variable, log-transformed), hypertension, unstable angina, history of myocardial infarction, beta-blocker therapy, and vitamin B_{12} and folic acid. Because of 77 missing values of ejection fraction in high-risk patients, no adjustment on ejection fraction was performed. For details see text.

CI = confidence interval; Hb = hemoglobin; HR = hazard ratio.

3.2-fold (95% CI 1.8 to 5.6; $p < 0.0001$) increase in cardiovascular risk. However, when formally tested, the interaction between GPx-1 and homocysteine did not reach statistical significance ($p = 0.16$). Figure 1 displays Kaplan-Meier survival curves for patients classified into subgroups according to the median value of homocysteine levels and Gpx-1 activity, showing a clear trend in risk from patients with low homocysteine/high GPx-1 to those with high homocysteine/low GPx-1.

To assess whether these biomarkers add to the predictive value of traditional risk factor screening, we computed the AUC associated with models based on traditional risk factors alone (age, diabetes, high-density lipoprotein cholesterol, and smoking) and compared it with the AUC obtained by adding homocysteine and GPx-1 to these risk factors (Fig. 2). Although these (anti)oxidative biomarkers had significant effects when added to classic risk factors, single determination added comparatively little information to traditional risk factors in the respective models. Simultaneous assessment of homocysteine and GPx-1 activity significantly increased the predictive value of the model (AUC 0.72 to 0.75).

DISCUSSION

In this prospective cohort of patients with documented CAD, plasma levels of homocysteine were related to future cardiovascular events. However, this relationship was predominantly present in patients with low erythrocyte intracellular GPx-1 activity and hence reduced antioxidative

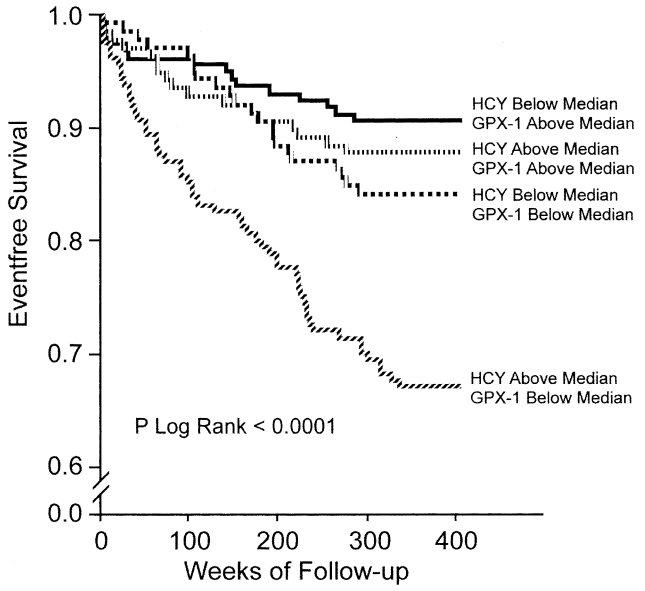

Figure 1. Kaplan-Meier curves showing cardiovascular events in the subgroups of patients classified according to median baseline levels of homocysteine (HCY) and baseline activity of glutathione peroxidase (GPx)-1.

defense. These patients revealed a three-fold increase of risk if their homocysteine level was above the median value. In patients with high GPx-1 activity, the effect associated with elevated homocysteine appeared twice lower, even though the difference was not significant due to a small sample size, suggesting that these patients are relatively protected against oxidative adverse effects induced by hyperhomocysteinemia. These results suggest that simultaneous assessment of both biomarkers provides additional information for cardiovascular risk stratification, and that interpretation of the homocysteine levels without knowledge of the GPx-1 activity might be misleading.

In this vein, serum creatinine was also a strong predictor of cardiovascular risk; however, it did not increase the AUC as compared with classic risk factors. It is well known that patients even with mild to moderate impaired renal function are at increased risk of cardiovascular complications (15). Creatinine might partly reflect the severity of vascular organ damage as a consequence of risk factor presence; alternatively, products of impaired excretion may have an additional adverse impact (16). Our findings confirm the recent data in mild renal impairment and can demonstrate the predictive strength of this routine marker.

A variety of cross-sectional and retrospective case-control studies have provided evidence for an association between mildly elevated plasma homocysteine and atherosclerotic cardiovascular disease (7). The results of prospective studies, however, have been controversial (17,18).

Our data may, at least in part, explain the overall modest association between elevated homocysteine plasma concentrations and cardiovascular risk. Patients with a high antioxidative capacity due to increased GPx-1 activity can cope with the oxidative stress induced by homocysteine. Accordingly, in subjects with low GPx-1 activity, homocysteine represents an important risk predictor for future cardiovascular events, independent of vitamin B_{12}, folic acid, and classic risk factors.

There is a growing body of evidence that the antioxidant enzyme GPx-1 plays a major role in the prevention of oxidative stress induced by cardiovascular risk factors, which renders it an important antiatherogenic enzyme. Heterozygous deficiency of GPx-1 has been demonstrated to cause endothelial dysfunction and to induce significant structural and cardiac abnormalities, presumably due to increased levels of ROS (19). Elevated homocysteine concentrations inhibit GPx-1 activity in endothelial cells in vitro as well as in mildly hyperhomocysteinemic mice in vivo (20,21). Deficiency of GPx-1 disturbs endothelial homeostasis in hyperhomocysteinemic mice and shows up the potentially toxic oxidative effects of homocysteine (22). Experimental

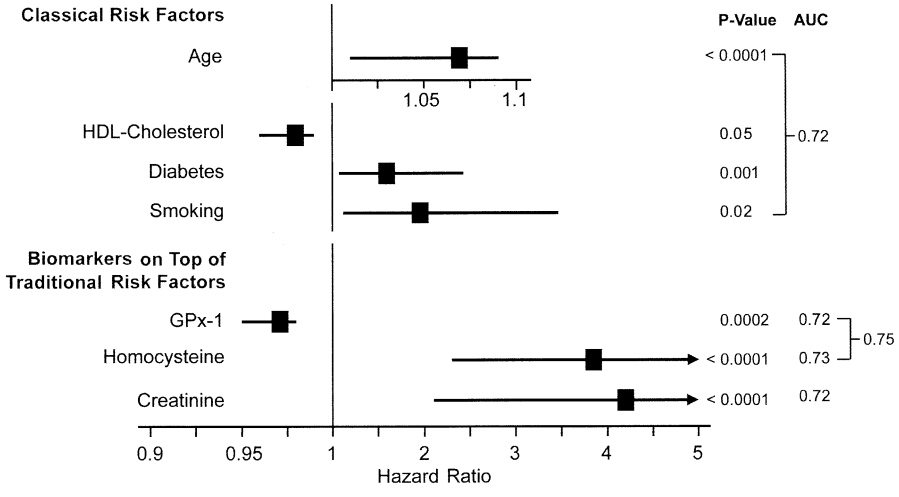

Figure 2. Hazard ratios (95% confidence interval) associated with classic risk factors in a multivariate model and with homocysteine and glutathione peroxidase (GPx)-1 (increase per one standard deviation) on top of classic risk factors (each marker added separately to classic risk factors). In addition, the area under the curve (AUC) of the corresponding multivariate model is provided. HDL = high-density lipoprotein.

studies have elegantly demonstrated the link between cellular thiol levels and reduced glutathione pools by showing that an increase in GPx-1 activity leads to a restoration of endothelial function (11). Thus, increased activity of cellular GPx-1 may rescue homocysteine-induced damages with far-reaching consequences if applicable in the clinical setting. Because they are explorative in nature, the present results that the effect of homocysteine on vascular events is modulated by GPx-1 activity have to be confirmed in large, prospective studies that primarily address the interaction between homocysteine and GPx-1 activity.

From a clinical point of view, we tested our primary hypothesis that both homocysteine and GPx-1 are likely to add incremental information to traditional cardiovascular risk factors. Interestingly, despite a highly significant association with future cardiovascular events, both markers alone provide comparatively little additional information to that obtained by traditional risk factors. Interestingly, the effect of GPx-1 varied over time; for example, incremental information on risk was observed by use of an earlier end point (median follow-up 4.7 years, AUC 0.72 to 0.75; data not shown). However, our results clearly indicate that predictive models, including traditional risk factors like diabetes, hypertension, smoking, age, gender, and plasma lipid levels, and both homocysteine plasma levels and GPx-1 activity have a significantly higher likelihood ratio and increased AUC as compared with models including traditional risk factors alone. Because recent data cast doubt on the value of homocysteine as an independent predictor for cardiovascular risk (4,23), these heterogeneous findings might be explained by the different antioxidative capacity. Our findings thus support the hypothesis of the great importance of oxidative stress in atherosclerotic disease and the important role of GPx-1 as a defense mechanism in this complex setting.

Biomarkers of oxidant stress confront clinicians with a substantial diagnostic challenge, as those markers often are at risk for auto-oxidation and tend to be unstable. In contrast to glutathione or lipid hydroperoxides, GPx-1 does not require immediate assay. So far, GPx-1 activity and plasma homocysteine levels provide independent information on the risk or prognosis, account for a large proportion of the risk, are reproducible measures, and jointly add information to that obtained from classic risk factors. Thus, some implications for patient care are fulfilled (24).

Because the activity of GPx-1 decisively determines whether risk factors may be translated into CAD, a next step would be the transfer of this knowledge into clinical practice (25,26). Subjects with high GPx-1 activity might profit less from vitamin supplementation than individuals with lower doses of this beneficial enzyme. On the other hand, substitution of selenium might reduce homocysteine-induced oxidative damage in humans by increasing the expression and/or activity of the selen-dependent GPx-1 (27,28).

Conclusions. In patients with CAD, the antioxidative enzyme GPx-1 seems to protect against adverse oxidative effects, in part induced by homocysteine. The simultaneous analysis of both GPx-1 activity and plasma homocysteine levels provides superior information on cardiovascular risk assessment compared with measurement of traditional risk factors alone.

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