Glutathione peroxidase activity and expression levels are significantly increased in acute coronary syndromes

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ABSTRACT

High levels of the antioxidant enzyme, glutathione peroxidase (GPx), have been associated with improved outcomes following acute coronary syndromes (ACS), suggesting a protective role. How GPx levels are altered with coronary disease is not clearly established. This study examined GPx activity, protein, and mRNA levels in healthy controls, patients with stable coronary artery disease (CAD), and patients with ACS. We studied 20 individuals from each of the healthy control, stable CAD, and ACS groups. GPx activity and protein levels, along with oxidized low-density lipoprotein (oxLDL) were assayed in plasma. GPx mRNA levels from whole blood were quantified using real-time PCR. Levels of GPx activity in the plasma were higher in ACS (109 ±7.7 U/mL) compared with patients with stable CAD (95.2±16.4 U/mL, p<0.01) and healthy controls (87.6±8.3 U/mL, p<0.001). Plasma GPx protein levels were also elevated in ACS (21.6±9.5 μg/mL) compared with patients with stable CAD (16.5 ±2.8 μg/mL, p<0.05) and healthy controls (16.3 ±5.3 μg/mL, p<0.05). Levels of *GPX1*, *GPX3*, and *GPX4* mRNA were significantly higher in the patients with ACS. Levels of oxLDL were also significantly higher in patients with ACS (61.9±22.2 U/L) than in patients with stable CAD (47.8±10.4 U/L, p<0.05) and healthy controls (48.9±11.9 U/L, p<0.05). Levels of oxLDL, GPx activity, protein, and mRNA are all significantly higher in patients with ACS compared with patients with stable CAD and healthy controls. These findings suggest that GPx may be upregulated in response to a change in oxidative stress during an ACS.

Significance of this study

What is already known about this subject?

- Glutathione peroxidase (GPx) is an antioxidant enzyme involved in the primary defense against oxidative stress.
- High activity levels have been associated with improved clinical outcomes following acute coronary syndromes (ACS).
- Unclear whether this elevation of activity is a product of differential capacity to respond to the acute event, or whether this variability occurs regardless of disease state.

What are the new findings?

- GPx activity, protein, and mRNA are significantly elevated in patients with ACS, compared with patients with stable CAD and healthy controls.
- Levels of oxidized low-density lipoproteins are significantly higher in patients with ACS, compared with patients with stable CAD and healthy controls.
- This is the first study to comprehensively examine GPx activity and corresponding protein and mRNA levels in a rigorously controlled patient population, in which potential confounders of oxidative stress were minimized.

How might these results change the focus of research or clinical practice?

- We suggest that GPx may be upregulated in response to the change in oxidative stress induced by the acute coronary vascular event.
- It is likely that a differential antioxidant capacity exists between patients following an ACS, and that those who lack the ability to upregulate GPx over a certain threshold are at an increased risk of poor clinical outcomes.

INTRODUCTION

Oxidative stress plays a role in the pathogenesis of atherosclerosis.[1 2] Consequences of high levels of oxidative stress include vascular remodeling, reperfusion injury, endothelial dysfunction, and plaque rupture,[3] all features commonly associated with the incidence of acute coronary syndromes (ACS). Antioxidant enzymes, such as glutathione peroxidase (GPx), function by reducing reactive oxygen species making them a primary defense mechanism against oxidative stress.

Deficiencies in GPx activity have been associated with abnormal vascular and cardiac structure and function in animal studies.[4 5] Human population studies have suggested that low levels of GPx activity are associated with the progression of coronary artery disease

(CAD),[6] and we have previously demonstrated that patients with ACS with lower levels of plasma-borne GPx activity are at a greater risk of adverse cardiovascular outcomes.[7] Taken together, this suggests that higher levels of GPx activity have a protective effect in a CAD setting.

What is not clear from the current literature, however, is whether levels of GPx activity differ in an ACS state compared with a healthy state, or even from patients with stable CAD. Previous studies on this topic give conflicting results, with reports of increases,[8] decreases,[9] and no change[10] all featuring when comparing levels of GPx activity. A common limitation in the current literature is lack of control for potential confounders that may influence GPx activity. Cardiac risk factors, such as diabetes and smoking, along with gender and age, have all been reported to alter GPx activity levels to some degree.[11–14] Controlling for these factors, and examining mRNA levels, protein levels, and GPx activity would provide a more complete picture of how GPx changes from healthy subjects to those with stable CAD and ACS. The aim of this study therefore was to undertake a comprehensive assessment of GPx mRNA, protein and activity levels in healthy controls, subjects with stable CAD and those with ACS.

PATIENTS AND METHODS

Study population

Twenty subjects were recruited into each of the healthy subject, stable CAD, and ACS cohorts, with a similar age and gender profile in each group. Subjects were included in the healthy cohort if they had no known cardiovascular disease and were not taking any cardiovascular medications. Patients were included in the stable CAD group if they had angiographically documented CAD with evidence of >70% stenosis in a major coronary artery but no ischemic symptoms within the preceding 4 weeks. Patients were included into the ACS cohort if they had symptoms suggestive of myocardial ischemia lasting >10 min, and either a troponin elevation, or ≥1 mm of new ST segment deviation or ≥1 mm T wave inversion on an electrocardiogram in at least two contiguous leads. Exclusion criteria for all three groups included diabetes mellitus, smoking, atrial fibrillation, heart failure, pregnancy, or an age outside the range of 45–65 years. The study was reviewed and approved by the Upper South A Regional and Lower Regional South Ethics Committee, and all patients provided written informed consent.

Data collection and blood sampling

Patient demographics, clinical characteristics, and medications were collected prospectively from review of the medical records and cardiac catheterization database. In the healthy and stable CAD cohorts, whole blood samples were collected in tubes anticoagulated with sodium citrate (0.109 M, BD Vacutainer; New Jersey, USA) from a peripheral vein using a 21-gauge needle. In the ACS cohort, whole blood was collected into sodium citrate tubes prior to angiography from either a peripheral vein using a 21-gauge needle or from the arterial sheath immediately after insertion and prior to heparin administration. In order to reduce variability between biochemical measures, sample preparation was standardized by following strict time protocols, in which plasma was separated from the cellular components by centrifugation at 1500 g for 12 min at 4°C within 30 min of collection. Plasma aliquots were stored at −80°C for later analysis.

GPx system

GPx activity assay

GPx activity was measured using a commercial colorimetric assay (Enzo Lifesciences; New York, USA) as per the manufacturer's instructions. The experimental protocol was based on a coupled reaction of GPx with the reduction of oxidized glutathione by glutathione reductase using nicotinamide adenine dinucleotide phosphate hydrogen (NADPH). The oxidation of NADPH to NADP+ accompanies a decrease in absorbance at 340 nm that is proportional to total GPx activity found in the plasma sample. Samples were measured in triplicate with absorbance read in a microplate reader (VersaMax, Molecular Devices; California, USA). GPx activity was defined as nanomoles of NADPH consumed per minute and expressed as units per mL of plasma. Intra-assay coefficient of variance was 9.9%, and interassay coefficient of variance was 8.1%.

GPx3 protein ELISA kit

Plasma GPx3 protein levels were measured according to the manufacturer's instructions using a sandwich ELISA kit (Adipogen International; California, USA). Samples were diluted 1:1000 with sample buffer and measured in duplicate, with absorbance read in a microplate reader (VersaMax). Intra-assay coefficient of variance was 2.5% and interassay coefficient of variance was 7.2%.

Quantitative real-time PCR (qPCR): GPx mRNA expression

Total RNA from whole blood was extracted using the QIAamp RNA blood mini kit (Qiagen; Hilden, Germany) following the manufacturer's instructions. RNA was quantified using a Bioanalyzer 2100 (Agilent Technologies; Santa Clara, USA). Total RNA was reverse transcribed (10 ng) using a SuperScript ViLo cDNA Synthesis Kit (Invitrogen; Auckland, NZ) following the manufacturer's instructions. Gene expression of *GPX3*, the isoform thought to be most abundant in the plasma,[15] along with *GPX1* and *GPX4*, both of which may be relevant for coronary artery disease,[6 16] were quantified on a Corbett Rotor-Gene 6000 machine (model no. 11754–050; Corbett Life Science; Sydney, Australia) using Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies). The thermal cycle conditions were as follows: 10 min at 95°C, then 40 repeats of 15 s at 95°C and 60 s at 60°C. The primers used are listed in table 1, including reference source and National Center for Biotechnology Information accession numbers for each gene investigated.[17 18] Two technical replicates were performed on all samples, with each replicate measuring all samples in duplicate. The mean cycle threshold (Ct) value of each duplicate was used for analysis. Any duplicates with Ct values differing by more than 0.5 were not used in the final data. Gene expression was normalized relative to the mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by means of the $2^{(-\Delta\Delta Ct)}$ method as described previously by Livak *et al*.[19]

Table 1 Oligonucleotide sequences

Primer target/ ref	NCBI accession no.	Forward (F)/ reverse (R)	Primer sequence 5′-3′
GPX1[17]	NM_000581	F	GCAACCAGTTTGGGCATCAG
		R	CGTTCACCTCGCACTTCTCG
GPX3[18]	NM_002084	F	CATCCCCTTCAAGCAGTATGC
		R	GCCCGTCAGGCCCTCAGTAG
GPX4[17]	NM_001039848	F	TGGGAAATGCCATCAAGTGG
		R	GGTCCTTCTCTATCACCAGGGG
GAPDH	NM_001289745	F	CGGATTTGGTCGTATTGG
Beacon Designer		R	GGTGGAATCATATTGGAACAT

Oligonucleotide sequences for forward and reverse primers used in SYBR Green qPCR reactions, including reference source and NCBI accession numbers for each gene investigated.
GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NCBI, National Center for Biotechnology Information.

Oxidized low-density lipoprotein (oxLDL) ELISA

Plasma oxLDL was measured by a sandwich ELISA based on monoclonal antibody 4E6 as the capture antibody (Oxidized LDL ELISA, Mercodia; Uppsala, Sweden) in accordance with the manufacturer's instructions. Samples were diluted 1:6561 with sample buffer and measured in duplicate with absorbance read in a microplate reader (VersaMax). Intra-assay coefficient of variance was 3.9% and interassay coefficient of variance was 9.9%.

Statistical analysis

Continuous variables are reported as mean±SD, and categorical variables are reported as frequencies and percentages. Statistical tests for continuous variables were performed using a student's unpaired t-test and one-way analysis of variance with Tukey's multiple comparisons test. χ^2 tests were used for the categorical variables. Relationships between parameters were determined by a Pearson's correlation coefficient. Differences in values corresponding to $p<0.05$ were taken as statistically significant. All statistical analyses were carried out in either GraphPad Prism Software V.6 (GraphPad Software; California, USA) or SPSS V.22 (IBM; Armonk, New York, USA).

RESULTS

Baseline characteristics

Baseline characteristics of the ACS, stable CAD, and healthy cohorts are presented in table 2. The patients with ACS had a significantly higher body mass index than the stable CAD and healthy populations ($p<0.001$). Table 3 lists the cardiovascular medications that the patients with stable CAD and ACS were on at the time of enrolment in the study. Medications were maintained throughout the study.

GPx measurements

GPx activity was significantly higher in patients with ACS (109±7.7 U/mL) compared with patients with stable CAD (95.2±16.4 U/mL, $p<0.01$) and healthy subjects (87.6 ±8.3 U/mL, $p<0.001$) (figure 1). No significant difference was detected between the patients with stable CAD and the healthy cohort.

Table 2 Baseline characteristics of patient groups

	Healthy group N=20	Stable group N=20	ACS group N=20
Male	15 (75)	17 (85)	16 (80)
Age (years)	57.1±6.5	59.4±5.0	59.0±6.6
BMI	24.1±2.8	28.0±4.8***	30±5.1***
Ethnicity			
European	20 (100)	20 (100)	18 (90)
Other	0 (0)	0 (0)	2 (20)
Cardiovascular risk factors			
Prior MI	0	13 (65)****	4 (20)*††
Prior PCI	0	11 (55)****	4 (20)*†
Prior CABG	0	9 (45)***	1 (5)††
Prior stroke	0	3 (15)	1 (5)
Hypertension	0	13 (65)****	9 (45)***
Dyslipidemia	0	16 (80)****	14 (70)****

Continuous variables expressed as mean±SD; categorical variables expressed as frequencies (percentages).
*Denotes significance in relation to healthy group, †denotes significance in relation to stable group, *†p<0.05, ††p<0.01, ***p<0.001, ****p<0.0001.
ACS, acute coronary syndrome; BMI, body mass index; CABG, coronary artery bypass graft; MI, myocardial infarction; PCI, percutaneous coronary intervention.

Table 3 Cardiovascular medication profile of patient groups

	Healthy group N=20	Stable group N=20	ACS group N=20
ACE inhibitor	0	7 (35)	9 (45)
β-blocker	0	15 (75)	14 (70)
Ca channel antagonist	0	3 (15)	3 (15)
Statin	0	16 (80)	18 (90)
Nitrates	0	3 (15)	1 (5)
Aspirin	0	20 (100)	20 (100)
Clopidogrel	0	0	20 (100)

Categorical variables expressed as frequencies (percentages).
ACS, acute coronary syndrome; Ca, calcium.

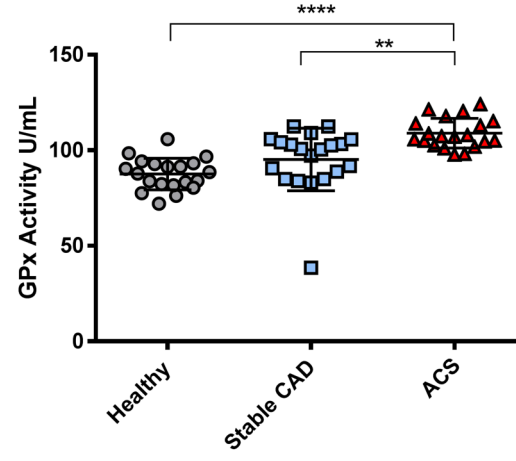

Figure 1 Levels of GPx activity. Levels of GPx activity (U/mL) were measured in healthy subjects, patients with stable CAD, and patients with ACS. Data are plotted for all 20 patients in each cohort as mean±SD, and one-way ANOVA with Tukey's multiple comparison test was used to compare differences, **p<0.01, ****p<0.0001. ACS, acute coronary syndromes; ANOVA, analysis of variance; CAD, coronary artery disease; GPx, glutathione peroxidase.

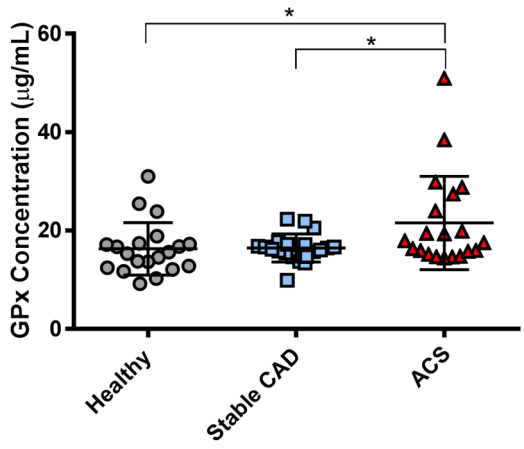

Figure 2 Levels of GPx3 protein concentration. Levels of GPx3 protein concentration (μg/mL) were measured in healthy subjects, patients with stable CAD, and patients with ACS. Data are plotted for all 20 patients in each cohort as mean±SD, and one-way ANOVA with Tukey's multiple comparison test was used to compare differences, *p<0.05. ACS, acute coronary syndromes; ANOVA, analysis of variance; CAD, coronary artery disease; GPx, glutathione peroxidase.

GPx3 protein concentrations were significantly higher in patients with ACS (21.6±9.5 μg/mL) compared with patients with stable CAD (16.5±2.8 μg/mL, p<0.05) and healthy subjects (16.3±5.3 μg/mL, p<0.05) (figure 2). No significant difference was detected between the patients with stable CAD and the healthy cohort.

GPX1, *GPX3*, and *GPX4* mRNA expressions were measured from whole blood samples collected from 19 healthy volunteers, 20 patients with stable CAD, and 20 patients with ACS. One sample from a healthy volunteer could not be used for qPCR analysis due to RNA degradation, and this sample was therefore excluded from analysis.

GPX1 mRNA expression was significantly higher in patients with ACS, with a 1.6-fold increase compared with healthy controls (p<0.0001). No difference was detected between patients with ACS and stable CAD (1.3-fold vs 1.1-fold change, respectively, p=0.12). Patients with stable CAD had a 1.4-fold increase in *GPX1* mRNA levels compared with healthy controls (p<0.01) (figure 3A).

GPX3 mRNA expression was significantly higher in patients with ACS, with a 1.9-fold increase compared with patients with stable CAD (p<0.01). Although the difference was not significant, the fold change in *GPX3*

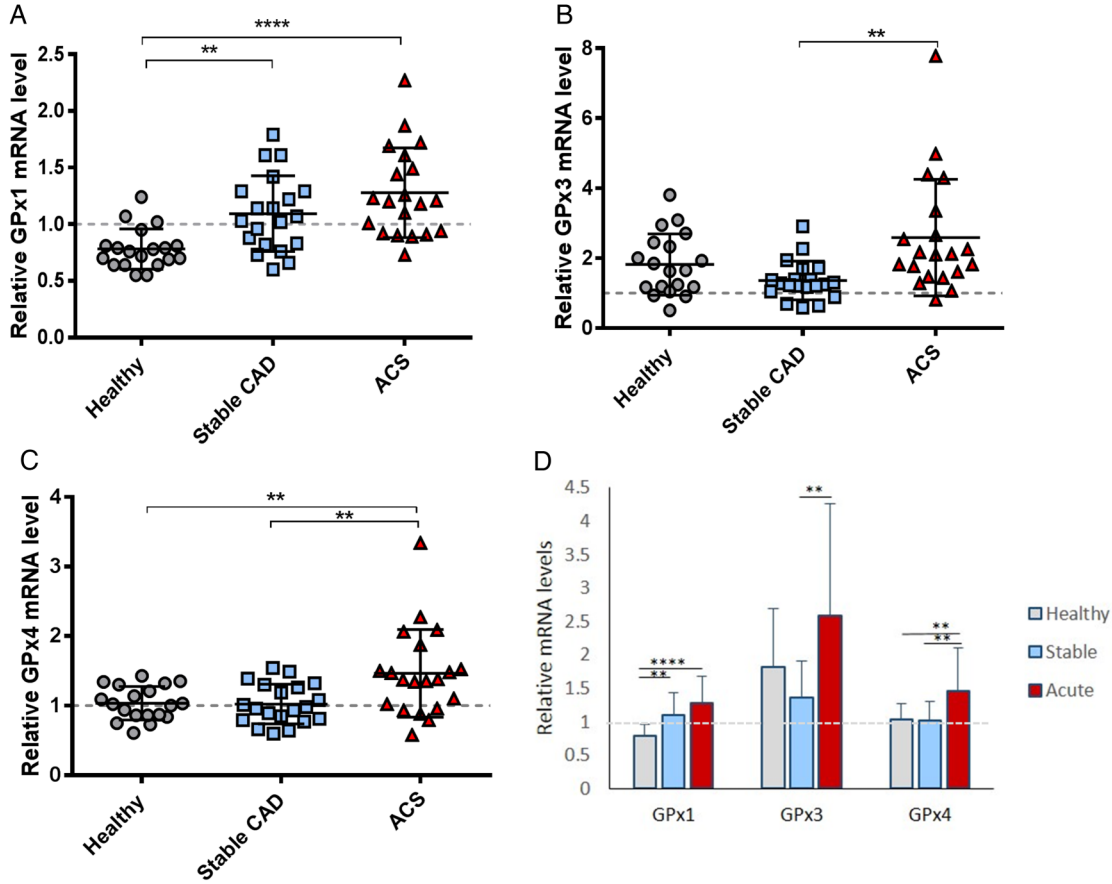

Figure 3 *GPX 1*, *3*, and *4* mRNA expression levels. Levels of *GPX1* (A), *GPX3* (B), and *GPX4* (C) mRNA expression were measured in healthy subjects, patients with stable CAD, and patients with ACS. (D) Bar graph displays all three isoforms on the same y-axis to compare the magnitude of change in each isoform for each cohort. mRNA expression is presented as arbitrary values expressed as fold change relative to *GAPDH* and normalized to the *GAPDH* calibrator control. Fold change is relative to the value 1.0. Data are plotted for 19 healthy subjects and 20 patients with stable CAD and ACS as mean±SD. One-way ANOVA with Tukey's multiple comparison test was used to compare differences, *p<0.05, **p<0.01, ****p<0.0001. ACS, acute coronary syndromes; ANOVA, analysis of variance; CAD, coronary artery disease; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

expression was numerically higher in patients with ACS compared with healthy controls (2.6-fold vs 1.8-fold change, respectively, p=0.09). No difference was detected between the stable CAD and the healthy cohorts in *GPX3* mRNA expression (1.4-fold vs 1.8-fold change, respectively, p=0.42) (figure 3B).

GPX4 mRNA expression was significantly higher in patients with ACS, with a 1.4-fold increase compared with patients with stable CAD and healthy controls (p<0.01). No significant differences were detected between the stable CAD and the healthy cohorts (1.02-fold vs 1.03-fold change, respectively, p=0.99) (figure 3C).

Oxidative stress biomarkers

OxLDL was significantly higher in the patients with ACS (61.9±22.2 U/L) compared with the stable CAD cohort (47.8±10.4 U/L, p<0.05) and healthy cohort (48.9 ±11.9 U/L, p<0.05) (figure 4). No significant difference was detected between the stable CAD and the healthy cohorts.

DISCUSSION

This study demonstrated that overall, levels of GPx mRNA, protein, and enzymatic activity were all significantly higher in patients with ACS compared with patients with stable CAD and healthy subjects. These results are consistent with the hypothesis that there is an upregulation of GPx expression in ACS leading to a higher activity level of the enzyme. Additionally, levels of oxLDL were significantly higher in patients with ACS compared with patients with stable CAD and healthy subjects. Although this study cannot determine a causative relationship between changes in oxLDL and GPx, it is plausible that the increase in GPx levels is a defense response to the acute increase in oxidative stress—demonstrated by elevated oxLDL—in the patients with ACS. This is the first study to comprehensively examine GPx activity and corresponding protein and mRNA levels in a rigorously controlled patient population, in which potential confounders of oxidative stress were minimized.

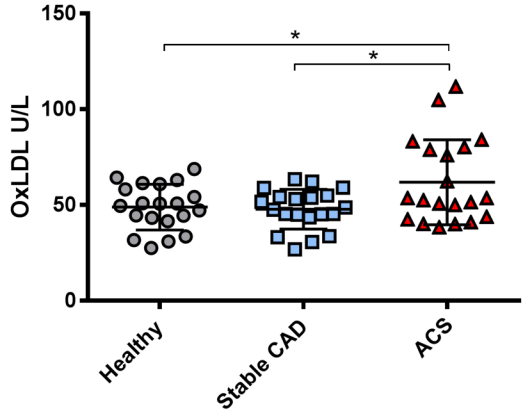

Figure 4 Levels of oxLDL. Levels of oxLDL (U/L) were measured in healthy subjects, patients with stable CAD, and patients with ACS. Data are plotted for all 20 patients in each cohort as mean ±SD, and one-way ANOVA with Tukey's multiple comparison test was used to compare differences, *p<0.05. ACS, acute coronary syndromes; ANOVA, analysis of variance; CAD, coronary artery disease; oxLDL, oxidized low-density lipoprotein.

There are previous studies examining GPx activity in cell fractions and plasma that have demonstrated increased levels in patients with ACS compared with healthy controls.[8 20 21] While some contradictory studies do exist, these have fewer than 10 patients with acute myocardial infarction per study[10 21] and the measurements were performed following reperfusion therapy.[9 22] We found no significant differences in GPx activity levels between stable CAD and healthy controls. Exclusion of confounding factors such as smoking and diabetes,[11–14] along with the medications being taken by those in the stable CAD cohort may account for differences from the prior literature that suggests GPx activity is altered in patients with stable CAD compared with healthy controls.[9]

Most of the prior literature focused on quantifying the level of GPx activity alone, and has not examined protein levels or mRNA levels. GPx3 is reported to be the predominant isoform in the plasma.[15] The present study was designed to quantify GPx3 activity levels in the plasma, using a commercially available ELISA kit (Adipogen) that provided a polyclonal antibody specific for GPx3. A significant increase in the GPx3 protein was detected in the ACS cohort, with no differences detected between the stable CAD and healthy cohorts. In addition to GPx3, GPx1 and GPx4 may be important contributors to overall GPx activity levels and were therefore included in this study. Patients with ACS demonstrated consistently higher levels of mRNA for all three of these isoforms of GPx. Taken together, these data support the notion that levels of GPx activity are regulated through an increase in mRNA and protein levels. The signaling responsible for a change in GPx mRNA levels remains largely uncharacterized. In addition to protein levels, GPx requires several secondary enzymes and cofactors[23] that influence the performance of the protein as an active enzyme.[24] The measurement of these cofactors, such as glutathione and selenium, was beyond the scope of this study, but such information would be useful for future regulatory studies of the GPx enzyme.

The increased level of oxLDL in our ACS cohort is consistent with the view that an acute coronary event represents a state of elevated oxidative stress,[2 25] with our results in line with studies indicating a positive correlation between oxLDL and the incidence of an ACS.[26] While there is evidence to suggest that levels of oxLDL and GPx activity are directly associated,[27 28] the results from our study cannot determine whether there is a causative relationship between the two factors during an ACS. Our results, however, are consistent with the view that antioxidant defense systems tend to respond to some form of acute challenge.[29] In the context of an ACS event, this challenge could encompass an increase in oxidative stress[30] and an increase in the overall proinflammatory environment associated with a myocardial infarct.[31] Although our study cannot determine the net change in redox status that is ultimately responsible for oxidative damage, it is likely that a differential antioxidant capacity exists between patients, and that those who lack the ability to upregulate GPx over a certain threshold are at an increased risk of poor clinical outcomes. This hypothesis is supported by our previous findings where patients with ACS with lower

levels of GPx activity were at an increased risk of developing cardiac events following their event.[7]

No difference in oxLDL levels were detected between the stable CAD cohort and healthy controls. While this is consistent with some previous studies that report no difference between the two groups,[32 33] it is in direct contrast with other studies that demonstrate that patients with stable CAD have elevated oxLDL levels when compared with healthy controls.[34 35] Similar to the reports with GPx activity, it is possible that such discrepancy exists due to inadequate control for cardiac risk factors known to influence levels of oxidative stress. The present study was carefully designed in order to look for differences in oxLDL between the patient populations. Rigorous controls were applied for conditions known to have high levels of oxidative stress, such as diabetes, smoking, and heart failure.[11 13 36] These conditions have been variably controlled for in previous studies, and may account for some of the discrepancies. Chronic statin therapy has been shown to reduce the level of LDL,[37 38] and may also account for the lower levels of oxLDL seen in patients with stable CAD compared with patients with ACS.

Limitations

This study was designed to examine changes in the plasma measures of GPx and the corresponding oxidative stress levels. It is possible that we were underpowered to detect some differences between the groups due to the moderate study size.

The medication profiles differed between the three study groups, with the healthy control group on no cardiovascular medications, and the stable CAD and ACS groups on guideline-recommended medical therapy for their disease states. We therefore cannot exclude the possibility that these medications may have some influence on the measured parameters.

Although we employed frequently used, commercially available kits for analytical detection of our markers, these methods still have limitations. All the methods used to assess oxLDL and GPx are indirect, and there is conflicting evidence on how well these circulating markers reflect oxidative stress in vivo.[39] Differences in analytical methodologies between laboratories make it difficult to directly compare the results obtained across different studies. Although most laboratories use methods based on the same methodological principles, the specific assay conditions with regard to concentration of reagents, recording time, and temperature differ. Sample handling is also extremely important for accurate measuring of oxidative stress. For example, if samples are not immediately centrifuged at 4°C after collection and stored appropriately, lipids have been shown to auto-oxidize.[40]

We controlled for multiple cardiovascular risk factors that may influence oxidative stress status in our three cohorts. This may partially explain why our results differ from some previous studies, but our results should be more representative of the true oxidative stress levels due to the more rigorous control. Other factors, such as the type of ACS recruited and the timing of blood samples may introduce an unknown variance to measures of GPx and oxLDL. For example, in our study, we collected blood within a mean time of 2–3 days post ACS. Another study collected blood from patients who were admitted with a history of 5 hours of chest pain. Further investigation into how GPx and oxLDL change over the course of an ACS event is required. This would potentially allow identification of an optimal sampling window.

CONCLUSION

This study demonstrated that patients with ACS experience an increase in GPx mRNA protein levels and enzymatic activity compared with stable CAD and healthy subjects, as well as an increase in levels of oxLDL. We suggest that GPx may be upregulated in response to the change in oxidative stress induced by the acute coronary vascular event. We observed no differences in GPx expression between patients with stable CAD and healthy controls.

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