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Plasma glutathione levels are independently associated with γ-glutamyltransferase activity in subjects with cardiovascular risk factors

VALENTINA SEDDA[1], BENEDETTA DE CHIARA[1], MARINA PAROLINI[1], RAFFAELE CARUSO[1], JONICA CAMPOLO[1], GIULIANA CIGHETTI[2], RENATA DE MARIA[1], ALDO SACHERO[3], LUIGI DONATO[1], & OBERDAN PARODI[1]

[1]*CNR Clinical Physiology Institute-Milan and Pisa, Cardiology Department, Niguarda Cà Granda Hospital, Milan, Italy,* [2]*Department of Preclinic Sciences LITA Vialba, University of Milan, Italy, and* [3]*Cardiology Department, Niguarda Cá Granda Hospital, Milan, Italy*

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Abstract

To investigate whether GGT (γ-glutamyltransferase) is associated to specific redox patterns. GGT, total and reduced aminothiols and malondialdehyde, were measured in 150 subjects (83 males, 48 (39–56) years), with none, one or more risk factors. By univariable analysis GGT was positively associated with age ($p = 0.001$), male gender ($p < 0.001$), risk factor number ($p < 0.001$), ACE-inhibitors ($p = 0.008$), anti-platelet agents ($p = 0.029$), atherothrombotic events ($p = 0.001$), glucose ($p = 0.013$), malondialdehyde ($p = 0.029$), plasma total cysteine ($p = 0.046$) and inversely associated with plasma total glutathione ($p = 0.001$). By multivariable analysis only male gender ($p < 0.001$), risk factor number ($p < 0.001$) and glutathione ($p < 0.001$) were independently associated with GGT activity. These findings suggest that an ongoing redox imbalance, in terms of decreased plasma glutathione, is associated with raised GGT activity in subjects with a greater risk factor burden.

Keywords: *γ-Glutamyltransferase, glutathione, cardiovascular risk factors*

Introduction

Serum γ-glutamyltransferase (GGT) activity has been associated with most cardiovascular risk factors and proved to be a predictor of ischemic heart disease and stroke, hypertension and type 2 diabetes [1–3]. Although the mechanisms underlying the above associations still remain unclear, oxidative stress has been suggested to play a key role in this process [4,5]. Conditions able to increase serum GGT, such as the presence of established cardiovascular risk factors [6], may lead to increased free radical production. Additionally, products of the GGT reaction themselves may augment oxidative stress, particularly in the presence of transition metals [4]. Finally, experimental studies have demonstrated that active GGT is present in the atherosclerotic plaques of diseased coronary arteries [5,7].

Chronic over-production of reactive oxygen species leads to redox imbalance, favouring depletion of glutathione (GSH), the major non-enzymatic antioxidant [8]. The GSH-antioxidant system has several physiological functions such as maintenance of protein–SH groups in a reduced state, detoxification from oxygen radicals, enzymatic degeneration of

Correspondence: Oberdan Parodi, MD, CNR Clinical Physiology Institute Niguarda Cà Granda Hospital P.za Ospedale Maggiore, 3 20162 Milano, Italy. Tel: +39-02-6473407. Fax: +39-02-66116990. Email: ifcnig@tin.it

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endogenous peroxides and formation of bioactive molecules. Furthermore, reduced levels of plasma GSH have been associated with the development of cardiovascular disease [9].

Until now, no study addressed the relationship between a raised GGT activity and GSH concentrations in cardiovascular diseases. Since GGT affects GSH catabolism, a potential link between raised GGT activity and redox state imbalance can be hypothesized in subjects with cardiovascular risk factors. Recognition of this association might lead to correction of these abnormalities in pre-clinical atherosclerosis, with important clinical implications for the prevention of cardiovascular events.

The present study was designed to investigate the relationship between serum levels of GGT and concentration of GSH, aminothiols, malondialdehyde (MDA), markers of redox state and the total burden of atherosclerotic risk factors.

Materials and methods

Study population

The study population included 150 consenting subjects, with or without cardiovascular risk factors, selected among 223 outpatients at the Cardiology Department of Niguarda Cà Granda Hospital (83 males, aged 20–75 years, median 48, interquartile range 39–56 years) who had been referred to our research laboratory from January to December 2006 for measurement of plasma homocysteine levels.

Subjects who had had atherotrombotic events (acute coronary syndromes, stroke/transient ischemic attack) within 12 months before the study were excluded to minimize a potential confounding influence of sub-acute inflammation. Subjects with the following conditions, known to be associated with an altered redox pattern, were also excluded: severe renal dysfunction (estimated glomerular filtration rate < 30 mL/min according to the Cockroft-Gault equation), severe uncontrolled hypertension, autoimmune diseases, neoplasia, advanced liver or pulmonary disease, acute or chronic inflammation and alcohol abuse meeting the criteria of DSM-IV American Psychiatric Association [10].

Information on common risk factors, general dietary-medical history and lifestyle were obtained from each subject using a questionnaire. Assessed cardiovascular risk factors were: smoking habit, hypertension, hypercholesterolaemia, diabetes, overweight and hyperhomocysteinaemia. Participants were considered smokers based on the current smoking status (10 cigarettes per day for at least 1 year without interruption). Hypertension was defined as systolic blood pressure > 140 mm Hg and/or diastolic blood pressure > 90 mm Hg or the need for anti-hypertensive drugs [11]; hypercholesterolaemia as LDL cholesterol level ≥ 160 mg/dL or the need for lipid-lowering medication [12]; diabetes mellitus as fasting glucose levels ≥ 126 mg/dL or the need for insulin or oral hypoglycaemic agents [13]; overweight as a body mass index of 25.0–29.9 Kg/m [14]; hyperhomocysteinaemia as plasma fasting homocysteine values ≥ 15 μM [15]. Subjects were required to fast overnight and refrain from the use of prescription drugs for at least 24 h. No patient was under antioxidant vitamin supplements, N-acetylcysteine or cysteine supplements for at least 2 months.

The study was approved by the Institutional Review Board of Niguarda Hospital.

Chemical analysis

Fasting blood samples were treated for blood and plasma reduced and total aminothiols (homocysteine, cysteine, GSH, cysteinylglycine) and free MDA analysis immediately after collection. For total aminothiols, an aliquot of whole blood was diluted with an equal volume of distilled water and frozen in liquid nitrogen; the remaining blood was centrifuged (2000 g for 10 min at 4°C) within 10 min of collection to obtain plasma, which was aliquoted, frozen and then stored at -80°C and analysed within 1 week. The total forms measured in our laboratory include the oxidized aminothiols (disulphides), all conjugated forms (among them protein-bound aminothiols and mixed aminothiols-disulphides produced through oxidative processes or thiol-disulphide exchange reactions) and reduced free aminothiols. Blood and plasma reduced aminothiols were determined by prompt acidification according to methods previously described [16]. Because plasma levels of reduced GSH are low (1–2%), blood reduced GSH concentrations may come close to GSH content inside the circulating cells (red and white blood cells, platelets). Aminothiol concentrations were determined by HPLC with fluorescence detection (ProStar, Varian, Surrey, UK) according to methods validated in our laboratory and previously reported [17]. Free MDA levels, the reactive non-conjugated MDA form, were assessed in stored plasma by gas chromatography–mass spectrometry with isotope dilution technique [18]. Plasma α-tocopherol and ascorbic acid levels were analysed by isocratic HPLC separation, as previously described [19].

A chemiluminescent competitive immunoassay (Roche Diagnostic GmbH, Mannheim, Germany) was used to measure folate levels and vitamin B_{12}, whereas glucose, creatinine, fibrinogen, total cholesterol and triglycerides were determined using standard laboratory methods. HDL cholesterol was measured after precipitation with dextran sulphate-magnesium and LDL cholesterol was calculated using the Friedewald's method.

Serum GGT activity was assessed by a kinetic colourimetric assay at 37°C and expressed as unit per

litre (laboratory cut-points for abnormal are 35 U/L in women and 50 U/L in men).

Statistical analysis

Continuous variables are presented as median and interquartile ranges [I–III]. Biochemical variables with a not normal distribution were logarithmically transformed and presented as geometric means (95% confidence interval). The association between GGT activity and the clinical, biochemical variables and aminothiol concentrations was evaluated by linear regression analysis in the entire cohort. Significant variables by univariable analysis ($p < 0.05$) were entered into a stepwise multivariable linear regression analysis to identify those independently associated with GGT levels. Associations of plasma total GSH with demographic parameters and risk factors were tested by Pearson correlation test.

Statistical significance was settled at a p value < 0.05. Statistical analyses were carried out with the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL) release 10.0 for Windows and S-PLUS (S-PLUS 2000 MathSoft, Seattle, WA).

Results

The study population included 37 subjects without and 113 with one or more cardiovascular risk factors. Clinical and biochemical characteristics and the redox state profile of the overall population are listed in Tables I and II, respectively.

None of the study subjects had diabetes. Fasting glucose levels, serum folate and vitamins concentrations fell within the normal range in the overall population, whereas values of GGT activity were out of range in 18% of men and in 6% of women.

In order to consider the burden of risk factors present in our series, subjects were categorized into four groups based on the number of cardiovascular risk factors (group 0, 1, 2, and 3 if $\geq$ 3 risk factors), irrespective of type (Table I).

A history of atherothrombotic events was present in 12%, 8% and 32% of subjects in groups 1, 2 and 3, respectively, and in none of group 0 subjects. Consistent with current guideline recommendations, subjects with risk factors were under specific pharmacological treatment based on the assessment of their global cardiovascular risk: 25% of group 1, 45% of group 2 and 68% of group 3 patients took at least one cardiovascular drug. Drug classes are detailed in Table I.

Linear regression analysis was performed in the entire cohort to identify demographic, clinical and redox state variables significantly associated to a raised GGT activity (Table III). Taking into account the potential antioxidant properties of many cardiovascular agents, drug classes administered were also

Table I. Clinical and biochemical variables in the overall population.

	All cases ($n = 150$)
Age (years)	48 [39–56]
Gender (male)	83 (55%)
BMI (kg/m^2)	24.3 [21.7–26.7]
Diabetes	0
Current smokers	47 (31%)
Hypertension	40 (27%)
Hypercholesterolemia	21 (14%)
Hyperhomocysteinemia	42 (28%)
History of atherothrombotic events	16 (11%)
Total cholesterol (mg/dL)	195 [174–229]
HDL-cholesterol (mg/dL)	57 [49–67]
LDL-cholesterol (mg/dL)	117 [93–142]
Triglycerides (mg/dL)	96 [69–138]
Gamma-glutamyltransferase (U/L)†	21.7 (19.6, 23.7)
Fasting glucose (mg/dL)†	89 (87, 91)
Estimated GFR (ml/min)	98 [81–115]
Serum folate (ng/mL)†	5.73 (3.69, 7.77)
Vitamin B_{12} (pg/mL)	411 [288–538]
*Number of risk factors	
None	37 (25%)
1	40 (26%)
2	51 (34%)
$\geq$3	22 (15%)
ACE-inhibitors	13 (9%)
Beta-blockers	10 (7%)
Calcium-channel blockers	15 (10%)
Anti-platelet agents	23 (15%)
Statins	11 (7%)
Angiotensin II receptor antagonists	10 (7%)

Note: Data are expressed as median value and interquartile range [I–III], number of patients (percentage) or, where indicated (†), as geometric mean (95% confidence interval).
BMI, body mass index; GFR, glomerular filtration rate estimated by the Cockroft-Gault equation.
* Number of risk factors, sum of all risk factors reported above in the table, irrespective of type.

Table II. Redox state variables in the overall population.

	All cases ($n = 150$)
Plasma total thiols	
Homocysteine (μmol/L)	10.1 [7.6–16.4]
Cysteinylglycine (μmol/L)†	33 (31, 35)
GSH (μmol/L)†	5.77 (3.73, 7.80)
Cysteine (μmol/L)	244 [203–287]
Plasma reduced thiols	
Homocysteine (μmol/L)	0.16 [0.11–0.33]
Cysteinylglycine (μmol/L)†	3.53 (1.47, 5.58)
GSH (μmol/L)†	2.49 (0.37, 4.60)
Cysteine (μmol/L)†	7.05 (5.01, 9.09)
Blood total GSH (μmol/L)†	1099 (1097, 1101)
Blood reduced GSH (μmol/L)	679 [506–862]
Ascorbic acid (μmol/L)	55 [39–74]
α-tocopherol/total cholesterol (μmol/mg)†	0.09 (−1.93, 2.11)
MDA (μmol/L)†	0.70 (−1.42, 2.83)

Note: Data are expressed as median value and interquartile range [I–III] or, where indicated (†), as geometric mean (95% confidence interval).
α-tocopherol values were normalized for the corresponding total cholesterol values.
GSH, glutathione; MDA, free malondialdehyde.

Table III. Univariable linear regression analysis of parameters associated to a raised GGT activity.

	β	p
Demographic and clinical variables		
Age	0.006	0.001
Male gender	0.322	<0.001
BMI	0.042	<0.001
Number of risk factors	0.108	<0.001
Fasting glucose	1.129	0.013
Estimated GFR (ml/min)	−0.045	0.87
Serum folate (ng/mL)	−0.182	0.15
Vitamin B_{12}	0.00007	0.61
History of events	0.271	0.001
ACE-inhibitors	0.239	0.008
Beta-blockers	0.084	0.41
Anti-platelet agents	0.153	0.029
Calcium-channel blockers	0.045	0.59
Statins	0.114	0.24
Angiotensin II receptor antagonists	−0.039	0.70
Redox state variables		
Plasma total thiols		
Cysteinylglycine (μmol/L)	0.066	0.66
GSH (μmol/L)	−0.421	0.001
Cysteine (μmol/L)	0.0008	0.046
Plasma reduced thiols		
Cysteinylglycine (μmol/L)	0.051	0.69
GSH (μmol/L)	−0.006	0.93
Cysteine (μmol/L)	0.192	0.098
Blood total GSH (μmol/L)	−0.135	0.58
Blood reduced GSH (μmol/L)	−0.00008	0.35
Ascorbic acid (μmol/L)	−0.0017	0.15
α-tocopherol/total cholesterol (μmol/mg)	−0.096	0.90
MDA (μmol/L)	0.270	0.029

Note: β indicates linear regression coefficient.
GSH, glutathione; MDA, free malondialdehyde.

included in the analysis (Table III). All variables significantly associated with serum GGT activity by univariable analysis were entered into the stepwise multivariable model: male gender, number of risk factors and plasma total GSH remained independently associated with a raised GGT activity (Table IV, Figure 1).

No significant association was observed between plasma total GSH and either gender ($p = 0.83$, $\beta = -0.007$; geometric mean for males, 5.82 μmol/L; 95% confidence interval (CI), 2.77–8.87; geometric mean for females, 5.73 μmol/L; 95% CI, 2.66–8.79) or number of risk factors ($p = 0.42$, $\beta = -0.012$; 0 risk factor, geometric mean: 6.13 μmol/L; 95% CI, 3.29–8.96; 1 risk factor, geometric mean: 5.47 μmol/L; 95% CI, 2.36–8.57; 2 risk factors, geometric mean: 5.98 μmol/L; 95% CI, 2.81–9.16; 3 or more risk factors, geometric mean: 5.26; 95% CI 2.22–8.30), whereas a negative correlation was found with age ($p = 0.002$, $\beta = -0.25$).

Table IV. Stepwise multivariable linear regression model of parameters independently associated to a raised GGT activity.

	β	p
Male gender	0.274	<0.001
Number of risk factors	0.075	<0.001
Total plasma GSH (μmol/L)	−0.382	<0.001

GSH, glutathione.

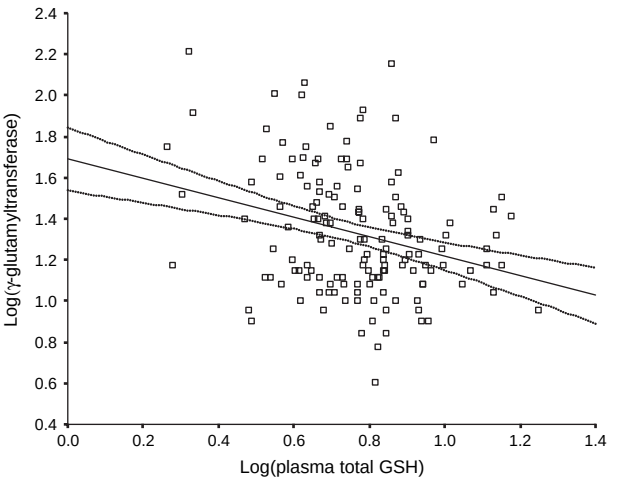

Figure 1. Scattergram, linear regression line and 95% confidence bands of gamma-glutamyltransferase against plasma total glutathione (GSH), adjusted for gender and number of risk factors.

Discussion

The major novel finding of the present study is the association between GGT activity, the levels of plasma total GSH and the number of risk factors, irrespective of type.

Recent clinical evidences have shown that serum GGT is strongly associated with several cardiovascular risk factors and overt disease, independently of alcohol consumption [1–6]. Mechanisms underlying these associations are still unclear. Previous experimental studies reported that GGT activity is accompanied by pro-oxidant processes, with production of reactive oxygen species and oxidation of cellular protein thiols [20]. Interestingly, after coronary revascularization both by the percutaneous and surgical approach, GGT prognostic value was lost, confirming the intrinsic link between raised GGT and plaque evolution [21].

In a large prospective series, Lee et al. [3] demonstrated that GGT values correlated with C-reactive protein and F2 isoprostanes, markers of inflammation and lipid peroxidation, respectively, in a level-dependent manner. Moreover, they found a negative association between serum GGT levels and the dietary antioxidants carotenoids and tocopherols [22]. These authors hypothesized that GGT might be a sensitive marker of oxidative stress [23].

Our findings confirm the expected association of GGT with a higher number of risk factors. The major novel finding of this study is the significant inverse association between GGT and the redox profile, in

terms of extracellular GSH, which represents a good marker of the pro-oxidant/anti-oxidant balance. Interestingly, this association was not influenced by lifestyle factors, such as smoking habit (data not shown), vitamins or medications (Tables III and IV), whereas it was much stronger in males and subjects with a higher number of risk factors (Table IV). Moreover older age was associated with higher GGT activity and lower plasma GSH levels in agreement with the recognized prognostic role of GGT in the development of cardiovascular disease [1] and with the proposed role of plasma GSH as risk factor [9].

We explore three possible explanations for the association between GGT and plasma GSH: increased extracellular GSH degradation, increased extracellular GSH depletion and/or increased GSH-related detoxification mechanisms of endo- and xenobiotics.

GSH catabolism: The good

Extracellular GSH and glutathione conjugates are substrates for GGT. This enzyme catalyses the transfer of the gamma-glutamyl moiety from GSH or a GSH conjugate to an alfa-amino acid to give gamma-glutamyl amino acid and the dipeptide cysteinylglycine. Cysteinylglycine is further metabolized to cysteine and glycine by dipeptidases. As a result of this gamma-glutamyl cycle, the extracellular space contains an available pool of cyst(e)ine which is the delimiting substrate for intracellular GSH biosynthesis.

By univariable analysis, we found a significant direct correlation between a raised GGT activity and plasma total cysteine levels (including both free cysteine, cystine and the protein-bound cysteine forms); however, this association did not reach the statistical significance by multivariable analysis. This may be explained by the fast dynamics of cysteine and its transport inside the cells in order to preserve the intracellular balance of GSH recycling. Accordingly, no significant correlation between GGT and blood reduced GSH, index of GSH concentration in circulating cells, was found.

Unexpectedly, we did not find any direct correlation between free MDA, index of lipid peroxidation and the increase of risk factors number. In an experimental study, Liu et al. [24] demonstrated that 4-hydroxy-2-nonenal, another lipid peroxidation metabolite, causes an increase in glutamate-cysteine ligase activity and GSH synthesis. It is conceivable that the raised GGT activity and the possible increase of glutamate-cysteine ligase activity by lipid peroxidation products (4-hydroxy-2-nonenal and, likely, MDA) are adaptive responses during oxidative stress. This effect might favourably control lipid peroxidation in subjects with increased GGT activity and higher number of risk factors. In agreement with this finding, we showed that, in hyperhomocysteinemic subjects, experimental acute hyperhomocysteinemia induced by methionine loading is associated with higher intracellular GSH content and lower lipid peroxidation, with respect to values observed in normohomocysteinemic subjects [25].

Preservation of intracellular GSH content has been recently confirmed by our group even in the presence of glutamate-cysteine ligase-129 C/T polymorphism in subjects with cardiovascular risk factors [26].

Thus, induction of GGT activity may reflect the activation of body defence mechanisms in maintaining adequate intracellular GSH level during oxidative stress; on the other hand, it might affect the extracellular concentration of GSH through its enhanced catabolism.

GSH catabolism: The bad

At a cellular level, the pro-oxidant effects of GGT activity are normally balanced by its role in favouring GSH re-synthesis, thus allowing the maintenance of intracellular antioxidant defences. However, the physiological function of GGT activity as a source of peptide precursors for intracellular GSH re-synthesis has been challenged. A growing body of evidence suggests that GSH catabolism can play a pro-oxidant role through its degradation via GGT [4,22]; in fact, GGT-mediated cleavage of GSH gives rise to the thiol dipeptide cysteinylglycine, whose -SH group is much more reactive than the GSH one and is provided in particular with a much stronger iron-reducing ability. This iron-dependent redox-cycling process may result in free radical generation and lipid peroxidation [20]. Accordingly, previous studies consistently hypothesized that GGT might be one of the enzymes related to oxidative stress and atherosclerosis development [5,7]. In line with these findings, the present study suggests that the presence of higher number of risk factors, associated to a raised GGT activity, might lead to conditions in which the pro-oxidant effects of soluble GGT activity in serum become predominant. In fact, the decreasing GSH content observed in the extracellular space might derive from its enhanced consumption to counteract the excessive amount of pro-oxidant species produced.

Recently, we found that in subjects with risk factors increased concentrations of plasma total cysteine and reduced concentrations of plasma total GSH after methionine loading were independently associated with abnormal flow-mediated dilation [27]. Additionally, in a different population, we found that baseline plasma GSH content was reduced in subjects with previous cardiovascular events, reinforcing the role of these scavengers in the protection against atherosclerosis [26]. In agreement, Shimizu et al. [9] suggested that reduced levels of plasma total GSH are

a risk factors *per se* for cardiovascular disease development.

GSH catabolism: The cleaner

Either by spontaneous conjugation or by reduction, GSH provides the bulk of available sulphydryl groups for binding and detoxification of reactive endogenous and exogenous compounds [28,29]. These GSH conjugates are formed by the interaction of an electrophilic compound, such as those produced by bioactivation of drugs by cytochrome P450, with the endogenous GSH pool. The resulting oxidized GSH and GSH adducts are removed from cells to maintain an intracellular thiol reduction potential by several transport mechanisms such as the multidrug resistance proteins [30]. The GSH adducts may then be excreted, usually into the bile, while the conjugates may undergo further metabolism via the mercapturic acid pathway. In subjects with established risk factors, who are frequently under pharmacological treatment, these detoxification mechanisms are enhanced and might contribute to GSH consumption.

On the basis of the evidence provided here, the most likely mechanism for the extracellular GSH decrease is both enhanced catabolism for subsequent intracellular *de novo* GSH synthesis and enhanced detoxification mechanisms to form less reactive metabolites and conjugates readily available for excretion.

Taking into account the constellation of biochemical pathways that take place in the extracellular compartment and see GSH involvement, the lack of association between this extracellular metabolite and the risk factor burden, reflecting only a potential condition of increasing oxidative stress, comes as no surprise. Risk factors influence the endothelial function, acting on the derangement of vascular nitric oxide pathway and increasing oxidant stress, both directly and indirectly.

As maintaining optimal GSH concentration in the cell is critical to survival, tight regulation of the system is imperative [24–26]. In agreement with these observations, we did not observe any significant change of the intracellular GSH concentration in our series. Likely, chronic ongoing vascular oxidative stress, due to the presence of risk factors, activates several intracellular GSH salvage pathways among which we focused on the GSH catabolism, in terms of a raised GGT activity. Enzymes are usually the first site of regulation in response to stimuli and, for this reason, much more sensitive than their substrate or other metabolites to physiologic or pathophysiologic processes and the ways in which these processes interact with cardiovascular risk. As such, a strong and independent relationship exists between GGT (the enzyme) and risk burden, while the association between the GGT metabolite (extracellular GSH) and risk burden is lacking.

Finally, as suggested by previous studies, GSH might itself be a risk factor for vascular disease development [9,27]; the lack of association between GSH with either the single type of risk factor or their cluster, is in line with the evidence that single risk factors may be independent from one another and that the clustering of risk factors substantially increases the risk of cardiovascular disease. Accordingly, no association was found between the risk factors considered in our series (data not shown), except for overweight and hypercholesterolemia.

Some limitations in this study need to be mentioned:

1. We did not examine the dietary intake of our study population. However, plasma concentrations of ascorbic acid, α-tocopherol and folate that may affect plasma levels of total GSH fell within the normal range in all subjects.
2. This was not a randomized trial of drug treatment, but an observational study and, therefore, following current guideline recommendations, most subjects in group 2 and 3 were prescribed cardiovascular drugs with antioxidant properties. Despite drug treatment, GGT concentrations were higher in patients with a greater risk factor burden and no drug class was independently associated to GGT activity.

In conclusion, our results indicate that male gender and a greater risk factor burden are directly and independently associated with a raised GGT activity. The strong inverse association found between extracellular GSH levels and GGT activity suggests that these circulating biomarkers can be usefully employed to prospectively assess therapies potentially influencing redox state imbalance in subjects with atherosclerotic risk burden. Both GGT activity and reduced levels of plasma GSH in fact represent early markers of oxidative stress.

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