Supplemental Material

Supplemental Material

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

Riyaz S. Patel MD,[1,2] Nima Ghasemzadeh MD,[1] Danny J Eapen MD,[1] Salman Sher MD,[1] Shawn Arshad MD,[1] Yi-an Ko PhD,[3] Emir Veledar PhD,[4,5] Habib Samady MD,[1] A. Maziar Zafari MD, PhD,[1,6] Laurence Sperling MD,[1] Viola Vaccarino MD, PhD,[1,5] Dean P Jones, PhD,[1] Arshed A. Quyyumi MD[1*]

[1]Dept of Medicine, Emory University School of Medicine, Atlanta, GA, USA
[2]Institute of Cardiovascular Science, University College London, London, UK
[3]Department of Biostatistics and Bioinformatics, Rollins School of Public Health, Atlanta, GA, USA
[4]Dept of Medicine, Baptist Health South Florida, Fl, USA
[5]Dept of Epidemiology, Rollins School of Public Health, Atlanta, GA, USA
[6]Dept of Medicine, Atlanta Veterans Affairs Medical Center, Decatur, GA, USA

Contents

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

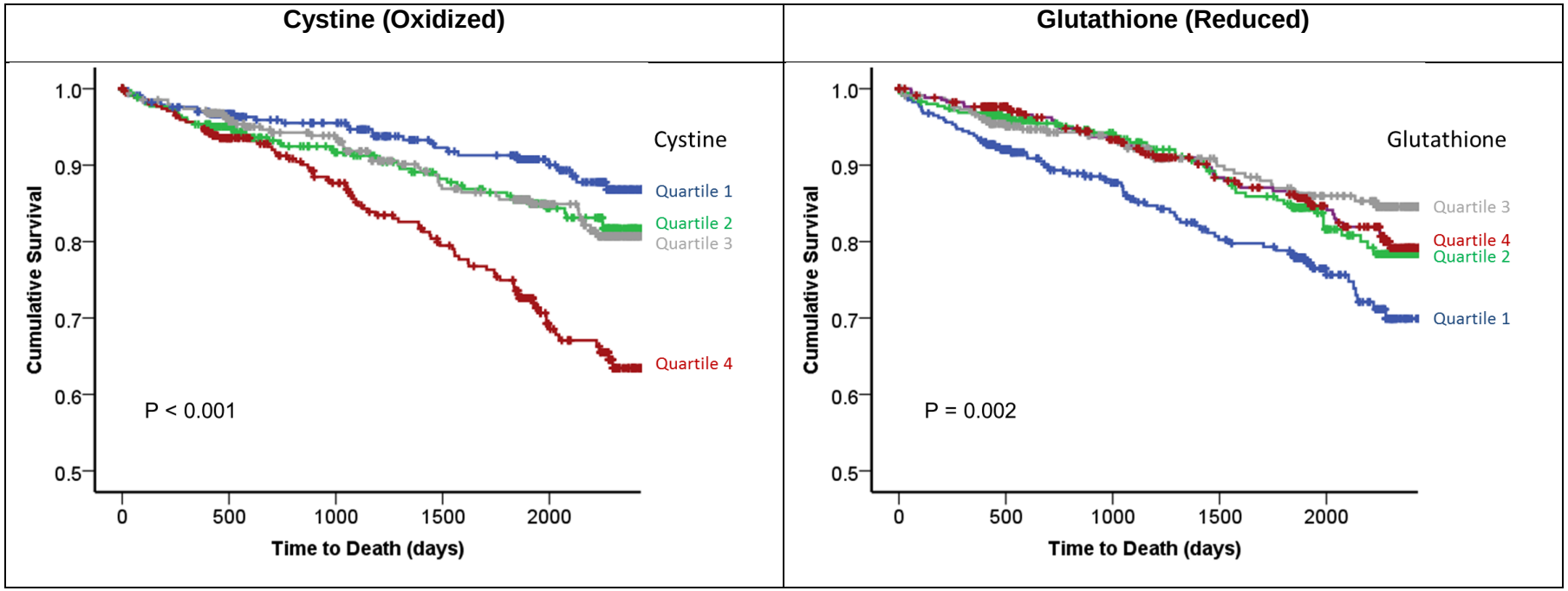
Multivariate model - Independent determinants of death using cox regression with all variables in the left column entered into the same model.
GFR – Glomerular Filtration Rate; MI –Myocardial Infarction; CAD – Coronary Artery Disease; LVEF –Left Ventricular Ejection Fraction; HDL – High Density Lipoprotein;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

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

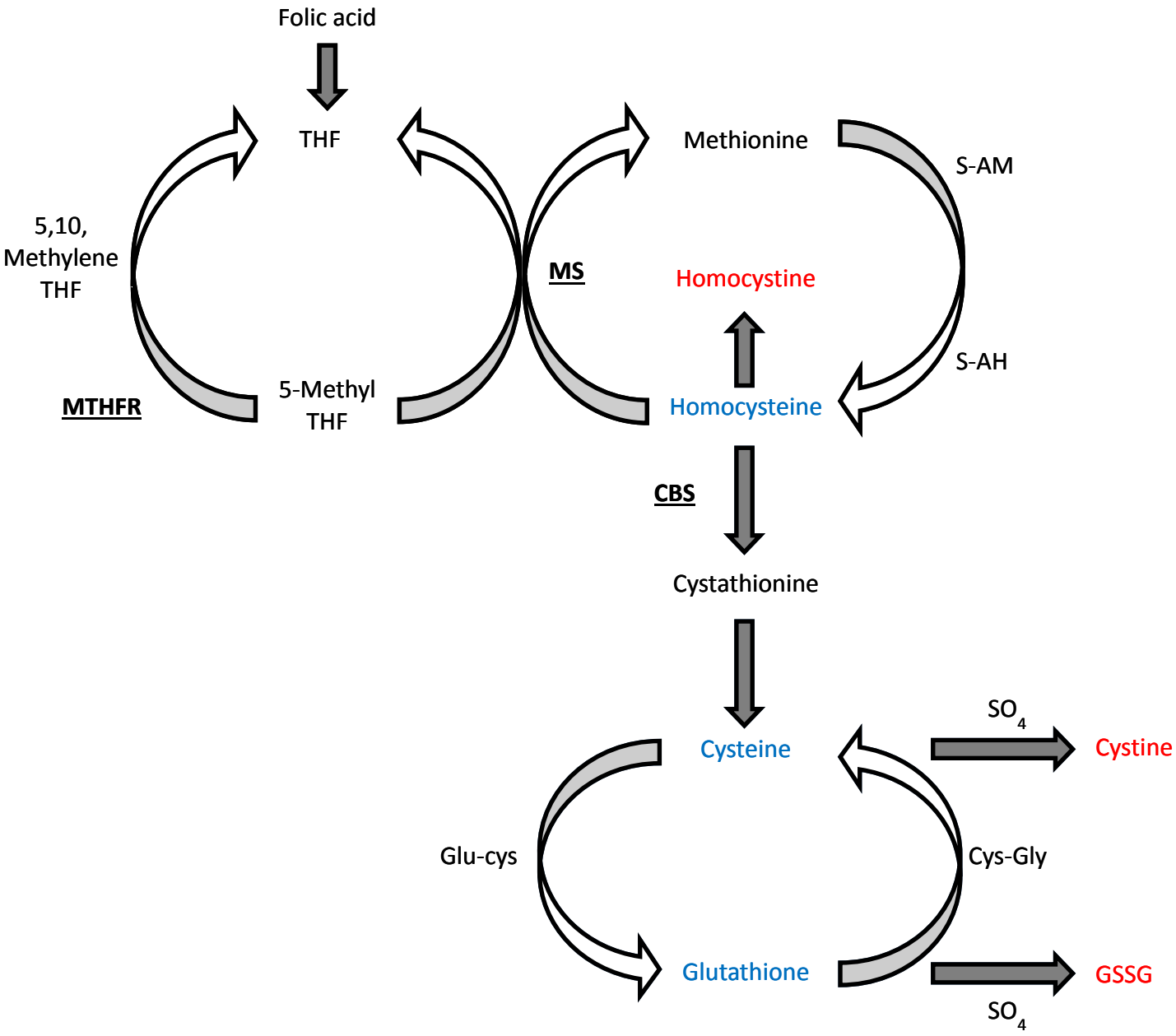
		Cystine		**Glutathione**	
		Q4 v Q1		Q1 v Q4	
		HR (95% CI)		HR (95% CI)	
Outcome		Age & gender adjusted	Fully adjusted model	Age & gender adjusted	Fully adjusted model
Death		2.15 (1.39-3.35)	1.82 (1.16-2.86)	1.64 (1.14-2.40)	1.40 (0.97-2.03)
Death/MI		1.98 (1.37-2.85)	1.72 (1.18-2.51)	1.47 (1.07-2.03)	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