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Abstract

Endothelial dysfunction is known to precede the development of atherosclerosis and results primarily from increased oxidative degradation of NO. We hypothesized that assessment of oxidative stress in the bloodstream will reliably predict endothelial function in healthy adults. A total of 124 healthy nonsmokers had endothelial function assessed using ultrasound measurement of brachial artery flow-mediated vasodilation. Plasma oxidative stress was estimated by measuring the levels of the reduced and oxidized forms of thiols, including glutathione (reduced glutathione and oxidized glutathione) and cysteine (cysteine and cystine), respectively, and the mixed disulfide. Among the traditional risk factors, there were significant and independent correlations between flow-mediated vasodilation and high-density lipoprotein level, body mass index, gender, and the Framingham risk score. Among the thiol markers, plasma cystine ($r=-0.23$; $P=0.009$) and the mixed disulfide ($r=-0.23$; $P=0.01$) levels correlated with endothelium-dependent but not endothelium-independent vasodilation, even after adjusting for the Framingham risk score and high-sensitivity C-reactive protein level. A higher level of oxidized metabolites was associated with worse endothelial function. In conclusion, the oxidative stress markers, cystine, and the mixed disulfide are independent predictors of endothelial function. These markers, in combination with the Framingham risk score, may help in the early identification of asymptomatic subjects with endothelial dysfunction who are at potentially increased risk for future atherosclerotic disease progression.

Keywords

risk factors; endothelial function; flow mediated vasodilation; antioxidants; oxidative stress

Clinical assessment of endothelial function involves the measurement of dilation of conductance arteries during periods of acute increases in shear stress, believed to be almost entirely mediated by NO release, or measurement of agonist-induced vasodilation.^{1–3} The magnitude of endothelial dysfunction is an important and independent predictor of future development of cardiovascular risk factors, such as hypertension and diabetes, and of

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cardiovascular events.^{4–8} Thus, assessment of endothelial function quantifies subclinical vascular damage and is a valuable prognostic tool.^{3,4} The available clinical techniques for estimating endothelial function require substantial expertise and are not suitable for use in routine clinical practice. There is, thus, a critical need for simpler tests, potentially biomarkers, that would provide an accurate index of vascular endothelial function.

The bioavailability of NO from the vascular endothelium is exquisitely modulated by reactive oxygen species that degrade NO, uncouple NO synthase, and inhibit synthesis.⁹ It is, therefore, possible that measurement of oxidative stress in the circulation would provide an index of endothelial function. In support of this hypothesis, a recent study has found a correlation between oxidized low-density lipoprotein and vascular endothelial function,¹⁰ although other markers have failed to confirm this.^{11–13}

Quantification of the major intracellular and extracellular aminothiols in plasma provides one potential measure of oxidative stress in vivo. Intracellularly, glutathione (GSH) is the major antioxidant that helps eliminate peroxides and other oxidants.¹⁴ GSH and its oxidized form (GSSG) that are released into plasma can be reliably measured, and lower levels of GSH and/or higher levels of GSSG are indicators of increased intracellular oxidative stress.

Extracellularly, cysteine (Cys) constitutes the major thiol pool that helps eliminate oxidants and results in conversion to its oxidized disulfide form, cystine (CySS).¹⁵ Because Cys is more reactive and present at a higher concentration in plasma than GSH, oxidants preferentially oxidize Cys to CySS, and redox balance is preserved by the supply of GSH from tissues. Released GSH reacts with CySS to form a mixed disulfide (CySSG) and Cys.¹⁶ The intermediary product released from this reaction, CySSG, appears uniquely positioned to assess overall body oxidative stress, because it is a product of a reaction between the predominant extracellular disulfide (CySS) and the predominant intracellular thiol (GSH). Recent experimental data also indicate that intracellular proatherogenic events and cell adhesion are modulated in part by extracellular thiol/disulfide redox state.¹⁷ The steady-state balance of GSH and GSSG and of Cys and CySS can be expressed as the redox state (E_h) of the GSH/GSSG and Cys/CySS couple, respectively, calculated using the Nernst equation.¹⁸ We hypothesized that these aminothiol levels (Cys, CySS, CySSG, GSH, and GSSG) would be predictive of vascular endothelial function assessed by flow-mediated vasodilation (FMD) of the brachial artery and tested this hypothesis in a relatively healthy cohort of nonsmoking subjects free of cardiovascular disease.

Methods

A total of 124 healthy nonsmoking volunteers between the ages of 30 and 65 years without any known cardiovascular risk factors, such as hypertension, diabetes, or hypercholesterolemia, and without clinically evident atherosclerosis were recruited by advertisement. Subjects were excluded if they were known to have a history of diabetes (fasting glucose of >126 mg/dL or hemoglobin A1c of >7%), hypertension (elevated systolic [>140 mm Hg] or diastolic blood pressure [>90 mm Hg] on 3 separate measurements), or hyperlipidemia requiring treatment; were smoking in last 3 months; or were on any vasoactive medications, vitamins, or supplements. Pregnant women and those with acute or chronic illnesses were also excluded. The study was approved by the Emory University Institutional Review Committee. Informed consent was obtained from all of the subjects.

After answering a questionnaire and a routine physical examination, overnight fasting blood samples were obtained. Plasma levels of total, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol; triglycerides; and glucose were measured. High-

sensitivity (hs) C-reactive protein (CRP) was measured by immunonephelometry (Dade Behring).

Measurement of Thiol and Disulfide Forms of Glutathione and CySS and the CySSG

Detailed procedures for measurements of blood GSH, GSSG, Cys, CySS, CySSG, E_h GSH/GSSG, and E_h Cys/CySS have been described previously.^{15,19–21} Samples were collected directly into specially prepared tubes containing a preservative to reduce auto-oxidation, centrifuged, and the supernatant frozen at –80°C, which shows no significant loss for 1 year. Analyses by high-performance liquid chromatography were performed after dansyl derivatization on a 3-aminopropyl column with fluorescence detection.¹⁹ Metabolites were identified by coelution with standards and quantified by integration relative to the internal standard, with validation relative to external standards. Issues of sample collection, stability, analysis, and standardization have been extensively studied, and the method has been used in several clinical studies.²² The coefficient of variation for GSH was 5%, and the coefficient of variation for GSSG was 9.7%. The SD for week-to-week variation among individuals for GSH redox potential was 3.22 mV. The reproducibility values were similar for Cys, CySS, CySSG, and E_h Cys/CySS.

Measurement of Brachial Artery FMD

Endothelium-dependent brachial artery FMD was determined as described previously after the blood sample for biomarker evaluation was obtained.^{2,23} Briefly, ultrasound images were obtained at baseline under standardized conditions and ≈60 seconds after induction of reactive hyperemia by 5-minute cuff occlusion of the forearm. After a 15-minute period to re-establish baseline conditions, endothelium-independent dilation of the brachial artery was assessed from images obtained before and 3 to 5 minutes after administration of 0.4 mg of sublingual nitroglycerin. Images were digitized online, and arterial diameters were measured with customized software (Medical Imaging Applications, Inc) by individuals blinded to the clinical status and laboratory status of the subjects. FMD and endothelium-independent vasodilation were expressed as the percentage increase in diameter from baseline. In our laboratory, the mean difference in FMD between 2 consecutive assessments performed in 11 subjects an average of 8 days apart was 1.26±0.76%, with a correlation coefficient of 0.75. The mean difference in the FMD between 2 readings of the same 11 measurements was 0.82±0.48% ($r=0.97$).

Statistical Considerations

Study variables are described as the mean±SD for continuous variables or as proportions for categorical variables. Age, body mass index (BMI), hs-CRP, LDL, HDL, total cholesterol, and triglyceride levels were used as continuous variables. Newly diagnosed hypertension or diabetes mellitus, smoking history, family history of coronary artery disease, and gender were categorical variables. Continuous variables were tested for normality using the Kolmogorov-Smirnov criterion. Skewed variables were log transformed before any parametric analysis.

Unadjusted linear regression and multivariate conditional linear regression analyses were performed to ascertain whether any subject characteristics, presence of conventional risk factors, or thiol levels were related to FMD or nitroglycerin-mediated vasodilation (S-Plus version 6.0.3, Insightful Corporation). Separate and combined multivariable models were run for each of the thiol markers that, on univariate analysis, demonstrated significant correlation with FMD. Only *P* values <0.05 were considered significant.

Results

The baseline characteristics of the 124 subjects (mean age: 44 ± 8.4 years; 40% male) are shown Table 1. Although unaware at recruitment, 11.2% were found to have new-onset hypertension, and 4.8% were found to have diabetes. Serum cholesterol levels ranged between 130 and 303 mg/dL. Their mean FMD was $7.5\pm 3.7\%$, and nitroglycerin-mediated dilation was $19.6\pm 7.7\%$.

Relationship Between FMD and Traditional Risk Factors

The univariate predictors of abnormal endothelial function were age, gender, elevated BMI, and hypertension. Thus, there was a significant inverse correlation between FMD and age ($r=-0.22$; $P=0.01$) and BMI ($r=-0.18$; $P=0.046$). Compared with nonhypertensive subjects, those with hypertension had lower FMD ($7.8\pm 3.8\%$ versus $5.3\pm 2.1\%$; $P=0.001$), and women had higher FMD compared with men ($8.1\pm 4.3\%$ versus $6.5\pm 2.3\%$; $P=0.007$). After multivariate analysis that included age; gender; BMI; levels of LDL, HDL, and triglycerides; and history of hypertension, diabetes, previous smoking, or family history, only gender, elevated BMI, and HDL cholesterol were independent predictors of FMD.

The mean Framingham risk score for the population was 0.2 ± 6.4 , underscoring the low risk profile of the population. There was a significant negative correlation between FMD and the Framingham risk score ($r=-0.22$; $P=0.01$).

Relationship Between Markers of Oxidative Stress

There were significant correlations observed between some of the thiol markers. Thus, CySS and GSH were inversely correlated, ($r=-0.24$; $P=0.008$), and CySSG was correlated with both GSH ($r=0.66$; $P<0.0001$) and GSSG ($r=0.58$; $P<0.0001$). Moreover, CySS correlated with both E_h GSH/GSSG ($r=0.35$; $P<0.001$) and E_h Cys/CySS ($r=0.28$; $P=0.001$), and CySSG correlated with E_h GSH/GSSG ($r=-0.25$; $P<0.005$).

Relationship Between Markers of Oxidative Stress and Risk Factors

Plasma GSH, GSSG, CySSG, Cys, CySS, E_h GSH/GSSG, and E_h Cys/CySS levels were within the ranges observed in previous studies in healthy subjects (Table 1).^{15,20} There was a significant correlation between CySS levels and age ($r=0.28$; $P=0.0001$), elevated BMI ($r=0.22$; $P=0.02$), and presence of hypertension ($r=0.21$; $P=0.02$). Similarly, there was a significant relationship between CySSG and plasma triglycerides ($r=-0.26$; $P=0.003$), HDL cholesterol ($r=0.22$; $P=0.01$), and hypertension ($r=0.20$; $P=0.03$). There was also a significant correlation between the Framingham risk score and CySS ($r=0.23$; $P=0.005$) but not with CySSG ($r=-0.15$; $P=0.10$). Thus, increased age and risk factor burden were associated with greater systemic oxidant stress measured as higher levels of CySS and CySSG.

Relationship Between Markers of Oxidative Stress and Endothelial Function

Correlations between markers of oxidative stress and endothelium-dependent vasodilation, measured as FMD, are shown in Table 2. Linear regression analyses demonstrated significant inverse correlations between FMD and CySS and CySSG, indicating that a higher level of oxidative stress measured by these markers was associated with lower FMD. The levels of CySS and CySSG in tertiles of FMD are shown in the Figure. There was no correlation between FMD and any of the other thiol markers or inflammation (hs-CRP) in this population.

A multivariate analysis was performed to determine predictors of FMD and included the traditional risk factors measured as the Framingham risk score and the thiol markers. The

Framingham risk score ($\beta=-0.23$; $P=0.01$) and the thiols, CySS ($\beta=-0.19$; $P=0.03$) and CySSG ($\beta=-0.27$; $P=0.002$), were determinants of FMD. Both markers remained determinants of FMD even after adjustment for each other and for the hs-CRP level ($\beta>-0.21$, $P=0.01$ for CySSG and $\beta=-0.22$, $P=0.01$ for CySS).

Finally, we analyzed the group of subjects who had incidental hypertension, diabetes, or a BMI ≥ 30 ($n=41$) and compared them with the remaining 83 who did not. The FMD was significantly lower among the patients with risk factors compared with those without ($5.8\pm 0.5\%$ versus $8.3\pm 0.4\%$; $P=0.0002$). Similarly, CySS level was higher (89.2 ± 3.1 versus 81 ± 1.4 ; $P=0.006$), but the CySSG levels were similar. Exclusion of subjects with new-onset diabetes or hypertension did not alter the relationship between these thiol markers and FMD (data not shown).

Relationship of Endothelium-Independent Vasodilation With Markers of Oxidative Stress and Traditional Risk Factors

There was a significant correlation between FMD and endothelium-independent vasodilation mediated with nitroglycerin ($r=0.42$; $P<0.0001$). Among traditional risk factors, there were univariate correlations between nitroglycerin-mediated vasodilation and several risk factors including BMI ($r=-0.27$; $P=0.03$), HDL cholesterol ($r=0.23$; $P=0.009$), and LDL cholesterol ($r=-0.22$; $P=0.02$). Compared with nonhypertensive subjects, those with hypertension had lower nitroglycerin-mediated dilation ($20.1\pm 7.8\%$ versus $15.8\pm 5.8\%$; $P=0.02$), women had higher dilation compared with men ($22.2\pm 7.6\%$ versus $15.6\pm 5.9\%$; $P<0.0001$), and ex-smokers had lower dilation compared with nonsmokers ($15.7\pm 6.5.6\%$ versus $20.8\pm 7.6\%$; $P=0.0003$). After multivariate analysis, only gender, BMI, and a smoking history correlated with endothelium-independent vasodilation. There was also a significant correlation between nitroglycerin-mediated vasodilation and Framingham risk score ($r=-0.32$; $P=0.0003$). After adjusting for traditional risk factors, none of the thiol markers correlated with endothelium-independent brachial arterial response (Figure and Table 2).

Discussion

By releasing NO and other autocrine and paracrine factors, the normal endothelium modulates smooth muscle tone and growth, platelet activation and thrombogenesis, inflammation, and plaque formation. Impaired NO bioavailability is characteristically observed in individuals exposed to risk factors for atherosclerosis, including smoking, hypertension, hyperlipidemia, diabetes, and advanced age. NO bioavailability is also reduced by environmental and genetic risk factors.²⁴ Because increased production of oxygen free radicals, such as superoxide, causes oxidation of NO and inhibition of its synthesis, we considered that an alternative method of estimating endothelial function would be to measure the degree of oxidation of endogenous substrates in vivo.

Our findings demonstrate that, in a relatively healthy group of subjects without overt atherosclerosis, there is a significant correlation between endothelial function and oxidized thiol species. We found that CySS and CySSG were significant predictors of FMD even after adjusting for the Framingham risk score, CRP level, and each other. Predicting individuals at high risk of future cardiovascular events remains a challenge, particularly in subjects with low or intermediate Framingham risk scores. Inflammatory markers such as CRP have been shown to be of some value in this regard, but in this study of relatively low-risk healthy subjects, we found that thiol markers of oxidative stress were better predictors of endothelial function.

The relationship between FMD and thiol markers of the plasma redox supports the concept of an etiologic link between increased oxidative stress in vivo and endothelial dysfunction.

Both markers have been shown previously to reflect intracellular and/or extracellular oxidative stress. Cys is an important extracellular antioxidant that can be measured in human plasma where it exists in an oxidized state as CySS.¹⁵ The CyS/CySS redox state can modulate cellular events, including cell proliferation rate,^{20,25} cellular resistance to apoptosis,²⁶ and cell adhesion.¹⁷ In this study, the plasma CySS level was an important predictor of FMD supporting the notion that endothelial dysfunction is at least in part dependent on the overall extracellular oxidant/antioxidant balance. GSH is the major intracellular small molecule antioxidant. After release from cells into the blood, GSH undergoes oxidation after reacting with extracellular CySS to CySSG. This reaction appears to be the predominant mechanism for GSH metabolism.¹⁵ In this study, as in previous investigations, CySSG levels were strongly correlated with both GSH and GSSG levels, the predominant intracellular thiols, but not with Cys or CySS levels, the major extracellular thiols. This suggests that it may be a more accurate reflection of intracellular rather than extracellular oxidant/antioxidant balance. Clinical studies have demonstrated increased oxidative stress with reduced circulating GSH and increased GSSG levels with aging beyond the age 45 years,^{20,23,27} type 2 diabetes,²³ high dose chemotherapy,²⁸ cigarette smoking,²⁹ and increased carotid thickening.³⁰

Comparison With Previous Studies

In the comparatively older-aged Framingham study group, with an average age of 61 years, the mean FMD was 3.3% in women and 2.4% in men, far lower than in our relatively younger healthy group. As in our healthy cohort, significant correlations were reported with several traditional risk factors, such as age, gender, BMI, presence of hypertension, lipid-lowering medication use, smoking, and the Framingham risk score.³¹ Previous studies have also noted significant correlations, as we found, between endothelium-independent responses and some risk factors and FMD, as observed in our population.^{32,33}

Because of the difficulties in establishing reliable biomarkers that accurately reflect intracellular or extracellular oxidative stress in vivo, there has been a dearth of studies comparing them with functional measures, such as endothelial function. Recent studies have shown that urinary and serum isoprostane levels^{11,34} and oxidized LDL levels^{12,13,35} predict the presence of underlying coronary artery disease in high-risk populations, but whether they can also predict FMD is not known and will need to be further studied. Myeloperoxidase, an enzyme typically found in the leukocytes that may also play a pathophysiological role in atherogenesis, has been shown recently to independently predict FMD in a higher risk population, but whether it will also be predictive in a healthy cohort is not known.³¹ Finally, in a recent investigation in a similar relatively healthy cohort of non-smokers, we found that serum GSH levels and GSH redox were independent predictors of carotid intima-media thickness, an estimate of early structural atherosclerosis, thus providing further evidence in favor of the value of thiol redox measures as estimates of early structural and functional vascular disease.³⁰ This is the first study to assess the relationship between thiol markers and endothelial function. In this relatively healthy population, it is remarkable to note that the 2 markers appeared to be additive when used in combination with traditional risk factors, particularly in individuals with a low Framingham risk score. When subjects with incident hypertension and diabetes and those with BMI >30 were compared with those without these risk factors, endothelial function was depressed, and CySS levels were greater in this cohort. Finally, we have analyzed CySS levels in subjects with multiple risk factors and those with coronary atherosclerosis and found that the levels are significantly greater in the latter populations compared with healthy subjects, and endothelial function (FMD) was also lower in those with risk factors compared with healthy subjects (unpublished study).

Limitations

The study by nature of its cross-sectional design demonstrates a correlation between endothelial function and thiol markers of oxidative stress but does not establish causality. The observed correlation between the oxidized thiol species and abnormal endothelial function may imply either that oxidative stress is a precipitating factor for the development of abnormal endothelial function or that abnormal endothelial function causes elevation of these markers.

We also did not observe a significant correlation between GSH or the Cys redox and FMD. This may have been partly because of the fact that CySS, which did predict FMD, is also highly correlated with these redox measures. Acute treatment with vitamin C, which is known to improve endothelial function, may also be worth investigating. Moreover, in this investigation, we were unable to study the relationship between these thiol redox measures and temporal progression of abnormal endothelial function to overt atherosclerosis or to the occurrence of future cardiovascular events. These issues need to be further addressed in other populations, including those with more advanced disease.

Perspectives

Elevated levels of systemic oxidative stress, measured as increases in plasma oxidized thiol levels, CySS and the CySSG, help to identify individuals with abnormal endothelial function who may be at increased risk of developing hypertension, atherosclerosis or its' other risk factors. The relationship between endothelial function and these thiol markers was independent of traditional risk factors and presence of inflammation. Whether elevated oxidative stress in a relatively healthy low risk population should be an indication for specific therapeutic intervention needs to be further investigated.

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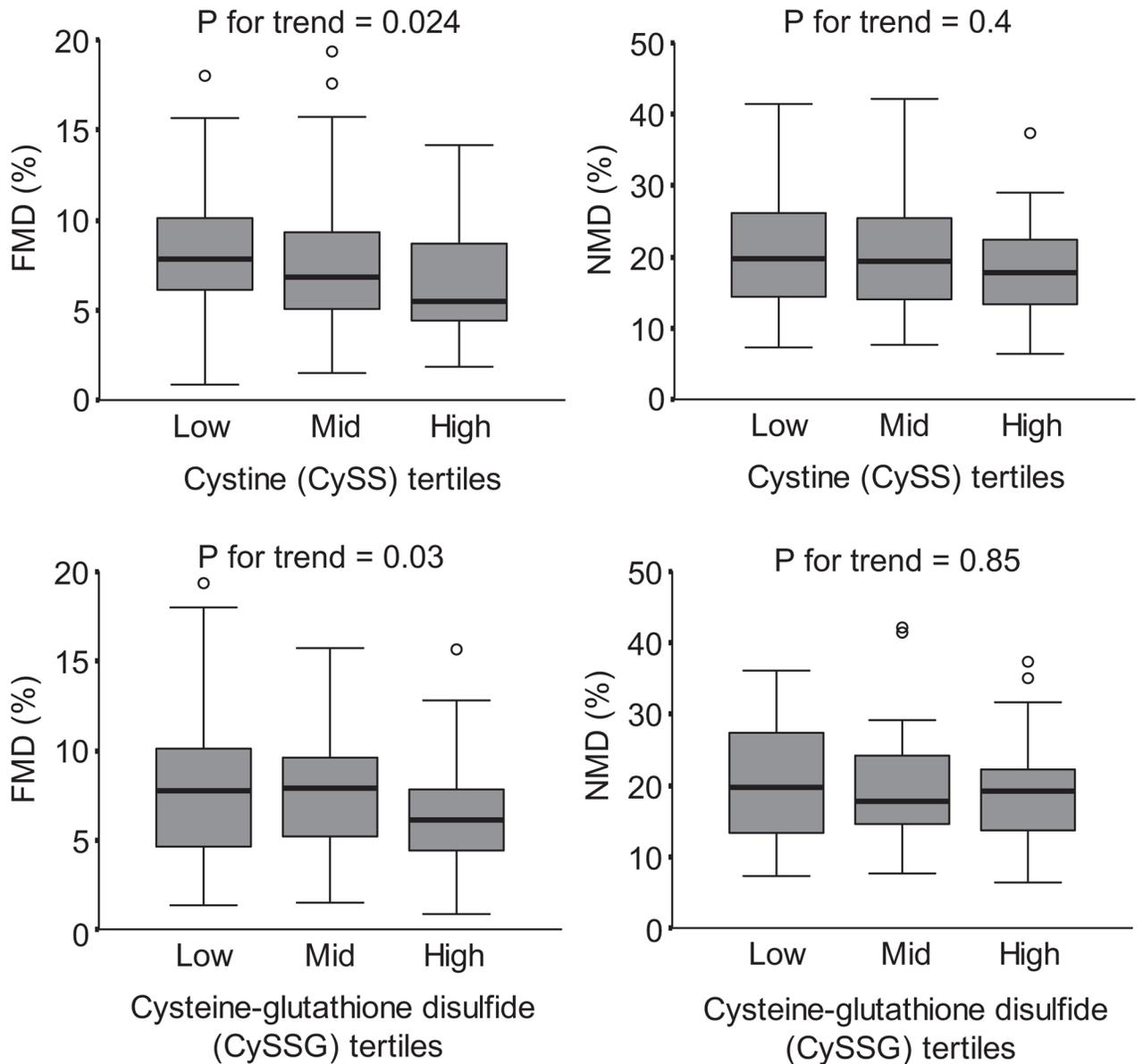
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**Figure.**

Box-and-whisker plots of FMD and nitro-glycerin-mediated dilation (NMD) according to tertiles of the thiols, CySS, and cysteine-glutathione disulfide. The centerline of the box denotes the median value, the extremes of the box denote the interquartile range, and the bars indicate the upper and lower limits of 95% of the data. The circles represent outlying data. *P* values are by analysis for linear trend after ANOVA.

Table 1

Population Characteristics

Demographics	Totals
Age, mean±SD	44±8.4
Male sex, %	39.5
LDL, mean±SD, mg/dL	122±35
HDL, mean±SD, mg/dL	50±14
Triglycerides, mean±SD, mg/dL	112±67
Cholesterol, mean±SD, mg/dL	192±33
BMI, mean±SD	27±6.0
Hypertension, %	11.2
Diabetes, %	4.8
Previous smoking, %	23.4
Markers, mean±SD	
Cys, $\mu\text{mol/L}$	9.9±2.6
CySS, $\mu\text{mol/L}$	83.7±15.9
GSH, $\mu\text{mol/L}$	1.65±0.54
CySSG, $\mu\text{mol/L}$	2.92±1.09
GSSG, $\mu\text{mol/L}$	0.098±0.5
E_h GSH/GSSG, mV	-126±7.4
E_h Cys/CySS, mV	-71.3±7.8
CRP, mg/L	3.3±5.3

Table 2

Pearson Correlation Between Endothelium-Dependent (FMD) and Nitroglycerin-Mediated Vasodilator Function and Plasma Thiol Levels

Biomarker	Correlation (r) With FMD	P	Correlation (r) With NMD	P
Cys, $\mu\text{mol/L}$	0.08	0.40	0.10	0.30
CySS, $\mu\text{mol/L}$	-0.23	0.009	-0.14	0.12
CySSG, $\mu\text{mol/L}$	-0.23	0.01	-0.04	0.67
GSH, $\mu\text{mol/L}$	0.08	0.50	0.20	0.02
GSSG, $\mu\text{mol/L}$	-0.003	0.96	0.083	0.36
E_h GSH/GSSG, mV	-0.12	0.17	-0.14	0.12
E_h Cys/CySS, mV	-0.14	0.11	-0.1	0.28
hs-CRP, mg/L	-0.11	0.24	-0.028	0.96

NMD indicates nitroglycerin-mediated dilation.