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A Novel Biomarker of Oxidative Stress is Associated with Risk of Death in Patients with Coronary Artery Disease

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A Novel Biomarker of Oxidative Stress is Associated with Risk of Death in Patients with Coronary Artery Disease

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Abstract

***Background*—**Free radical scavengers have failed to improve patient outcomes promoting the concept that clinically important oxidative stress (OS) may be mediated by alternative mechanisms. We sought to examine the association of emerging aminothiol markers of *non-free radical* mediated oxidative stress with clinical outcomes.

***Methods and Result*—**Plasma levels of reduced (cysteine and glutathione) and oxidized (cystine and glutathione disulphide) aminothiols were quantified by high performance liquid chromatography in 1411 patients undergoing coronary angiography (mean age 63 years, male 66%). All patients were followed for a mean of 4.7±2.1 years for the primary outcome of all-cause death (n=247). Levels of cystine (oxidized) and glutathione (reduced) were associated with risk of death ($p<0.001$ both) before and after adjustment for covariates. High cystine and low glutathione levels (>+1 SD & <-1 SD respectively) were associated with higher mortality (adjusted HR 1.63 (95% CI 1.19-2.21; HR 2.19 (95% CI 1.50-3.19), respectively) compared to those outside these thresholds. Furthermore, the ratio of cystine/glutathione was also significantly associated with mortality (adjusted HR 1.92 (95% CI 1.39-2.64) and was independent of and additive to hs-CRP level. Similar associations were found for other outcomes of cardiovascular death and combined death and myocardial infarction.

***Conclusions*—**A high burden of OS, quantified by the plasma aminothiols, cystine, glutathione and their ratio is associated with mortality in patients with CAD, a finding that is independent of and additive to the inflammatory burden. Importantly, this data supports the emerging role of non-free radical biology in driving clinically important oxidative stress.

Key words: oxidative stress; redox; biomarker; coronary artery disease; mortality; cystine; glutathione; prognosis; cardiovascular outcomes; cardiac biomarker

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Introduction

Oxidative stress (OS) is implicated in the pathophysiology of multiple conditions including cardiovascular disease (CVD).[1] Although the harmful cellular effects of free radical species in vitro remain undisputed, observational evidence along with clinical trials of free radical scavengers has been uniformly disappointing.[2, 3] This has promoted the concept that free radicals may not constitute *clinically important* sources of oxidants and that non-free radical species may be of equal or greater importance.[4]

Proteins are susceptible to oxidation through alterations of reactive aminothiol residues such as cysteine and glutathione. These covalent modifications serve to alter the cellular signaling activity of the proteins, thereby coupling redox modifications of aminothiols to functional activity.[5] Importantly these aminothiols can be quantified in plasma to assess the oxidant burden in vivo.[6] Of these, cysteine constitutes the major aminothiol pool *extracellularly* that reacts readily with oxidants to form its oxidized disulphide cystine. *Intracellularly*, glutathione is a major antioxidant that helps eliminate peroxides and maintain cellular redox, and its oxidized form is glutathione disulfide.[4] Increased OS, measured as higher levels of cystine, lower levels of glutathione, or altered ratios of oxidized to reduced aminothiols, is associated with cellular dysfunction, aging, risk factors for CVD and subclinical vascular disease, and are likely to be reliable markers of systemic OS and antioxidant defence.[7-13]

However, there remains a need to determine if oxidant burden as indicated by alterations in the levels or ratios of these aminothiols is clinically relevant and determines adverse outcomes. This would support the use of these aminothiols as biomarkers of OS and potentially promote development of novel anti-oxidant therapies. We thus sought to determine whether the major aminothiols and their respective ratios would be associated with increased mortality and

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cardiovascular events in a prospectively followed high risk population.

Methods

Study population

Study participants aged 20-90 years were recruited as part of the Emory Cardiovascular Biobank, an ongoing prospective cohort of patients enrolled prior to undergoing coronary angiography for investigation or management of CAD across three Emory Healthcare sites with collection of extensive data on demographic characteristics, medical history, medication use, behavioral habits and risk factor prevalence.[14, 15]

Recruited patients were stable at the time of enrollment and undergoing an elective procedure, although stable patients with non-ST elevation myocardial infarction, defined using international criteria were also included and classified as "Acute MI".[16] Coronary artery disease (CAD) burden was quantified using the semi-quantitative Gensini score, as previously described.[16] Left ventricular function was expressed using ejection fraction (EF).[17] Finally glomerular filtration rate (GFR) was estimated using the CKD-EPI formula.[18] Subjects were excluded if they had a history of heart transplantation, recent transfusion, immunosuppressant use, malignancy, or significant infections or any vitamin supplements in the previous 6 weeks. Specific dietary intake patterns were not documented but blood samples and measurements were taken after an overnight fast prior to planned coronary angiography, except in <1% of patients who underwent angiography emergently on the same day. The study was approved by the Institutional Review Board at Emory University and all subjects provided written informed consent.

Follow-up and Outcomes

The cohort was prospectively followed for determination of the primary outcome of all-cause

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death and the secondary outcomes of cardiovascular death and the composite of death/or non-fatal MI. This was performed by personnel blinded to aminothiol data, through telephone interview, chart review and linkage with the Social Security Death Index and State records. Cardiovascular death was defined as death attributable to an ischemic cardiovascular cause (fatal MI, ischemic stroke, peripheral arterial disease) or sudden death due to an unknown but presumed cardiovascular cause in high risk CAD patients. Medical records were accessed or requested to validate all self-reported events including MI, which was defined using standard criteria as above.[16] Fifteen patients (1%) were lost to follow up and were excluded from analysis, leaving 1411 patients with complete biomarker and follow-up data.

Measurement of aminothiols and CRP

We measured plasma cysteine (CyS), its oxidized form cystine (CySS), glutathione (GSH), and its oxidized form glutathione disulphide (GSSG) in all subjects using high performance liquid chromatography (HPLC) mass spectrometry. A full methods and protocol paper has been published previously outlining sample collection, processing and analysis steps in detail.[6] Summary details are also presented in supplementary materials, but briefly, arterial blood samples were drawn via syringe immediately after placement of a femoral arterial sheath (prior to heparin or saline flush or any coronary intervention) and transferred into pre-prepared Eppendorf tubes containing preservatives to retard auto-oxidation, centrifuged, and stored at −80 °C for no more than 2 months prior to transfer to the laboratory. Sample collection and storage conditions in this way have been previously verified.[6] Analyses by HPLC were performed after dansyl derivatization on a 3-aminopropyl column with fluorescence detection. Metabolites were identified by co-elution with standards and quantified by integration relative to the internal standard, with validation relative to external standards as previously described.[6]

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Ratios of oxidized to reduced aminothiols (cystine/cysteine and glutathione disulphide/glutathione) are expressed directly. The coefficients of variation for each of the aminothiols were as follows: cysteine 3.8%; cystine 3.2%; glutathione 5% and glutathione disulphide 9.7%. High sensitivity CRP levels were quantified using a sandwich immunoassay (R&D Systems, Minneapolis, Minnesota). Minimum detectable hs-CRP concentrations were 0.1mg/l.

Statistical methods

Continuous variables are presented as means ± SD or as median (IQR) and categorical variables as proportions (%) with one way analysis of variance and chi-squared tests used to determine differences between groups.

Prior to analysis, aminothiol measures were non-normally distributed and were natural log +1 transformed. Furthermore, to make the effects comparable between markers, the log-transformed variables were standardized to have mean 0 and standard deviation (SD) 1. They were assessed as continuous and categorical traits, initially by per unit log increase and per SD increment and then by a 1x SD cut off to classify "high" and "low" values. Survival analysis was performed using Kaplan Meier curves as well as Cox proportional-hazards regression in models adjusted first for age, gender and then additionally for body mass index (BMI; kg/m^2), glomerular filtration rate (GFR; l/min), presence of diabetes, hypertension, total cholesterol (mg/dl), High Density Lipoprotein (HDL; mg/dl), current smoking, statin use, acute MI at enrollment, LV function (EF; %), Gensini score and plasma hs-CRP at baseline. Given inflammation and oxidative stress are biologically inter-related, an interaction term between hs-CRP and each of the markers (including their ratios) was initially included in the model. Interaction between age and each marker was also considered to examine any potential age-modifying effects. Missing covariate

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data (range 0-3%) was imputed and sensitivity analysis with un-imputed data found results to be similar. The proportional hazards assumption for Cox models was evaluated by plots of Schoenfeld residuals and formal testing (a chi-square test calculated as the sum of Schoenfeld residuals), with no significant violations of the assumption found.

The incremental value of the aminothiol markers for risk prediction was tested before and after their addition to a clinical model with traditional risk predictors (age, gender, BMI, GFR, diabetes, hypertension, total cholesterol, HDL, current smoking, statin use, acute MI, LV function, Gensini score). The C-statistic and category-free net reclassification improvement (NRI) as well as integrated discrimination improvement (IDI) that can account for censored data were calculated as a measure of risk discrimination.[19-22] We set the truncation time at 5 years. The resulting risk discrimination metrics indicate the performance of the given model in predicting events that occurred in the time range from baseline to 5 years. P values of <0.05 from two-sided tests were considered to indicate statistical significance. Statistical analyses were performed using SPSS 20.0 (Chicago, IL), SAS (Cary, NC) and R (3.1.0).

Results

Baseline characteristics of the 1411 patients are presented in **Table 1** and were reflective of a typical population recruited at coronary angiography. The mean age of the cohort was 63.2 (±11.3) years, 66% male, 32% with diabetes, 69% with hypertension and or hyperlipidemia, and 16% were current smokers. Approximately 72% had significant CAD (>50% luminal stenosis) on angiography, 14% had presented with evidence of acute myocardial infarction (all stable NSTEMI), and 46% were treated with revascularization during the admission at which they were enrolled, **Table 1**.

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Relationship between aminothiols

The oxidized aminothiol, cystine was almost 8-fold more abundant than its reduced form cysteine, while the reduced aminothiol glutathione was 40-times more abundant than its oxidized form glutathione disulphide, **Table 1**. There were modest correlations between the various aminothiols, while hs-CRP was only marginally associated with the aminothiol markers (**Supplementary Table 1**).

Relationship between aminothiols and demographic and clinical features

In univariate analyses, higher plasma cystine levels (high OS) were associated with older age, female gender, higher BMI, impaired renal function (lower GFR), presence of diabetes, hypertension, lower total cholesterol levels, statin use, impaired LV function (lower EF), greater CAD burden (Gensini), and greater inflammation (hs-CRP). Of these, only age, gender, BMI, GFR, diabetes and hypertension were independently associated with plasma cystine in a multivariate model. Higher glutathione levels (less OS) were associated with younger age, lower GFR, absence of diabetes and hypertension, lower CAD burden and higher total cholesterol. Of these only age, GFR, CAD burden, and total cholesterol remained independently associated with plasma glutathione. Higher cysteine levels also correlated independently with GFR, diabetes, CAD burden and total cholesterol while glutathione di-sulphide did not show any associations aside from an inverse association with GFR (**Supplementary Tables 2A and 2B**).

Relationships between individual aminothiols and outcomes

During a mean follow up of 4.7 (±2.1) years (median 5.3; IQR 3.1 - 6.2), representing 6570 person-years of follow-up, 247 patients experienced the primary outcome of death, of which there were 169 cardiovascular deaths and 314 composite outcomes of death/MI. Patients who experienced the primary outcome, were generally older and had more risk factors and disease

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burden as shown in **Table 1**. Independent clinical predictors of outcomes are presented in **Supplementary Table 3**.

The baseline cystine (p<0.001) and glutathione (p=0.002) levels were both associated with risk of future death after adjustment for age and gender (log values, **Table 2**). These associations persisted after further adjustment for important covariates (see methods) including hs-CRP (p=0.001 and p=0.006 respectively). After standardization, to permit marker comparisons, a one SD increment in cystine and a one SD decrease in glutathione was associated with a 26% and 20% increase in risk of death after adjustment for all risk factors, respectively, **Table 2**.

This association was also evident when cystine and glutathione levels were categorized into quartiles (Kaplan Meier log rank p<0.001 and p=0.002 respectively; **Supplementary Figure 1** & **Supplementary Table 4**). Examination of the Kaplan Meier plots revealed a possible threshold effect, especially for glutathione.

We further explored this by using a 1 SD cut point to define high and low levels of aminothiol markers (see methods). Survival analysis confirmed a worse prognosis for patients with high cystine (>+1SD; >129.8μM) and for those with low glutathione (<-1SD, <0.68μM) levels (log rank p<0.001 for both, **Figure 1**). Both a high cystine level and low glutathione level were each associated with a 2 to 3-fold increase in age and gender adjusted risk of death (HR 2.05 (1.53-2.75); 3.16 (2.20-4.54) respectively). After adjustment for all covariates, a high cystine level was associated with a HR of 1.63 (1.19-2.21) and a low glutathione level of 2.19 (1.50-3.19).

Importantly, both cystine and glutathione were independently associated with the primary outcome of death, when entered into the same multivariate model. Furthermore, both of these

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aminothiols were also associated with the secondary outcomes of cardiovascular death and the composite of death and MI (**Table 2**). Glutathione in particular showed a greater effect size for cardiovascular death compared to all-cause death. However, their respective couples, cysteine (reduced) and glutathione disulphide (oxidized) were not associated with the outcomes examined (data not shown).

Relationship between aminothiol ratios and outcomes

We also examined the ratio of cystine to glutathione, as a novel measure of *extracellular* oxidation to *intracellular* reducing capacity and demonstrated a highly significant association with the primary outcome ($p<0.001$) (**Table 3, Figure 1**). Patients with a >+1SD level of cystine/glutathione ratio, reflecting a high extracellular oxidant burden and low intracellular reducing capacity, demonstrated a HR of 1.92 (1.39-2.64) for death after full adjustment for all covariates (**Table 3**). Similar significant associations were noted for the secondary outcomes of cardiovascular death (HR 1.91 (1.34-2.72) and the composite of death/MI (HR 1.88 (1.40-2.52)).

In contrast, although the direct ratios within the extracellular and intracellular compartments, of cystine (oxidized) to cysteine (reduced) and glutathione disulphide (oxidized) to glutathione (reduced) were associated with the studied outcomes in adjusted models, the associations were attenuated compared to those for cystine, glutathione, or the cysteine/glutathione ratio (**Table 3**).

CAD and MI Subgroup Analyses

There was no significant heterogeneity in the association between the cystine/glutathione ratio and adverse events based on baseline characteristics. Thus, among patients with obstructive CAD, those with high cystine/glutathione ratio had a HR of 1.80 (95% CI 1.27-2.54)) compared to 3.11 (95% CI 1.19-8.15) for those with non-obstructive CAD. While there was no significant

interaction, the risk of a high cystine/glutathione ratio was higher in those with acute MI compared to those without; HR 3.87 (1.74-8.63) and HR 1.82 (1.26-2.62), respectively.

Inflammation and oxidant stress

Given that no significant interaction between hs-CRP and each of the markers (including their ratios) was found (results not shown) and both hs-CRP and the cystine/glutathione ratio were independently associated with risk of death, we devised a simple multi-marker score, using high/low categories to identify the potential value of combining inflammation and OS measures for predicting future events. A score of 0 (n=647) represented both low inflammation (low hs-CRP, defined as <3mg/L (median))[23] and low OS (low cystine/glutathione ratio (by SD as above)), while a score of 2 reflected both high hs-CRP and high cystine/glutathione (n=84). A score of 1 was given to the remaining 661 subjects (**Figure 2**). Compared to those with a score of 0, those with a score of 1 had a covariate adjusted HR of 1.46 (1.08-1.97) for risk of death while those with a score of 2 had a HR of 3.26 (2.17-4.90). Thus, patients with a score of 0, 1 or 2 experienced a one year death rate of 1.1%, 4.9% or 14.5% or a 5-year event rate of 9.7%, 17.3% and 41.5%, respectively.

Discrimination testing

When compared to a standard model for risk of death, consisting of traditional risk factors (see methods), the addition of hs-CRP, hs-CRP + the ratio of cystine/glutathione, and the combination of these two biomarkers as a multi-marker score improved the risk discrimination significantly, including C-statistic, NRI, and IDI (**Table 4**). Specifically, while addition of the individual aminothiols cystine or glutathione did not improve risk discrimination, the ratio of cystine/glutathione improved both the NRI (0.109, 95% CI=[0.011, 0.176]) and IDI (0.012, 95% CI=[0.001, 0.029]) (**Table 4**).

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Discussion

Herein we demonstrate that the plasma aminothiols, cystine and glutathione associate with risk of future death in a high risk population with CAD. This effect is independent of, and additive to that of inflammation as assessed by hs-CRP. Quantification of plasma aminothiol markers may thus represent an important advance for in-vivo assessment of clinically important OS.

Specifically, we show that patients with high OS captured as (1) a high level of oxidized cystine, representing greater extracellular oxidant burden, (2) a low level of reduced glutathione, representing low intra-cellular reducing capacity, or (3) a high ratio of the two, have a 2-fold increase in risk of mortality over a mean of 5 years independent of age and other risk factors including inflammation. While previous attempts at quantifying aminothiol mediated OS have utilized the redox potential of cystine or glutathione disulphide using the Nernst equation,[6] we found that the directly combined, cross compartment ratio of cystine to glutathione is simple and practical to calculate and able to discriminate risk, thus representing an improved approach to capturing the overall burden of OS in vivo.

Control of protein redox state via thiol-disulfide switching is critical for normal cellular activities and for maintaining physiological and pathophysiological functions including promotion of CVD. This includes (1) experimental evidence for effects on pro-inflammatory signalling, mitochondrial oxidation, nuclear factor KB activation and elevated expression of genes for monocyte recruitment to endothelial cells;[5, 7, 8, 24] (2) association with clinical risk factors such as aging, obesity and smoking;[9, 25, 26] (3) translational studies confirming association between aminothiols and worse endothelial function, carotid intima media thickness and arterial stiffness;[11, 27, 28] and (4) prospective outcome data in patients at high CVD risk as presented here. In totality, these findings support the use of plasma levels of oxidized and reduced aminothiols as

key biomarkers of OS and cellular health and potentially as new therapeutic targets.

The utility of these aminothiols in clinical practice requires further testing. While addition of cystine and glutathione individually did not improve risk discrimination beyond a standard clinical model, addition of the cystine/glutathione ratio did improve both risk reclassification metrics. Given the interplay between inflammation and OS at a molecular level, and with additive effect on risk, we devised and tested a simple multi-marker score combining the biomarkers of each, for ease of clinical use. This simple 3-point score clearly stratified risk and when added to a clinical model also improved the C-statistic and metrics of risk reclassification. Thus, a combination of biomarkers, in this case reflecting aminothiol mediated OS and inflammation quantified by CRP, may offer a valuable approach for clinical risk stratification as has been recently described.[14]

Mechanistically, these findings may have implications for understanding other observations. Homocysteine, an important aminothiol, is biosynthesized from dietary methionine, and in the presence of folate and B vitamins converts to cysteine by cystathione synthase (**Supplementary Figure 2**). While patients with genetic hyperhomocystinemia are prone to severe atherosclerosis, folate supplementation and homocysteine reduction does not appear to reduce CVD risk.[29, 30] This may be because cysteine is independently maintained from homocysteine and represents a more abundant and reactive aminothiol that on oxidation forms cystine, which is 30-times more abundant than homocysteine and perhaps is a more pathological component. While some studies have shown association between total cysteine and CVD,[31, 32] none until now, have examined the individual oxidized and reduced components or their respective contribution to the oxidant burden. This data may thus offer a partial explanation for the failure of homocysteine targeted therapy and possible new treatment opportunities.

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Although experimental data supports the role of free radical biology in OS, clinical attempts at improving outcomes with free radical scavengers (vitamins C, E etc) have been uniformly disappointing.[2, 33] Our findings support the hypothesis that in-vivo, OS may also be driven by non-free radical processes, raising the possibility that alternative anti-oxidative therapies may be more effective. In humans there is no currently known pathway to reduce cystine to cysteine, although cystine levels are in part controlled by the Xc system acting as a highly efficient glutamate-cystine transporter.[34] Cellular expression of this transporter declines with age, potentially explaining the association with cystine and age that we and others have observed. In contrast zinc enhances expression of this system and could represent a therapeutic option to reduce plasma cystine and OS. Indeed a recent pilot study in patients with macular degeneration has revealed reductions in plasma levels of oxidized cystine with zinc supplementation, suggesting that levels can be manipulated with therapeutic interventions.[35]

Strengths & Limitations

Strengths of our study include its prospective design, large sample size, exploration of both reduced and oxidized aminothiols, long follow up, use of robust clinical outcomes and exploration of the interaction with inflammation assessed by hs-CRP. We did not have dietary information on our subjects and ingestion of sulphur-rich amino acids may influence plasma aminothiol levels. However, after a meal, cysteine shows rapid distribution and cystine levels increase for 2-3 hrs and almost all of our patients were fasting for >8 hours which minimized the likely dietary changes on aminothiol levels. We did not have detailed drug information to explore whether thiol containing medications impacted on measured levels and it is possible that some patients taking these drugs may have non representative levels. Our population is also not representative of all populations, and thus our findings may not be generalizable, and require

further validation in different groups.

Finally, as an observational study we cannot infer causality and confounding by CAD remains possible. Nonetheless, oxidative stress is an accepted mechanism for plaque development and previous associations with upstream risk factors and subclinical phenotypes in those without CAD, as well as sensitivity analysis in those with and without CAD showing a consistent effect in both groups, suggests reverse causation is less likely. However confounding can never be fully excluded without interventional studies, but even if causality is not confirmed, this does not limit the value of these thiols for use as biomarkers of intracellular and extracellular oxidative stress, analogous to use of CRP as a biomarker of systemic inflammation.

Conclusions and Implications

A high extracellular oxidant burden and/or reduced intracellular anti-oxidant capacity quantified through assessment of plasma aminothiols, is associated with higher mortality in patients with CAD. As well as representing potentially novel therapeutic targets, OS measured in this way could complement risk stratification in conjunction with assessment of inflammation assessed by hs-CRP. Further studies will evaluate non-HPLC methods of aminothiol assessments to facilitate their wider use as biomarkers and to investigate whether therapies such as zinc supplementation, seeking to reduce plasma cystine can alter OS and improve outcomes.

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Conflict of Interest Disclosures: None.

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Clinical Perspective

Although oxidative stress is a critically important process in atherosclerosis, observational evidence and clinical trials of free radical scavengers have proven unfirmly disappointing. This has promoted the concept that clinically important oxidative stress may be mediated by non-free radical species. Proteins with reactive aminothiols are susceptible to oxidation and quantification of these reduced and oxidized (redox) residues offers an alternative means of quantifying in-vivo oxidative stress and oxidant burden. Having developed means to reliably collect and quantify these markers in plasma, we have previously shown associations with multiple risk factors for cardiovascular disease as well as with subclinical markers such as arterial stiffness and intima media thickness. However, whether these markers are clinically relevant has remained unknown. In this study, we now present long term outcome data demonstrating association between these redox markers and adverse cardiovascular outcomes and mortality. These findings are important as they support the use of these aminothiols as novel and reliable biomarkers of oxidative stress. Importantly, given that oxidation of these aminothiols leads to altered cellular signaling, these findings may offer new opportunities for therapeutic interventions for reducing the adverse clinical impact of oxidative stress in vivo.

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Table 1. Patient Characteristics.

	All (1411)	No Event (1164)	Event (247)
Clinical Characteristics			
Age, years	63.2 (11.3)	62.0 (11.1)	69.0 (11.0)
Male, %	66.3	66.2	66.8
Caucasian, %	85.7	85.2	88.3
Body Mass Index (BMI), kg/m2	29.9 (6.41)	30.2 (6.4)	28.5 (6.4)
GFR, ml/min	73.1 (21.6)	75.3 (20.2)	62.5 (24.6)
Hypertension, %	68.9	66.9	78.1
Hyperlipidemia, %	68.4	67.8	71.7
Total Cholesterol, mg/dL	172.2 (45.7)	174.4 (45.7)	161.4 (44.1)
LDL, mg/dL	99.6 (38.5)	101.4 (39.2)	91.2 (34.5)
HDL, mg/dL	41.2 (12.3)	41.4 (12.1)	40.3 (13.2)
Diabetes mellitus,%	32.2	29.9	43.3
Current Smoking,%	16.4	16.4	16.6
Acute MI, %	14.3	13.2	19.8
LV EF %	53.7 (11.9)	54.9 (10.8)	48.0 (15.3)
Angiographic CAD			
Significant >50%, %	71.5	69.8	81.9
Normal <10%, %	19.1	20.9	8.7
Median Gensini Score (IQR)	14.5 (1-51)	13.0 (0-44)	28 (5-121)
Revascularization at Enrollment, %	46	44.8	52.5
Medication use			
Statin use, %	74.4	74.6	73.1
Aspirin use, %	83	83.4	81.4
ACE or ARB use, %	62.7	62.3	64.9
Beta blocker use, %	63.8	62.2	71.5
Inflammation *(median, IQR)*			
C-Reactive Protein, mg/L	2.9 (1.2-7.1)	2.6 (1.2-6.4)	4.7 (1.75-14.0)
Oxidative stress *(median, IQR)*			
Cystine, μM	97.6 (83.3-115.0)	96.1 (82.3-112.3)	106.9 (90.4-130.6)
Cysteine, μM	12.2 (10.1-14.7)	12.2 (10.1-14.4)	12.3 (9.6-16.1)
Glutathione, μM	1.17 (0.92-1.47)	1.19 (0.94-1.48)	1.07 (0.82-1.43)
Glutathione disulphide, μM	0.02 (0.01-0.03)	0.02 (0.01-0.03)	0.02 (0.02-0.04)

Mean (SD) values, median (IQR) values and % shown unless stated
GFR – Glomerular Filtration Rate; MI –Myocardial Infarction; CAD – Coronary Artery Disease; LVEF –Left Ventricular Ejection Fraction; LDL – Low Density Lipoprotein; HDL – High Density Lipoprotein

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Table 2. Cox regression survival analysis for the individual aminothiol markers showing significant association with adverse events*.

		Continuous (log)		Standardized (per SD)		Categorized (High v Low)	
		HR (95% CI)		HR (95% CI)		HR (95% CI)	
Aminothiol	**Outcome**	Age & gender adjusted	Fully adjusted model	Age & gender adjusted	Fully adjusted model	Age & gender adjusted	Fully adjusted model
Cystine (oxidized)	Death (n=247)	4.02 (2.38-6.78) p<0.001	2.42 (1.40-4.17) p=0.001	1.44 (1.25-1.64) P<0.001	1.26 (1.09-1.45) P=0.001	2.05 (1.53-2.75) P<0.001	1.63 (1.19-2.21) P=0.002
	Death/MI (n=314)	3.15 (1.99-4.99) p<0.001	2.06 (1.28-3.32) p=0.003	1.35 (1.20-1.52 P<0.001)	1.21 (1.07-1.37) P=0.003	1.88 (1.44-2.46) p<0.001	1.54 (1.16-2.04) p=0.003
	CV Death (n=169)	3.90 (2.07-7.33) p<0.001	2.06 (1.07-3.96) p=0.031	1.42 (1.21-1.68) P<0.001	1.21 (1.02-1.43) P=0.031	2.16 (1.52-3.07) p<0.001	1.63 (1.12-2.36) p=0.010
Glutathione (reduced)	Death (n=247)	1.82 (1.42-2.33) P<0.001	1.57 (1.22-2.02) P=0.001	1.24 (1.08-1.43) P=0.002	1.20 (1.05-1.37) P=0.006	3.16 (2.2-4.54) P<0.001	2.19 (1.50-3.19) p<0.001
	Death/MI (n=314)	1.73 (1.38-2.17) P<0.001	1.51 (1.20-1.90) P<0.001	1.23 (1.08-1.39) P=0.001	1.20 (1.06-1.34) P=0.003	2.66 (1.89-3.74) p<0.001	1.89 (1.32-2.70) p<0.001
	CV Death (n=169)	1.88 (1.40-2.53) P<0.001	1.63 (1.20-2.20) P=0.002	1.21 (1.03-1.43) P=0.023	1.18 (1.01-1.38) P=0.041	3.63 (2.39-5.50) p<0.001	2.53 (1.63-3.92) p<0.001

Cox regression analysis of aminothiol markers as continuous and categorized measures, with risk of adverse outcomes; Cystine categorized as high if >+1 SD and low if ≤+1 SD; Glutathione categorized as low if <-1SD and high if ≥-1 SD; Cysteine (reduced) and Glutathione disulphide (oxidized) did not show any significant associations – data not shown; Full model includes adjustment for age, gender, body mass index, glomerular filtration rate, diabetes, hypertension, TC, HDL, current smoking, statin use, acute myocardial infarction, LV EF, Gensini score and LnCRP.

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Table 3. Cox regression survival analysis for oxidized to reduced **ratios** of aminothiol markers with the primary outcome of death.

		Continuous (log)		**Standardized (per SD)**		**Categorized (High v Low)**	
		HR (95% CI), p value		HR (95% CI), p value		HR (95% CI), p value	
Aminothiol Ratio	**Outcome**	Age & gender adjusted	Fully adjusted model	Age & gender adjusted	Fully adjusted model	Age & gender adjusted	Fully adjusted model
Cystine/Glutathione ratio	Death (n=247)	2.16 (1.65-2.82) p<0.001	1.74 (1.34-2.27) p<0.001	1.47 (1.29-1.68) P<0.001	1.32 (1.16-1.50) P<0.001	2.26 (1.65-3.09) P<0.001	1.92 (1.39-2.64) p<0.001
	Death/MI (n=314)	1.97 (1.56-2.49) p <0.001	1.65 (1.31-2.08) p <0.001	1.40 (1.25-1.58) P<0.001	1.29 (1.14-1.44) P<0.001	2.17 (1.63-2.90) p<0.001	1.88 (1.40-2.52) P<0.001
	CV Death (n=169)	2.13 (1.59-2.86) p<0.001	1.66 (1.21-2.28) p=0.002	1.45 (1.24-1.71) P<0.001	1.29 (1.10-1.51) P=0.002	2.31 (1.58-3.36) p<0.001	1.91 (1.34-2.72) P<0.001
Cystine/Cysteine ratio	Death (n=247)	1.87 (1.28-2.73) p<0.001	1.50 (1.05-2.15) p=0.027	1.23 (1.09-1.40) P=0.001	1.15 (1.02-1.29) P=0.027	1.72 (1.24-2.39) P=0.001	1.36 (0.97-1.91) P=0.07
	Death/MI (n=314)	1.78 (1.27-2.51) p=0.001	1.50 (1.08-2.08) p=0.015	1.21 (1.08-1.36) P=0.001	1.14 (1.03-1.28) P=0.015	1.57 (1.15-2.12) p=0.004	1.31 (0.96-1.80) P=0.09
	CV Death (n=169)	1.72 (1.09-2.71) p=0.021	1.32 (0.86-2.04) P=0.20	1.20 (1.03-1.39) P=0.021	1.10 (0.95-1.27) P=0.20	1.74 (1.12-2.58) p=0.006	1.30 (0.87-1.95) P=0.21
Glutathione disulphide/ Glutathione ratio	Death (n=247)	1.34 (1.12-1.60) p=0.001	1.21 (1.01-1.46) p= 0.035	1.22(1.10-1.37) P=0.001	1.14 (1.01-1.29) P=0.035	1.59 (1.10-2.29) P=0.014	1.36 (0.93-1.98) P=0.111
	Death/MI (n=314)	1.41 (1.20-1.65) p<0.001	1.31 (1.12-1.53) P=0.001	1.25 (1.13-1.39) P<0.001	1.20 (1.08-1.33) P=0.001	1.78 (1.29-2.45) p<0.001	1.55 (1.11-2.14) P=0.009
	CV Death (n=169)	1.37(1.07-1.699) p=0.004	1.22 (0.98-1.53) P=0.07	1.23 (1.07-1.42) P=0.004	1.14 (0.99-1.32) P=0.074	1.70 (1.10-2.62) P=0.017	1.43 (0.92-2.24) p=0.11

Cox regression analysis of aminothiol marker ratios as continuous and categorized measures, with risk of adverse outcomes; All ratios categorized as high if >+1 SD and low if ≤+1 SD; Full model includes adjustment for age, gender, body mass index, glomerular filtration rate, diabetes, hypertension, TC, HDL, current smoking, statin use, acute myocardial infarction, LV EF, Gensini score and LnCRP.

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Table 4. Estimates and the corresponding 95% confidence limits for the risk discrimination metrics (change in C-statistic, NRI, and IDI) for the primary outcome of death within five years of follow up.

Models	C-statistic	Δ C-statistic	Category free NRI	Relative IDI
Clinical Model	0.717 (0.674, 0.761)	-	-	-
+ CRP	0.736 (0.692, 0.779)	0.018 (0.003, 0.034)	0.109 (0.023, 0.203)	0.0202 (0.006, 0.04)
+ Cystine (CySS)	0.722 (0.678, 0.766)	0.005 (-0.003, 0.013)	0.046 (-0.041, 0.132)	0.006 (-0.001, 0.020)
+ Glutathione (GSH)	0.724 (0.688, 0.759)	0.006 (-0.004, 0.017)	0.084 (-0.026, 0.157)	0.005 (-0.001, 0.017)
+ CySS/GSH Ratio	0.728 (0.695, 0.762)	0.011 (-0.002, 0.024)	0.109 (0.011, 0.176)	0.012 (0.001, 0.029)
+ CRP + CySS/GSH Ratio	0.746 (0.708, 0.785)	0.029 (0.009, 0.049)	0.133 (0.044, 0.214)	0.032 (0.013, 0.059)
+ Multi-marker score*	0.736 (0.702, 0.769)	0.018 (0.002, 0.035)	0.124 (0.026, 0.191)	0.018 (0.004, 0.043)

Clinical Model – includes age; gender; BMI; GFR; diabetes; hypertension; current smoking; acute MI; Gensini score; LV EF; statin use; total cholesterol and HDL

*Multi-marker score is scored 0, 1 and 2 based on combinations of high or low CRP and high or low cystine/glutathione ratio

NRI = Net Reclassification Improvement; IDI = Integrative Discriminatory Improvement

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Figure Legends:

Figure 1. Kaplan Meier curves for association between high v low levels of cystine, glutathione and the cystine/glutathione ratio. High v low categorization was defined by SD cut off (see methods). Log rank p values and number of patients within each category is shown.

Figure 2. Covariate adjusted survival analysis for the multi-marker score by cox regression, combining low v high hs-CRP (inflammation) and low v high cystine/glutathione ratio (Oxidative Stress (OS)). A score of 0 (blue line, n=647) represents low inflammation and low OS, while a score of 2 (red line, n=84) represents high inflammation and high OS. A score of 1 (green line, n=661) represents either high inflammation or high OS only.

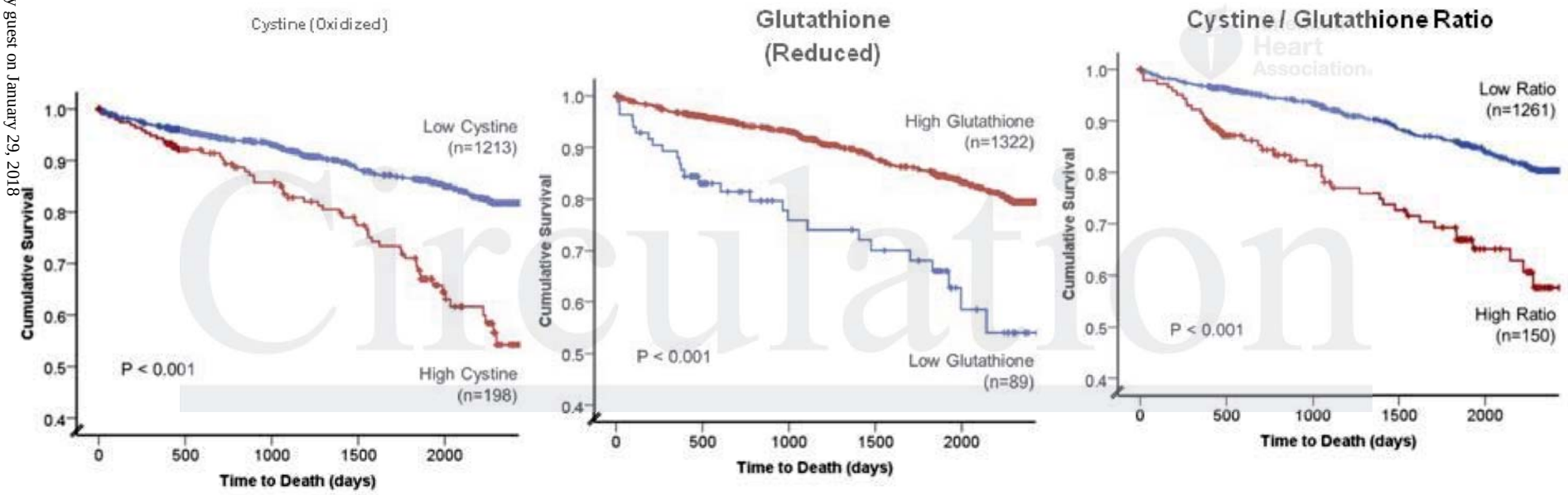

Figure 1

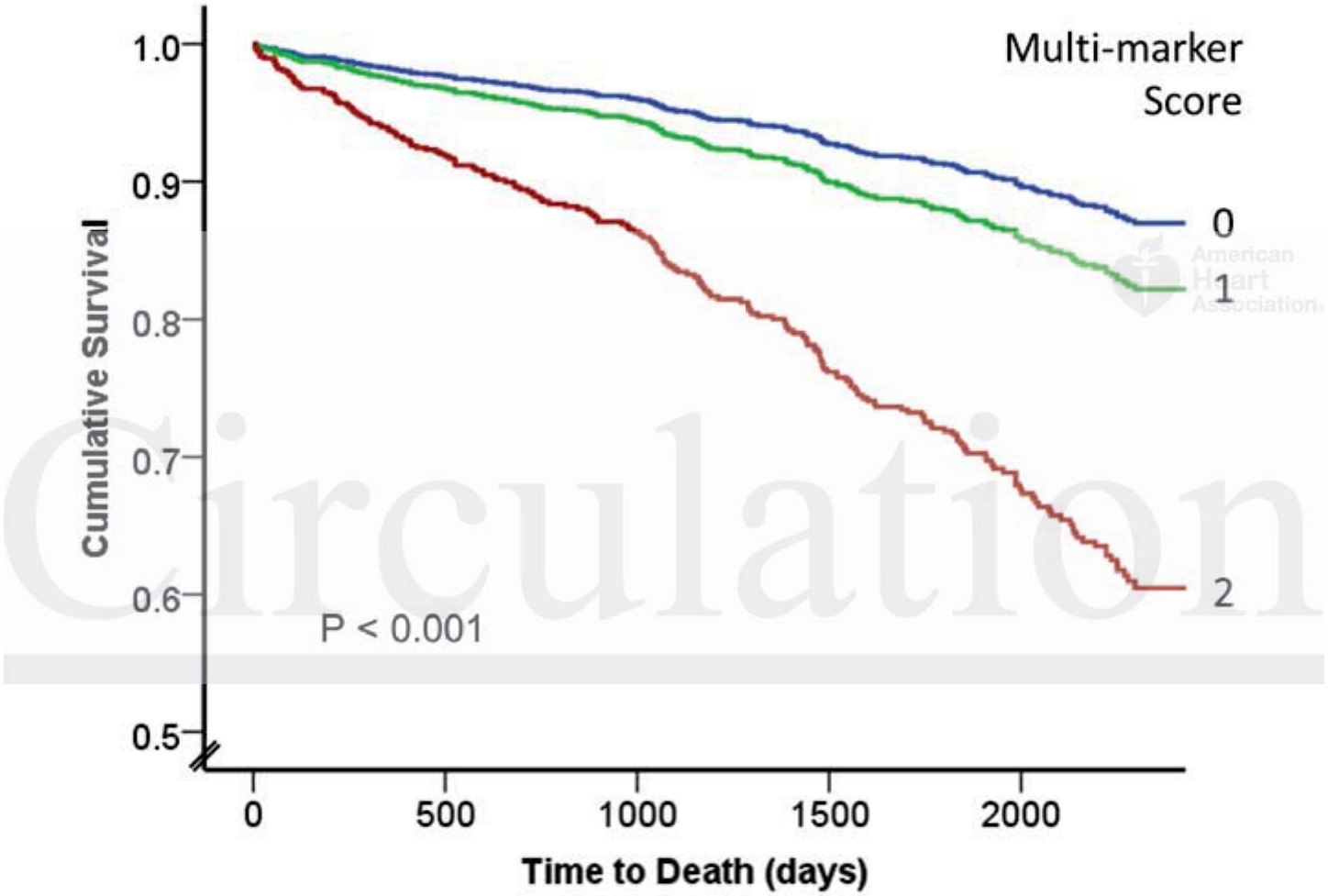

Figure 2

A Novel Biomarker of Oxidative Stress is Associated with Risk of Death in Patients with Coronary Artery Disease

Riyaz S. Patel, Nima Ghasemzadeh, Danny J. Eapen, Salman Sher, Shawn Arshad, Yi-an Ko, Emir Veledar, Habib Samady, A. Maziar Zafari, Laurence Sperling, Viola Vaccarino, Dean P. Jones and Arshed A. Quyyumi

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Supplemental Material

Supplemental Material

Title: A Novel Biomarker of Oxidative Stress is Associated with risk of Death in Patients with Coronary Artery Disease

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Supplementary Methods: Sample collection and storage details.

A full methods paper has been published previously which details each step of the protocol with key references and figures to replicate the procedure exactly (Jones 2009). Abbreviated key details for review are provided here:

Collection of samples and processing involves use of 2 sets of microcentrifuge (Eppendorf) tubes termed “N” and “S” tubes, prepared in advance and stored at -80C. The “N” tube consists of L serine, heparin, bathophenanthrolene disulfonate, iodoacetic acid in borate buffer with internal standard. Treatment with the first derivatizing agent (to block thiols) occurs during the sample collection and is complete by the time the plasma (from N tube) is transferred to S tube. The “S” tube contains boric acid in distilled de-ionized water.

Blood is drawn with a syringe through the arterial sheath at the time of catheterization as in this study or via butterfly needle if venous cannulation is performed. Blood is transferred to the “N” tube carefully bringing the level up to the 1.5ml line (to account for 1350ul of blood and 150ul of additive). The tube is inverted gently and then spun using a centrifuge to remove RBCs. Routine use of this method has only identified minor haemolysis in 2 of 600 samples by spectrophotometry. Next, 200ul of supernatant is transferred to the “S” tube. This should be done within 2 minutes after blood collection, although 5 minutes is acceptable. This tube is then inverted gently, labelled and placed on ice before transfer to a -80C freezer.

Within 1-2 months, samples are shipped to an onsite laboratory for analysis. Samples in the “S” tubes are thawed and the supernatant transferred to a fresh Eppendorf tube before addition of KOH to adjust the pH to 9.0, followed by addition of dansyl chloride. Chloroform is added to extract unreacted dansyl chloride and samples stored again until assay by HPLC, usually within a few days.

Reproducibility, stability and recovery tests for glutathione have indicated that non-derivatized samples stored at -80C were stable for 2 months without significant loss, while dansyl-derivatives were stable in the dark at 0-4 degrees for 12 months (Jones 1998). Similar findings have been documented for the cystine pool, with no evidence of loss demonstrated at 12 months (Johnson 2008).

Supplementary Table 1: Correlation coefficients between each of the thiols

	Cystine	**Cyste*i*ne**	**Glutathione**	**Glutathione disulphide**	**C-Reactive Protein**
Cystine		0.262	-0.111	0.075	0.057
Cysteine			0.330	0.095	-0.015
Glutathione				0.314	-0.025
Glutathione disulphide					0.038
C-Reactive Protein					

Spearman Rank coefficients (rho) for correlation between markers

Supplementary Table 2A: Univariate and multivariate associations between patient characteristics and the cysteine couple

	Cystine				Cysteine			
	Univariate		Multivariate		Univariate		Multivariate	
	beta	*p value*	*beta*	*p value*	*beta*	*p value*	*beta*	*p value*
Age, years	0.30	<0.001	0.164	<0.001	0.08	0.002	0.005	0.89
Male Gender	-0.12	<0.001	-0.095	0.001	-0.04	0.11	-0.056	0.053
Body Mass Index, kg/m2	0.13	<0.001	0.128	<0.001	-0.01	0.60	-0.016	0.523
GFR, ml/min	-0.39	<0.001	-0.261	<0.001	-0.13	<0.001	-0.115	<0.001
Acute MI	0.04	0.19	0.012	0.672	0.013	0.62	-0.006	0.79
Diabetes	0.17	<0.001	0.092	<0.001	0.03	0.20	0.020	0.47
Hypertension	0.18	<0.001	0.058	0.021	0.04	0.14	0.007	0.82
Current Smoking	-0.11	<0.001	-0.046	0.085	-0.026	0.33	-0.012	0.612
LV EF, %	-0.06	0.02	-0.044	0.042	-0.05	0.054	-0.047	0.099
Gensini Score (CAD burden)	0.13	<0.001	0.038	0.168	0.09	0.001	0.064	0.044
Statin use	0.07	0.006	0.008	0.753	0.06	0.022	0.035	0.169
HDL, mg/dl	-0.03	0.33	-0.037	0.31	-0.02	0.52	-0.036	0.197
Total Cholesterol, mg/dl	-0.08	0.003	-0.003	0.80	0.034	0.20	0.072	0.013
hs-CRP, mg/L (log)	0.06	0.03	-0.001	0.951	-0.01	0.69	-0.039	0.195

Multivariate model - Independent determinants of each aminothiol (natural log transformed), using linear regression with all variables in the left column entered into the same model. GFR – Glomerular Filtration Rate; MI –Myocardial Infarction; CAD – Coronary Artery Disease; LVEF –Left Ventricular Ejection Fraction; HDL – High Density Lipoprotein; hs-CRP – high sensitivity C Reactive Protein

Supplementary Table 2B: Univariate and multivariate associations between patient characteristics and the glutathione couple

	Glutathione				Glutathione disulphide			
	Univariate		Multivariate		Univariate		Multivariate	
	beta	*p value*	*beta*	*p value*	*beta*	*p value*	*beta*	*p value*
Age, years	-0.10	<0.01	-0.072	0.042	0.04	0.12	0.000	0.964
Male Gender	0.04	0.14	0.029	0.323	0.004	0.89	0.028	0.335
Body Mass Index, kg/m2	-0.05	0.058	-0.067	0.020	-0.024	0.41	-0.027	0.555
GFR, ml/min	0.06	0.021	0.037	0.353	-0.077	0.004	-0.074	0.020
Acute MI	0.08	0.76	0.007	0.810	0.044	0.10	0.037	0.171
Diabetes	-0.08	0.004	-0.039	0.152	-0.013	0.63	-0.006	0.782
Hypertension	-0.09	0.001	-0.041	0.124	0.018	0.51	0.014	0.617
Current Smoking	-0.012	0.66	-0.041	0.122	-0.014	0.60	-0.011	0.735
LV EF, %	-0.015	0.57	-0.029	0.292	-0.021	0.42	-0.013	0.633
Gensini score (CAD burden)	-0.06	0.016	-0.067	0.026	-0.023	0.38	-0.057	0.087
Statin use	0.014	0.6	0.055	0.052	0.014	0.64	0.032	0.562
HDL, mg/dl	-0.013	0.63	-0.032	0.223	0.035	0.19	0.039	0.168
Total Cholesterol	0.07	0.008	0.061	0.020	-0.012	0.66	-0.006	0.779
hs-CRP, mg/L (log)	-0.016	0.56	-0.005	0.843	0.037	0.17	0.035	0.242

Multivariate model - Independent determinants of each aminothiol (natural log transformed), using linear regression with all variables in the left column entered into the same model. GFR – Glomerular Filtration Rate; MI –Myocardial Infarction; CAD – Coronary Artery Disease; LVEF –Left Ventricular Ejection Fraction; HDL – High Density Lipoprotein; hs-CRP – high sensitivity C Reactive Protein

Supplementary Table 3: Univariate and multivariate predictors of death

	Univariate (beta, p)	Multivariate Effect (HR, 95 CI)	P value
Age, years	0.04 (0.008)	1.04 (1.025-1.058)	P<0.001
Male Gender	0.16 (0.15)	1.174 (0.87-1.59)	0.302
Body Mass Index, kg/m2	-0.025 (0.012)	0.98 (0.95-1.00)	0.049
GFR, ml/min	-0.014 (0.004)	0.986 (0.979-0.993)	<0.001
Acute MI	0.072 (0.170)	1.075 (0.77-1.501)	0.67
Diabetes	0.47 (0.14)	1.59 (1.21-2.09)	0.001
Hypertension	0.341 (0.163)	1.406 (1.02-1.94)	0.037
Current Smoking	0.280 (0.183)	1.32 (0.93-1.89)	0.125
LV EF, %	-0.027 (0.005)	0.97 (0.96-0.98)	<0.001
Gensini score (CAD burden)	0.044 (0.042)	1.05 (0.96-1.14)	0.30
Statin use	-0.290 (0.155)	0.75 (0.55-1.01)	0.061
HDL, mg/dl	0.001 (0.006)	1.001 (0.989-1.014)	0.84
Total Cholesterol	-0.001 (0.002)	0.99 (0.995-1.002)	0.45
hs-CRP, mg/L (log)	0.254 (0.052)	1.289 (1.16-1.428)	<0.001

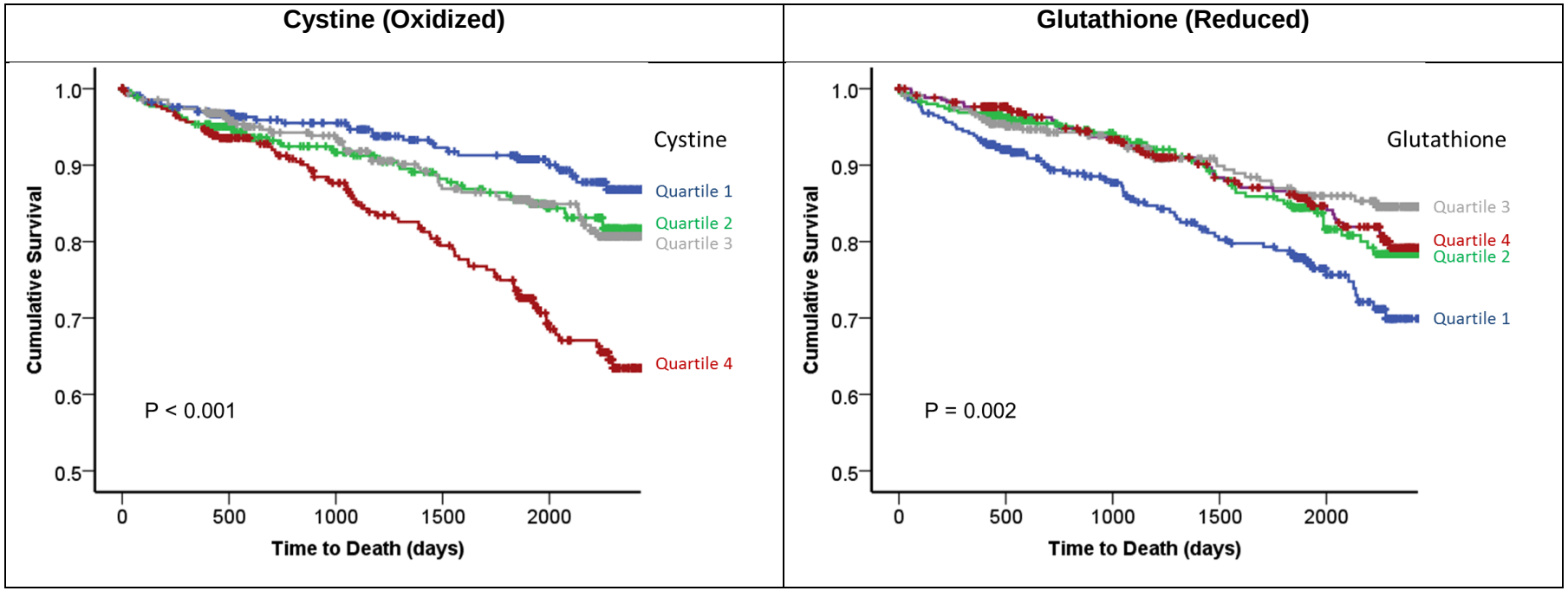
Multivariate model - Independent determinants of death using cox regression with all variables in the left column entered into the same model.
GFR – Glomerular Filtration Rate; MI –Myocardial Infarction; CAD – Coronary Artery Disease; LVEF –Left Ventricular Ejection Fraction; HDL – High Density Lipoprotein; hs-CRP – high sensitivity C Reactive Protein

Supplementary Table 4: Cox regression analysis of aminothiol association with death by quartiles

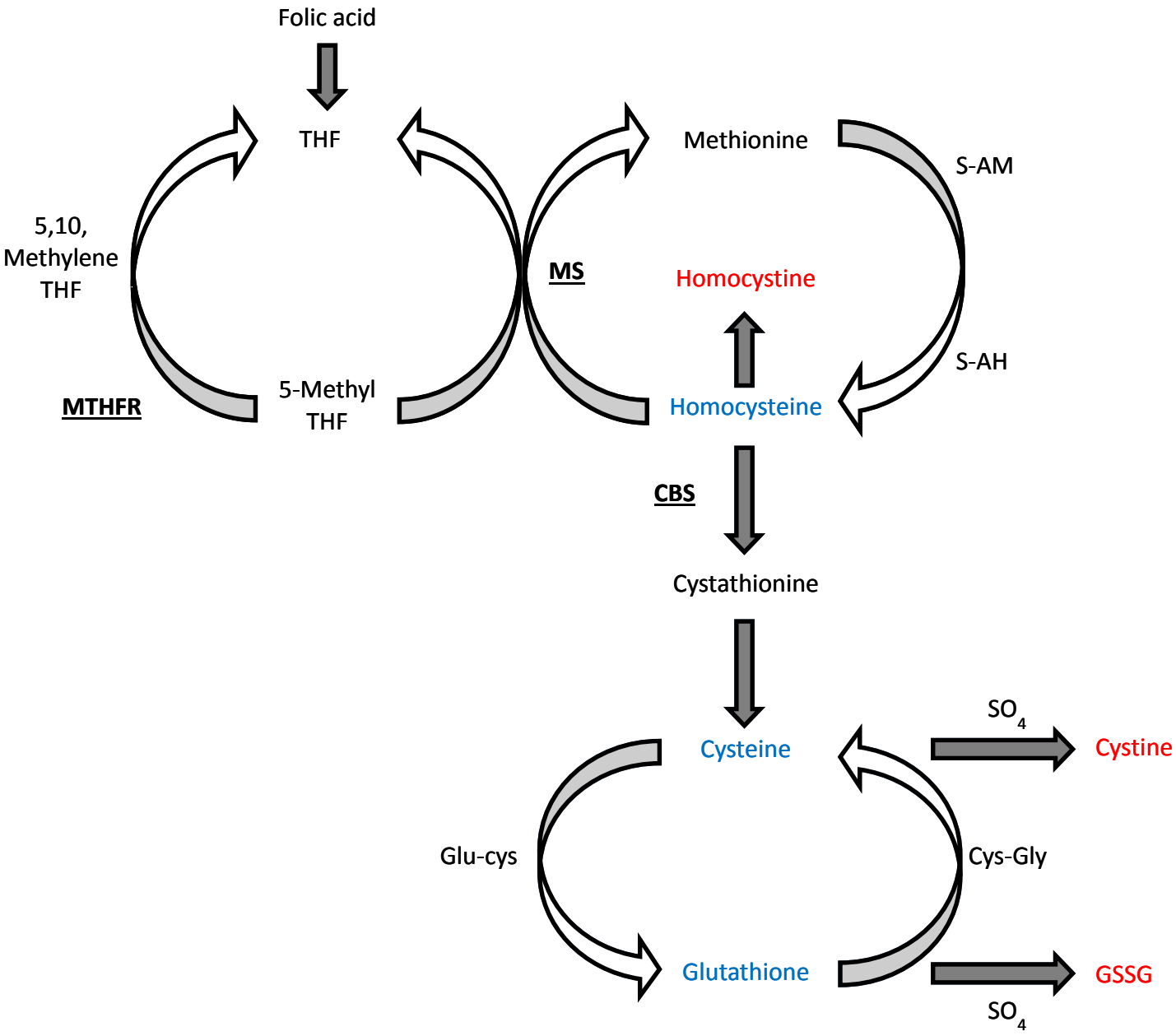
		Cystine		**Glutathione**	
		Q4 v Q1		Q1 v Q4	
		HR (95% CI)		HR (95% CI)	
Outcome		Age & gender adjusted	Fully adjusted model	Age & gender adjusted	Fully adjusted model
Death		2.15 (1.39-3.35)	1.82 (1.16-2.86)	1.64 (1.14-2.40)	1.40 (0.97-2.03)
Death/MI		1.98 (1.37-2.85)	1.72 (1.18-2.51)	1.47 (1.07-2.03)	1.29 (0.93-1.79)
CV Death		2.05 (1.25-3.39)	1.75 (1.05-2.91)	1.67 (1.11-2.53)	1.42 (0.93-2.16)

Cox regression analysis of aminothiol marker ratios as quartiles, with risk of adverse outcomes; Q1 = lowest quartile and Q4 = highest quartile; Full model includes adjustment for age, gender, body mass index, glomerular filtration rate, diabetes, hypertension, TC, HDL, current smoking, statin use, acute myocardial infarction, LV EF, Gensini score and LnCRP.

Supplementary Figure 1: Kaplan Meier analysis for cystine and glutathione by quartiles, for association with the primary outcome of Death



Supplementary Figure 2: Interplay between homocysteine and cysteine and glutathione



Supplementary Figure Legends:

Supplementary Figure 1: Kaplan Meier curves for association with quartiles of cystine and glutathione; Log rank p values and number of patients within each category is shown.

Supplementary Figure 2: Homocysteine pathway - Homocysteine may be converted to cystathionine by cystathionine beta synthase. This in turn may yield cysteine. Cysteine and glutathione may be generated from each other. As these thiols are reduced (blue) they may become oxidized (red) to generate the oxidized thiols cysteine and glutathione disulphide. There is no known pathway to reduce these oxidized thiols back to their reduced state.

MTHFR – methyl tetrahydrofolate reductase; MS - Methionine synthase; CBS – cystathinone beta synthase; GSSG – glutathione disulphide; THF – tetrahydrofolate; S-AH – S-adenosyl homocysteine; SAM -adenosyl methionine