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Title:

Plasma concentrations of thioredoxin, thioredoxin reductase and peroxiredoxin-4 can identify high risk patients and predict outcome in patients with acute coronary syndrome: a clinical observation.

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Contents

Abstract

Background: Oxidative stress is a pathological feature of acute coronary syndrome (ACS), a complex disease with varying clinical outcomes. Surrogate biomarkers of oxidative stress including, peroxiredoxin-2 (PRDX2), PRDX4, thioredoxin (TRX) and thioredoxin reductase (TRXR) were measured in ACS patients at presentation and follow-up, to assess their clinical utility in diagnosis and risk stratification.

Methods: Plasma from 145 participants (80 ACS and 65 healthy) at diagnosis, 1-3 month (first) and 6-month follow-up (second) was analysed by ELISA. ACS patients were monitored for 12-months.

Results: ACS patients at diagnosis had significantly higher concentrations of TRX ($p<0.05$), TRXR ($p<0.01$) and PRDX4 ($p<0.01$), compared to healthy donors. This was increase was driven by non-ST elevated myocardial infarction for TRX ($p<0.01$) and PRDX4 ($p<0.05$). For TRXR, ACS females were significantly higher than males ($p<0.05$). TRX was also higher in older females (>55 years) at diagnosis ($p<0.05$). At first follow-up, TRX had lowered, whereas PRDX4 remained significantly high ($p<0.05$). Stratification of ACS patients according to percutaneous coronary intervention (PCI) revealed that TRXR was significantly higher in patients receiving PCI to the right coronary artery ($p<0.05$). Whereas both TRXR ($p<0.01$) and PRDX4 ($p<0.01$) were significantly higher in patients receiving PCI to the left anterior descending (LAD) artery. ACS patients who had plasma TRX >13.40 ng/ml at second follow-up were at high risk of readmission ($p<0.05$), as were patients with TRXR of <1000 pg/ml at diagnosis having PCI to the LAD ($p<0.05$).

Conclusion: This study indicates that TRX, TRXR and PRDX4 may have clinical utility for ACS stratification.

Word count = 250 / 250

Keywords

ACS, NSTEMI, LAD, PRDX4, TRX, TRXR.

1. Introduction

Acute coronary syndrome (ACS) affects around 7 million individuals each year globally and refers to a group of pathologies that cause myocardial ischemia, and ultimately acute myocardial infarction (AMI) (1). There are three types of ACS, ST-segment Elevation Myocardial Infarction (STEMI), Non-ST-segment Elevation Myocardial Infarction (NSTEMI) and unstable angina, where the presence of high-sensitive cardiac troponin (hs-cTn) in the blood distinguishes an AMI ACS (2,3). Reperfusion is achieved via percutaneous coronary intervention (PCI), restoring blood supply to the myocardium at the respective culprit lesion i.e., right coronary artery (RCA), circumflex or left anterior descending (LAD) artery, and when performed within 2 hours for STEMI ACS can improve patient outcome (1). However, research indicates that LAD culprit lesions have a higher risk of long-term mortality compared with RCA (4). Moreover, for NSTEMI ACS the disease may be managed for some time before the best course of intervention is decided (5). Therefore, biomarkers that assist with diagnosis and risk stratification of ACS are warranted.

Previous research has identified a link between ACS, AMI, and oxidative stress (6–8). Oxidative stress manifests when the generation of reactive oxygen species (ROS) exceeds the antioxidant capacity of the cell, causing damage to macromolecules and organelles (9). Tissues including the myocardium produce an array of antioxidant enzymes such as peroxiredoxin (PRDX), thioredoxin (TRX) and thioredoxin reductase (TRXR), which protect against the damaging effects of ROS (10,11). Studies illustrate a protective role for TRX and TRXR in cardiomyocytes by promoting redox homeostasis (12). Expression of TRX is also noted in medial smooth muscle cells of the coronary arteries and plays a central role in regulating ROS and maintaining eNOS function (12,13). Using *in vitro* modelling it is shown that the expression of various PRDX isoforms (notably PRDX4) are increased in

cardiomyocytes following exposure to exogenous hydrogen peroxide (H_2O_2) (14). Research also illustrates that alterations in the intracellular redox environment mediates the secretion of TRX and PRDX via conventional and alternative secretion mechanisms (15), where it is suggested that this secretion may play a wider antioxidant role (16). Support for this notion comes from studies showing that secreted PRDX4 can bind to endothelial cells, which may protect these cells during periods of oxidative stress (17). Thus, extracellular TRX and PRDX may be central components in maintaining redox homeostasis during ischemia induced ROS, where crosstalk between cardiomyocytes and endothelial cells is important (18).

Previous research shows that PRDX2, PRDX4, TRX and TRXR are released into the blood during oxidative stress and may be used as surrogate biomarkers (19–21). Thus, the aim of this study was to quantify blood PRDX2, PRDX4, TRX and TRXR in patients presenting with ACS and at 1–3-month (first) and 6-month (second) follow-up and evaluate whether concentration changes of these biomarkers may be used to support diagnosis and aid ACS risk stratification.

2. Methods

2.1. Participant recruitment

This was a retrospective study of cardiac participants sponsored by Worcestershire Acute NHS hospital trust (WAHT). Participant recruitment was at the single site between July 2017 and July 2018. All participants, after gaining informed consent had a venous blood sample collected and completed a short demographic questionnaire. Diagnosis of STEMI and NSTEMI ACS patients admitted to WAHT were positive for hs-cTn conducive with standard diagnostic protocols. All ACS participants were followed-up at 1-3 months, and then at 6 months before a medical review at 12 months. On completion of the 12-month review, data was available to determine how many ACS participants had been readmitted to hospital during this period. Healthy volunteers had a single visit only.

Inclusion eligibility was designed to allow recruitment of a wide range of participants at risk of ACS. Exclusion criteria included aged <18 years, STEMI, NSTEMI or unstable angina complicated by trauma, gastrointestinal bleeding, co-morbidities with life expectancy less than 12 months, known liver or renal disease prior to admission, and inability to consent. Consenting procedures were carried out in accordance with the WAHT Standard Operation Procedure Version 1 (10/10/2016), and the declaration of Helsinki (2008). Participants were provided with information and could ask questions in line with the International Council for Harmonisation Guidelines (2016). Ethical approval for the study was given by the NHS Research Ethics Committee and Heath Research Authority, reference number 17/NS/0032.189016. All data collected was anonymised and treated in the strictest of confidence, in accordance with the Data Protection Act (2018). Table 1 presents participant characteristics.

2.2. Sample size calculation

Using previous data obtained by our group from a similar study evaluating plasma changes of TRX, TRXR, PRDX2 and PRDX4 in response to exercise (21), we observed that these biomarkers collectively had a standardised difference of 0.59, based on a target difference of 0.49 and standard deviation of 0.83. Therefore, a target sample size of 120 participants was required for $p<0.05$ at a power of 0.9 (22). The sample size selected also accounted for statistical uncertainties in the case of unequal sized groups should this have been the case at the end of the study (23). Participant recruitment exceeded this target at 145.

143 **Table 1: Participant Characteristics.**

Characteristic	Healthy Cohort (N = 65)	ACS Cohort (N = 80)
Age range — yr.	18 to 85	44 to 89
Male — N. (%)	32 (49%)	59 (74%)
Female — N. (%)	33 (51%)	21 (26%)
Body-mass index†*	27.2 ± 4.0	27.9 ± 4.5
Family history of CAD — N. (%)	19 (29%)	32 (40%)
Arterial hypertension — N. (%)	10 (15%)	41 (52%)
Diabetes mellitus – N. (%)	12 (19%)	23 (29%)
Fatality – N. (%)	0 (0%)	4 (5%)
Lipid Profile		
Total Cholesterol >4.0 mmol/L – N. (%)	~	41 (51%)
Total Cholesterol mmol/L* – N. (%)	~	4.33 ± 1.12
LDL* – N. (%)	~	2.48 ± 1.01
HDL* – N. (%)	~	1.21 ± 0.37
Smoking		
Previous – N. (%)	16 (25%)	23 (29%)
Current – N. (%)	12 (18%)	26 (32%)
Never – N. (%)	35 (54%)	31 (39%)
Unknown – N. (%)	2 (3%)	0 (0%)
Myocardial infarction		
Unstable angina — N. (%)	~	7 (9%)
NSTEMI — N. (%)	~	41 (51%)
STEMI — N. (%)	~	32 (40%)
Percutaneous coronary intervention		
Right Coronary Artery (RCA) – N. (%)	~	39 (49%)
Circumflex – N. (%)	~	11 (14%)
Left Anterior Descending (LAD) – N. (%)	~	30 (37%)

***** Values are means ± SD. Data on race/ethnic group are not reported due to low frequency.
† Body-mass index (BMI) is the weight in kilograms divided by the square of the height in meters.
~ Healthy Cohort was excluded from general laboratory ACS / CVD diagnostic tests.

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2.3. Blood sampling and processing

Non-fasted blood samples were taken at screening and scheduled visits at 1-3 months (first follow-up) and 6 months (second follow-up). A total of 4 ml venous blood was drawn into a Becton Dickinson EDTA vacutainer (BD, Crawley, UK). Samples were immediately homogenised by inversion and left to stand for 30 minutes. Blood samples were centrifuged at 500 x g for 5 min using a Kestrel MSE1191 centrifuge (MSE, East Sussex, UK), following which the plasma was transferred to a fresh sterile tube and centrifuged again at 2,000 x g for 15 minutes to deplete platelets and other contaminating cellular material. The plasma was subsequently stored at -80^{o}C until the analysis by ELISA was carried out.

2.4. TRX, TRXR, PRDX2 and PRDX4 analysis by quantitative ELISA

The plasma concentration of each biomarker was determined from an ELISA standard curve using the respective recombinant protein diluted in phosphate buffered saline (PBS). The recombinant proteins were sourced from AbCam (Cambridge, UK) and included TRX (ab51064), TRXR (ab168011), PRDX2 (ab85331) and PRDX4 (ab93947). All primary and secondary antibodies used in the ELISA were sourced from AbCam (Cambridge, UK). The primary antibodies used were rabbit monoclonal IgG anti-TRX (ab133524), rabbit monoclonal IgG anti-TRXR (ab124954), rabbit monoclonal IgG anti-PRDX2 (ab109367) and rabbit monoclonal IgG anti-PRDX4 (ab59542). The secondary antibody used was goat anti-rabbit IgG biotin-conjugate (ab207995). A 96-well Greiner ELISA plate (M6562, Sigma-Aldrich, Dorset, UK) was loaded with 100 µl of the standard curve in duplicate and 100 µl of participant plasma in duplicate and incubated overnight at 4^{O}C to ensure binding. Each well was washed x 3 in 200 µl PBS adjusted to contain 0.1% w/v casein and 0.05% v/v Tween-20

(PBSTwC). 200 µl of block solution (PBS adjusted to contain 1% w/v casein) was added to each well and incubated for 30 minutes at room temperature (RT). Following this, each well was washed x 3 in 200 µl PBSTwC before the respective titre of primary antibody was added diluted in PBSTwC and incubated for 1 hour at RT. This was repeated for the secondary antibody. Finally, 200 µl of streptavidin-HRP (SLS, Nottingham, UK) diluted 1:8000 in PBSTwC was added to each well and incubated for 1 hour at RT, before the addition of 100 µl 3,3',5,5' tetramethylbenzidine (TMB) solution (1 TMB tablet; Fisher Scientific, Loughborough, UK., plus 1 Phosphate Citrate Buffer tablet; Sigma-Aldrich in 100 ml dH_2O). The plates were protected from light and incubated for 30 - 45 minutes at RT with gentle agitation. The reaction was stopped by the addition of 50 µl 1.5M sulphuric acid. The ELISA plate absorbances were measured at λ450 nm using a Multiskan Ascent ELISA plate reader (Thermo-Labsystems, Cheshire, UK). For the ELISA the intra-assay CV ranged from 4.02% to 9.37%, with the inter-assay CV 6.08%. For individual duplicates the CV ranged from 0.03% to 18.86%, which is within acceptable limits according to the literature (24,25).

2.5. Statistical methods

Following a Shapiro-Wilk test for normality, a one-tailed unpaired t-test was used to analyse ACS patient plasma concentration relative to the healthy donor cohort. One-way ANOVA with Tukey's multiple comparison test was used to evaluate ACS type (STEMI, NSTEMI and unstable angina), ACS patient concentrations at point-of-diagnosis and follow-up, and ACS patient stratified according to PCI (RCA, circumflex, or LAD) relative to healthy controls. A two-tailed paired t-test was used to compare ACS patients at point-of-diagnosis with first and second follow-up respectively. Two-way ANOVA, with Bonferroni multiple-comparison test was used to evaluate ACS patients and healthy donors stratified according

to age (≤55 years or >55 years), and sex (males and females). Logistic regression analysis was used for each biomarker at various cut-off concentrations to compare true positives (specificity) with the false positive rate (1-specificity). Log-rank Mantel-Cox test was used to predict ACS readmissions. Refer to the supplemental material for raw data. Statistical significance was regarded where $p<0.05$. All analysis was performed using GrapPad Prism v10 (Dotmatics; Boston, MA, USA).

3. Results

3.1. Evaluation of TRX, TRXR, PRDX2 and PRDX4 between ACS and healthy donors

Initially, plasma TRX, TRXR, PRDX2 and PRDX4 concentration was measured for ACS patients at admission (n=80) and compared with the healthy cohort (n=65). The data presented in figure 1 shows that the average concentrations of TRX (12.37 SEM 0.95 ng/ml; $p<0.05$), TRXR (1.14 SEM 0.16 ng/ml; $p<0.01$) and PRDX4 (22.16 SEM 1.44 ng/ml; $p<0.01$) were significantly higher for ACS patients, compared with the healthy cohort (TRX: 10.06 SEM 0.64 ng/ml, TRXR: 0.56 SEM 0.14 ng/ml, PRDX4: 15.54 SEM 1.52 ng/ml). Stratification of the ACS patients into 'unstable angina' (n=7), 'NSTEMI' (n=41) and 'STEMI' (n=32) revealed that the average plasma TRX concentration was significantly higher for NSTEMI ACS patients (14.73 SEM 1.63 ng/ml), compared with healthy donors (10.06 SEM 0.64 ng/ml; $p<0.01$), unstable angina ACS (9.94 SEM 0.70 ng/ml; $p<0.01$) or STEMI ACS (9.88 SEM 0.91 ng/ml; $p<0.05$) see figure 1(e). For PRDX4, the average plasma concentration was higher for NSTEMI ACS patients (22.82 SEM 1.88 ng/ml) compared with the healthy cohort (15.54 SEM 1.52 ng/ml; $p<0.05$) see figure 1(h). Receiver operator curve (ROC) analysis based on NSTEMI ACS patients at admission and healthy donor controls illustrate clinical utility for plasma TRX (AUC=0.69; p=0.0009), TRXR (AUC=0.65; p=0.008) and PRDX4 (AUC=0.69; p=0.001) see figure 1(i), figure 1(j), and figure 1(l) respectively. No significant differences were detected between smokers and non-smokers (supplemental figure S1). Taken together, the data indicate that ACS patients have increased plasma TRX, TRXR and PRDX4 concentrations at admission. Stratification of the data reveal that NSTEMI ACS mediate the increase observed for TRX and PRDX4, with plasma TRX, TRXR and PRDX4 displaying clinical utility for NSTEMI ACS diagnosis.

Given the findings presented in figure 1, we next evaluated whether there were any differences in plasma concentrations between males and females according to age i.e., ≤55 years or >55 years. This analysis was important given that oxidative stress differs with age and sex (26,27). The data presented in figure 2(b) shows that ≤55 ACS females (n=3) had a significantly higher average plasma TRX concentration compared with ≤55 ACS males (n=13); 27.71 SEM 9.88 ng/ml vs 8.74 SEM 1.08 ng/ml ($p<0.05$). The data also reveal that as a whole ACS females (n=22) have significantly higher plasma TRXR concentration compared with the healthy female cohort (n=33); 1.72 SEM 0.08 ng/ml vs 0.61 SEM 0.03 ng/ml ($p<0.05$) see figure 2(c). Although similar trends were noted for the other biomarkers, the data did not reach significance. Taken together, these data indicate that plasma TRX and TRXR may be useful for differentiating ACS female subjects.

3.2. Evaluation of TRX, TRXR, PRDX2 and PRDX4 at admission and follow-up

Analysis was next performed to evaluate plasma concentrations for ACS patients at first follow-up (1-3 month following) and second follow-up (6 month following). Data presented in figure 3 shows that there was a significant reduction in ACS patient plasma TRX concentration at first follow-up (10.29 SEM 0.72 ng/ml) compared with diagnosis (12.37 ± 0.95 ng/ml; $p<0.05$), with no significant difference detected between diagnosis and second follow-up, see figure 3(a). The average PRDX4 concentration for ACS patients was significantly higher at first follow-up (22.49 SEM 1.62 ng/ml), compared with healthy donor controls (15.54 SEM 1.52 ng/ml; $p<0.05$) see figure 3(d). No other significant changes in plasma concentrations were noted. 'Follow-up' refers to time-dependent changes with no intervention beyond standard care.

3.3. Evaluation of TRX, TRXR, PRDX2 and PRDX4 according to PCI

We next evaluated whether there were any differences in plasma TRX, TRXR, PRDX2 and PRDX4 concentration for AMI ACS patients at diagnosis, stratified according to percutaneous coronary intervention (PCI). Here ACS patients were grouped as PCI to right coronary artery (RCA, n=39), circumflex (n=11) or left anterior descending artery (LAD, n=30). The data presented in figure 4(c) show that the average plasma TRXR concentration was significantly higher for patients receiving PCI to RCA (1.21 SEM 0.39 ng/ml; $p<0.05$) and LAD (1.59 SEM 0.26 ng/ml; $p<0.01$) compared with the healthy cohort (0.56 SEM 0.14 ng/ml), with no difference for circumflex ACS patients (0.81 SEM 0.29 ng/ml) and the healthy cohort. Since the blood samples were acquired at admission ROC analysis displays clinical utility for TRXR measurement in diagnosing an RCA ACS (AUC=0.701; p=0.016) and LAD ACS (AUC=0.699; p=0.0004) respectively see figure 4(d). The data presented in figure 4(g) shows that plasma PRDX4 was also significantly elevated in ACS patients receiving PCI to LAD (21.57 SEM 1.87 ng/ml, $p<0.01$) compared with the healthy cohort (15.54 SEM 1.52 ng/ml), with ROC analysis illustrating the clinical utility for measuring PRDX4 in diagnosing LAD ACS (AUC=0.647; p=0.009) see figure 4(h). PRDX4 was also elevated for ACS patients that received PCI to circumflex, however this data did not reach significance. Taken together, the data presented show that ACS patient TRXR and PRDX4 concentration at diagnosis is significantly elevated in patients receiving PCI to LAD. Plasma TRXR concentration was also significantly elevated in ACS patients receiving PCI to RCA.

3.4. Evaluation of TRX, TRXR and PRDX4 in predicting ACS readmission

Finally, assessment of whether plasma TRX, TRXR or PRDX4 concentration could predict ACS readmission was evaluated. PRDX2 was not included since previous analysis

suggested there was no clinical utility (figures 1-4). ACS patients were stratified according to the 'lower-quartile', 'inter-quartile range' and 'upper-quartile' concentration for TRX, TRXR and PRDX4 respectively. The analysis was performed on the admission blood sample, as well as the first (1-3 month) and second follow-up (6 month) blood samples. The data presented in figure 5 reveals that at second follow-up, approximately 50% of ACS patients with blood plasma TRX levels >13.40 ng/ml (upper-quartile, n=13) were readmitted with a secondary ACS event within 12-months ($p=0.0499$) compared with 0% of ACS patients with a blood plasma of <8.425 ng/ml (lower-quartile, n=8) see figure 5(d). Data also revealed that at first follow-up, approximately 55% ACS patients with blood plasma PRDX4 levels >31.93 ng/ml (upper-quartile, n=10) were readmitted with a secondary ACS event within 12-months, compared with approximately 25% of ACS patients with blood plasma <10.33 ng/ml (lower-quartile, n=7), however this was not statistically significant (supplemental figure S2). No other trends in the data were noted.

Given the previous observation that plasma TRXR and PRDX4 at ACS admission was differentially elevated according to PCI type, most notably LAD (figure 4), readmission predictive analysis was subsequently performed for ACS patients who received PCI to LAD. Due to low frequency of events, this analysis was performed on the admission blood sample only. The data presented shows that for TRXR, approximately 60% of ACS patients in the low-quartile concentration (<1,000 pg/ml, n=14) who received PCI to LAD were readmitted with a secondary ACS event within 12-months ($p=0.0443$), compared with approximately 25% of ACS patients in the upper-quartile concentration (>2.00 ng/ml, n=13) see figure 5(h). A similar trend was observed for plasma TRX; however, these data did not reach significance see figure 5(g). There were no significant predictions or trends for either biomarker noted

for ACS patients who received PCI to RCA (supplemental figure S3). Patients who received PCI to circumflex could not be analysed in this way due to the low frequency of events (n=11). Taken together, these data show that elevated TRX concentration at second follow-up is associated with an increased risk of ACS readmission. Whereas ACS patients who receive a PCI to LAD based on a plasma TRXR concentration of <1,000 pg/ml on admission have an increased readmission risk.

4. Discussion

To the best of our knowledge this study is the first to collectively assess changes in these specific plasma biomarker concentrations for ACS patients at admission, follow-up, and following stratification into ACS type, age, sex, and PCI.

The data presented in figure 1 shows that the average plasma concentration of TRX, TRXR and PRDX4 was significantly increased in ACS patients compared with the healthy cohort. Only a few studies have investigated these specific biomarkers in ACS. However, a TRX study involving 176 cardiac arrest syndrome ICU patients found similar trends (28). The authors reported median plasma TRX concentration of 10.7 ng/ml for healthy volunteers, compared with 22.0 ng/ml for ICU survivors (28), which is comparable to our findings. Whilst the cause of cardiac arrest syndrome was not defined (17), pathophysiology nonetheless is mediated by ischaemia and subsequent oxidative stress (29), which also underlines ACS pathogenesis (30). Since TRX is upregulated during oxidative stress (31), it is plausible that the increase in plasma TRX observed in our ACS cohort may be explained by a common mechanism, whereby myocardial injury mediates TRX release into the plasma along with other biomarkers, for example hs-cTn (32).

Elevation in PDRX4 is also linked to oxidative stress and ACS pathophysiology and was previously identified as a risk factor (33). However, our data show for the first time that the elevation in PRDX4 [and TRX] is likely driven by NSTEMI (figure 1). Moreover, our ROC analyses identify clinical utility for measuring plasma PRDX4 and TRX for NSTEMI diagnosis (AUC 0.69 for both, $p<0.001$). Since ACS includes STEMI, NSTEMI and unstable angina, tests that highlight a risk of NSTEMI at early clinical presentation may be advantageous, particularly considering recent guidelines from the European Society of Cardiology regarding

NSTEMI risk stratification (34). Recent studies are now confirming an associated risk between oxidative stress and NSTEMI (35), where co-morbidities such as renal disease exacerbate prognosis (36). ACS patients with renal disease were excluded from our study, therefore we are not able to comment on NSTEMI patients with renal disease in the context of the data presented herein, which is a limitation. Nevertheless, to our knowledge we are the first to show raised TRXR in ACS compared to healthy subjects (figure 1). Moreover, it was noted that following sex stratification, this increase in TRXR may be polarised by the female cohort who had significantly higher concentrations compared to males (Figure 2). Our data also indicate significantly higher levels of TRX in younger females (≤55 years) presenting with ACS, compared with younger ACS males (Figure 2). However, we treat this finding with caution due to the low frequency of young ACS females. Nonetheless, given that the incidence of younger females diagnosed with ACS has increased markedly over the past 20 years (37), measuring TRX at clinical presentation as our data indicate may help avoid misdiagnosis in this cohort, which has been challenging (37,38).

A unique aspect of this study was the ACS patient follow-up. Monitoring patients at 1-3 months (first follow-up) and 6 months (second follow-up), revealed that mean TRX levels decreased at first follow-up. We speculate that this may be explained by successful reperfusion, where TRX levels have been shown to become reduced in as little as 12-hours post-PCI and remain reduced for up to 1-month following PCI (39) (figure 3). Our findings may therefore present TRX as a possible biomarker for evaluating successful and sustained reperfusion. Moreover, stratification of ACS patients based on TRX concentration (figure 5), shows that ACS patients with low plasma TRX (<8.425 ng/ml) at second follow-up have a significantly reduced risk of ACS readmission compared with patients whose TRX remained

high (>13.40 ng/ml). The raised TRX levels at second follow-up could therefore indicate reemergence of an oxidative stress, thus measuring TRX at follow-up may help identify high risk patients who are experiencing certain conditions which may lead to a secondary ACS event such as 'silent ischemia' (40). Recent European guidelines based on minimising the risk of secondary ACS following PCI highlight a need to improve and standardise follow-up care, particularly the 1 to 6-month period following the initial event (41). Thus, monitoring plasma TRX during this period may be worth consideration.

Previous research indicates that LAD culprit lesions carry a worse prognosis, with patients having an increased risk of heart failure or mortality occurring within 1 year following PCI (4). Based on our findings, we were interested to investigate changes in TRX, TRXR and PRDX4 following RCA, circumflex, and LAD PCI stratification. Our analysis shows that TRXR was significantly increased in RCA and LAD ACS patients at first admission, with PRDX4 significantly raised in LAD patients (figure 4). The ROC analysis identifies utility for measuring plasma TRXR (AUC 0.70, $p<0.001$) and PRDX4 (AUC 0.65, $p<0.01$) at ACS admission for LAD diagnosis. Moreover, stratification of ACS patients based on low (<1,000 pg/ml), medium and high (>2.00 ng/ml) plasma TRXR at diagnosis shows that LAD patients with low TRXR have a significantly increase chance of readmission due to a second ACS event ($p<0.05$, figure 5). Several studies using various models note that a reduction or inhibition of TRXR leads to oxidative stress and cellular death (42–44). It is noted that in the myocardium, TRXR acts the final enzymatic step in cellular antioxidant redox reactions that involve PRDX and TRX, requiring NADPH to complete the catalytic cycle (45). Therefore, it appears that TRXR may act as a 'bottleneck', whereby ACS patients who express low levels post-PCI would be subjected to increased oxidative stress over time. This may in turn

impact negatively on readmission rates, particularly for ACS patients who received PCI to LAD. Given the negative prognosis associated with AMI due to LAD culprit lesions, evaluating plasma TRXR at diagnosis may therefore be clinically relevant for risk stratifying patients, and could be a useful addition to the GRACE and CRUSADE scoring systems (46).

5. Limitation

There are some limitations identified in the present study. First, PRDX exists as homodimers which oligomerise into a functional decamers under reduced conditions (47). Subsequent oxidation loosens the PRDX decamer structure favouring dissociation back into dimers (47). These effects are observed in preclinical studies examining the impact of oxidative stress in cardiac tissue, where PRDX oxidised dimers and over-oxidised (sulphinated or sulphonated) monomers are detected (48). Furthermore, *in vitro* studies show PRDX4 oligomerisation and mixed disulphide formation in epithelial cells (49). Overoxidation analysis and detection of oligomeric states was not conducted in this study. Knowledge of this would provide a more rounded picture, where detection of overoxidised isomers may indicate a more severe or prolonged oxidative stress, which may impact on ACS patient outcome.

Second, it is noted that oxidative stress may promote the degradation of PRDX. For example, several PRDX isoforms were found to be degraded via proteasome and autophagy pathways in MDA-MB-231 cells *in vitro* following 1 h exposure to exogenous H_2O_2, with new proteins formed during the recovery phase (50). Recently, preclinical models showed a link between oxidative stress and PRDX4 degradation in aged myocardial cells (51). These observations extend beyond PRDX, where *in vitro* studies using HUVEC endothelial cells showed that H_2O_2 mediated TRX degradation (52). Whilst preclinical and *in vitro* studies

have demonstrated biochemical and molecular intracellular heterogeneity, there is a gap in the literature exploring the oxidation and degradation state of secreted PRDX and TRX, particularly in the context of ACS. Therefore, further research to elucidate a comprehensive picture of this diverse landscape with respect to patient outcome and disease stratification in ACS is warranted.

Third, this study was limited by not being an 'event driven' study. Whilst our power calculation provided statistical significance for the holistic analyses, when ACS patents were stratified for the more specific analyses, the frequency of events decreased dramatically, and whilst trends in the data were observed, statistical significance was not always achieved.

6. Conclusions

In conclusion, this study shows that the mean plasma concentrations of TRX, TRXR and PRDX4 were elevated in ACS, compared with healthy subjects. For TRX and PRDX4, this elevation was likely due to NSTEMI, whereas for TRXR this elevation was likely due to female subjects. Moreover, TRXR appeared at higher concentrations for younger ACS females, offering a possible biomarker for future diagnosis. Our findings illustrate that mean TRX concentration was lower at first follow-up, which may reflect successful reperfusion. Moreover, high TRX at second follow-up was associated with a greater risk of a second ACS admission. Finally, ACS stratification based on PCI shows that LAD ACS patients with low TRXR at diagnosis was a risk factor for ACS readmission within 12 months. Taken together, this study reveals clinical utility for TRX, TRXR and PRDX4 in diagnosis and risk stratification of ACS.

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Author contributions

Conceptualisation: AD, AJW and SJC. Acquisition of data: AD. Methodology: AD, GK and SJC. Data curation: AD. Analysis and interpretation of data: AD, BM and SJC. Drafting of original manuscript: AD and SJC. Revising: GK, AJW, BM and AAB. Supervision: SJC. All authors approved the final version submitted. Artificial Intelligence was not used in any aspect of the study, writing or otherwise.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. Analysis of plasma TRX, TRXR, PRDX2 and PRDX4 in healthy volunteers and ACS patients. Comparison of average blood plasma concentration for a) TRX, b) TRXR, c) PRDX2 and d) PRDX4, between healthy volunteers (n=64) and ACS patients (n=78). Data analysed by one-tailed unpaired t-test. Comparison of average plasma concentration for ACS patients separated by type to include unstable angina (n=5), NSTEMI (n=41) and STEMI (n=32) for TRX (d), TRXR (e), PRDX2 (f) and PRDX4 (g). Data analysed by one-way ANOVA. Logistic regression analysis was performed on each biomarker for NSTEMI ACS patients and corresponding receiver operator curves are presented for TRX (h), TRXR (i), PRDX2 (j) and PRDX4 (k), with area under the curve (AUC) and p values denoted. Statistical significance on bar charts is reported at *p<0.05 and **p<0.01. Data presented are mean +/- SEM.

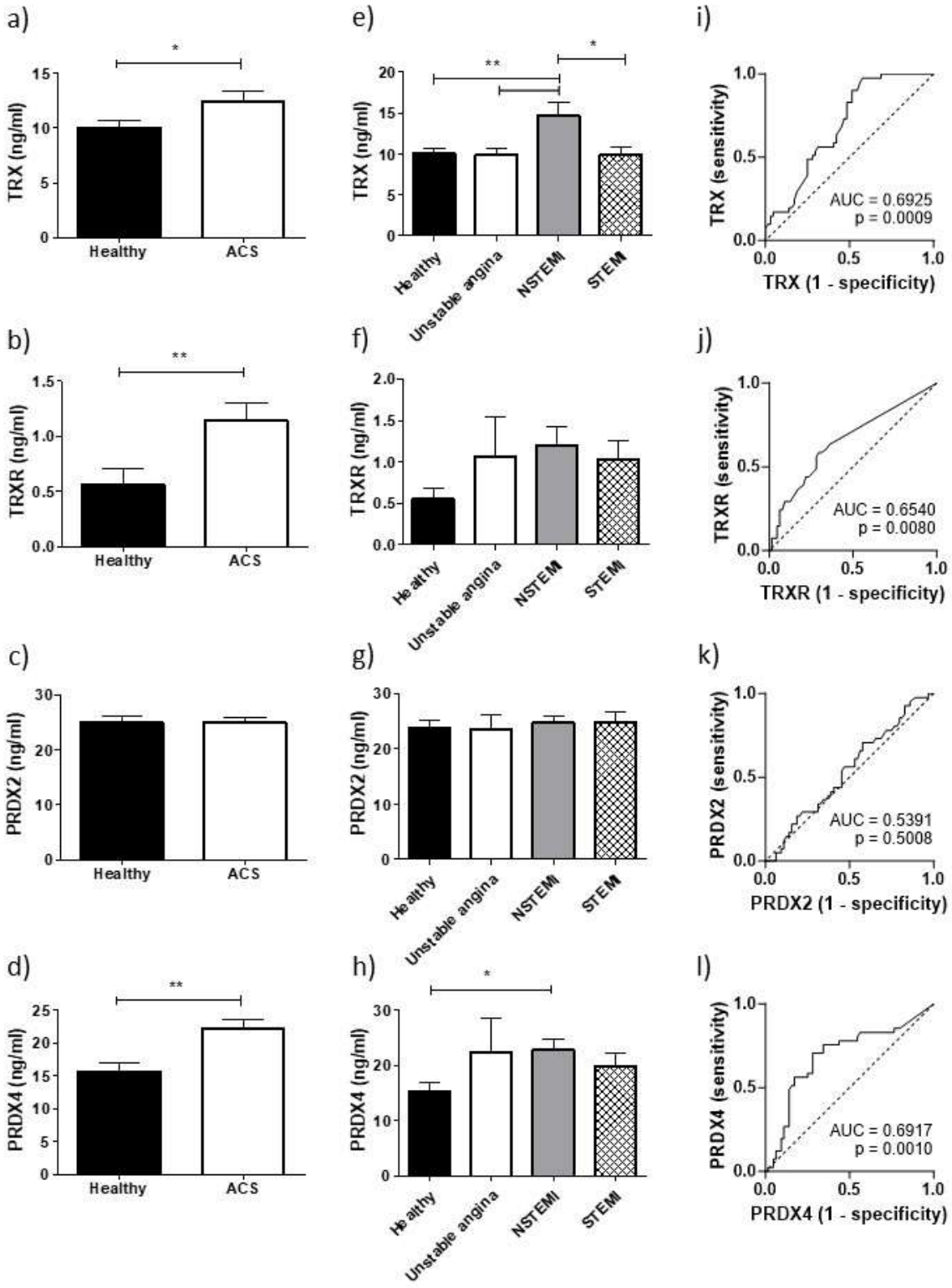
Figure 2. Comparison of plasma TRX, TRXR, PRDX2 and PRDX4 in healthy volunteers and ACS patients for males and females by age. Bar charts illustrating average blood plasma concertation for males (black bars) and females (white bars) for healthy volunteers and ACS patients. The data presented are for a) TRX for all age groups including healthy male (n=32), ACS male (n=58), healthy female (n=33) and ACS female (n=22), b) TRX for <55 year olds vs >55 year olds. c) TRXR for all age groups and d) TRX for <55 year olds and >55 year olds. e) PRDX2 for all age groups and f) PRDX2 for <55 year olds and >55 year olds. g) PRDX4 for all age groups and h) PRDX4 for <55 year olds and >55 year olds. Data analysed by two-way ANOVA and presented as mean +/- SD. Statistical significance is reported at *p<0.05.

Figure 3. Evaluation of plasma TRX, TRXR, PRDX2 and PRDX4 in healthy volunteers and ACS patients at diagnosis and follow-up. Bar charts display average plasma concentration for healthy volunteers and ACS patients at diagnosis, with corresponding 1st and 2nd follow-up concentrations for a) TRX, b) TRXR, c) PRDX2 and d) PRDX4. Follow-up refers to time-dependent changes with no intervention beyond standard care. Data presented are mean +/- SEM and analysed by one-way ANOVA (*p<0.05 and **p<0.01). Data analysed by two-tailed paired t-test ($^{\#}$p<0.05).

Figure 4. Evaluation of plasma TRX, TRXR, PRDX2 and PRDX4 in ACS stratified according to PCI. Bar charts display average plasma concentration for ACS patients stratified according to PCI for a) TRX, c) TRXR, e) PRDX2 and g) PRDX4. Data presented are mean +/- SEM and analysed by one-way ANOVA (*p<0.05, **p<0.01 and ***p<0.001). The horizontal solid line on each bar chart represents the corresponding average concentration for healthy volunteers, with the SEM as a dotted line. Logistic regression analysis was performed on each biomarker for ACS patients stratified according to PCI and corresponding receiver operator curves are presented for TRX (b), TRXR (d), PRDX2 (f) and PRDX4 (h), with area under the curve (AUC) and p values denoted. PCI to RCA (sold line), PCI to LAD (fine dotted line) and PCI to circumflex (thick dotted line).

Figure 5. Impact of blood plasma TRX, TRXR and PRDX4 concertation on readmission rate for ACS patients at diagnosis, second follow-up and LAD PCI stratification. To evaluate the impact of plasma concertation with respect to a secondary ACS readmission, patients were stratified according to their plasma concentration i.e., upper quartile (solid line), interquartile range (fine dotted line) and lower quartile (thick dotted line) for TRX, TRXR and PRDX4. Plots display the time to readmission (days) due to a secondary ACS event based on the admission blood sample for TRX (a), TRXR (b) and PRDX4 (c), second follow-up blood sample for TRX (d), TRXR (e) and PRDX4 (f) and ACS patients who received PCI to LAD for TRX (g), TRXR (h) and PRDX4 (i). Significant p values are denoted. Data analysed by log-rank Mantel-Cox test.

669 Fig 1



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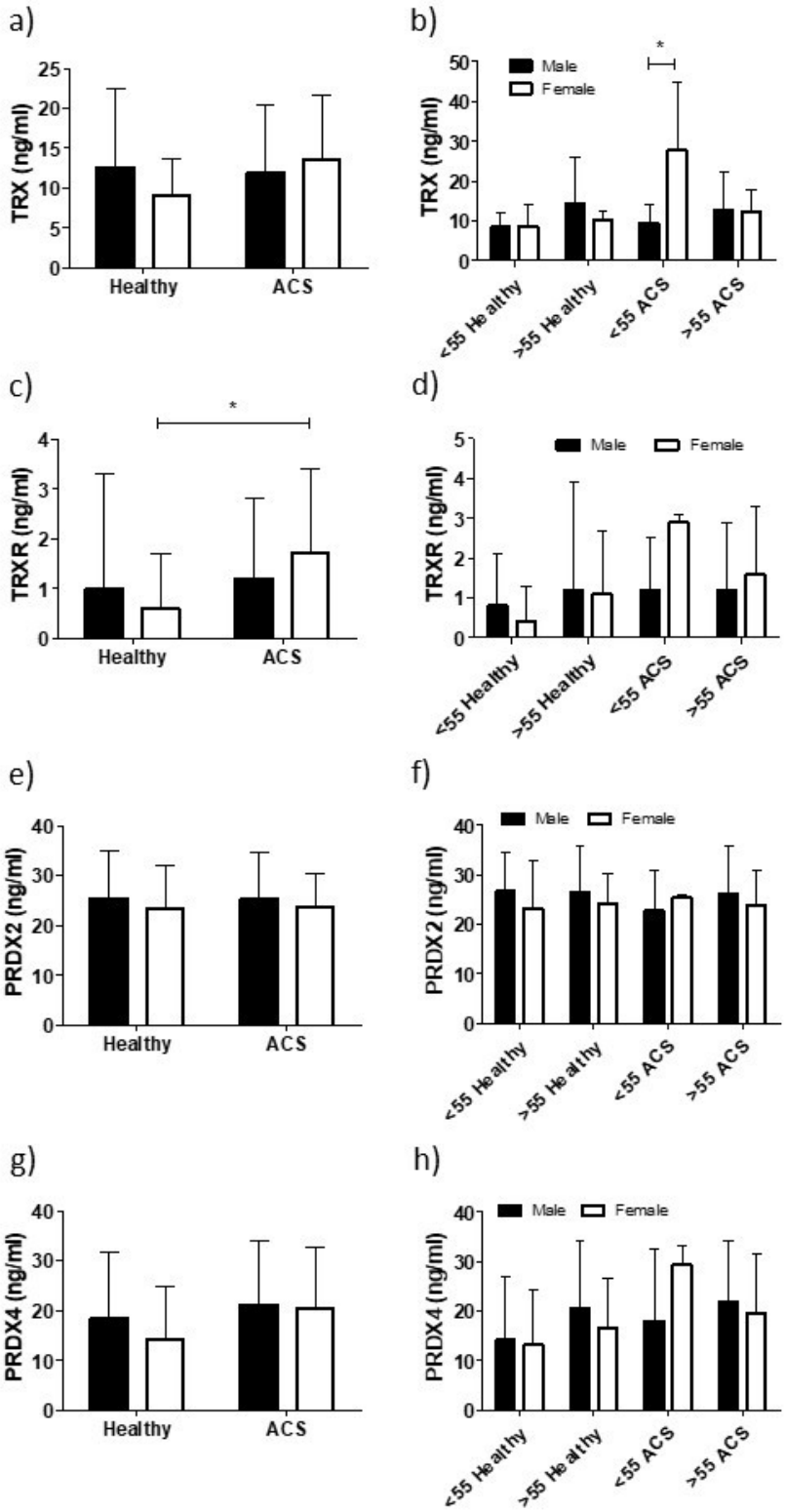
672 Fig 2



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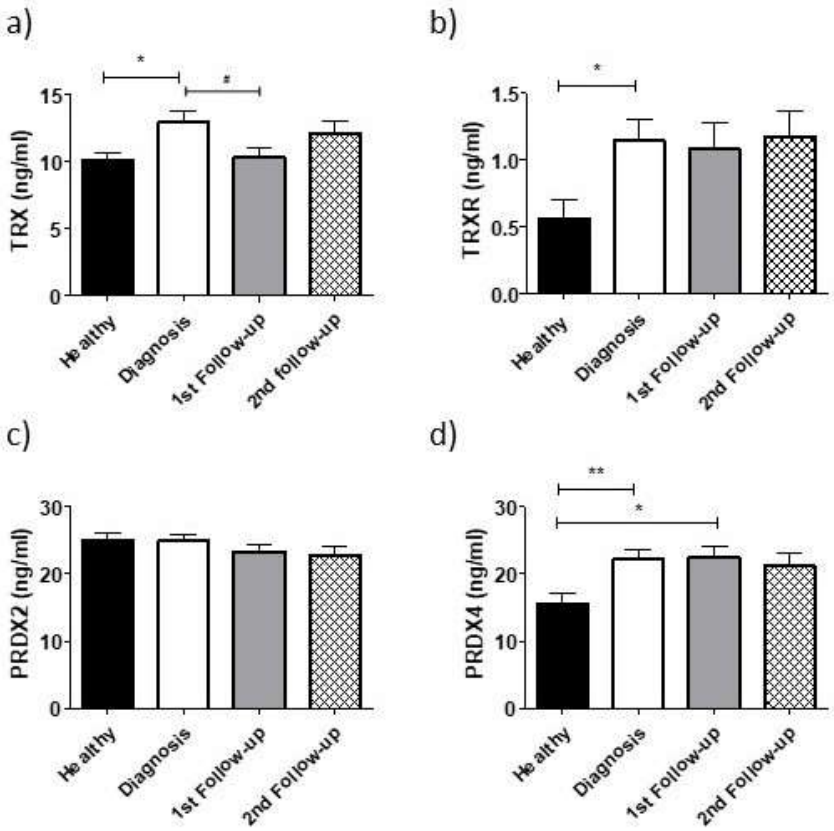
675 Fig 3



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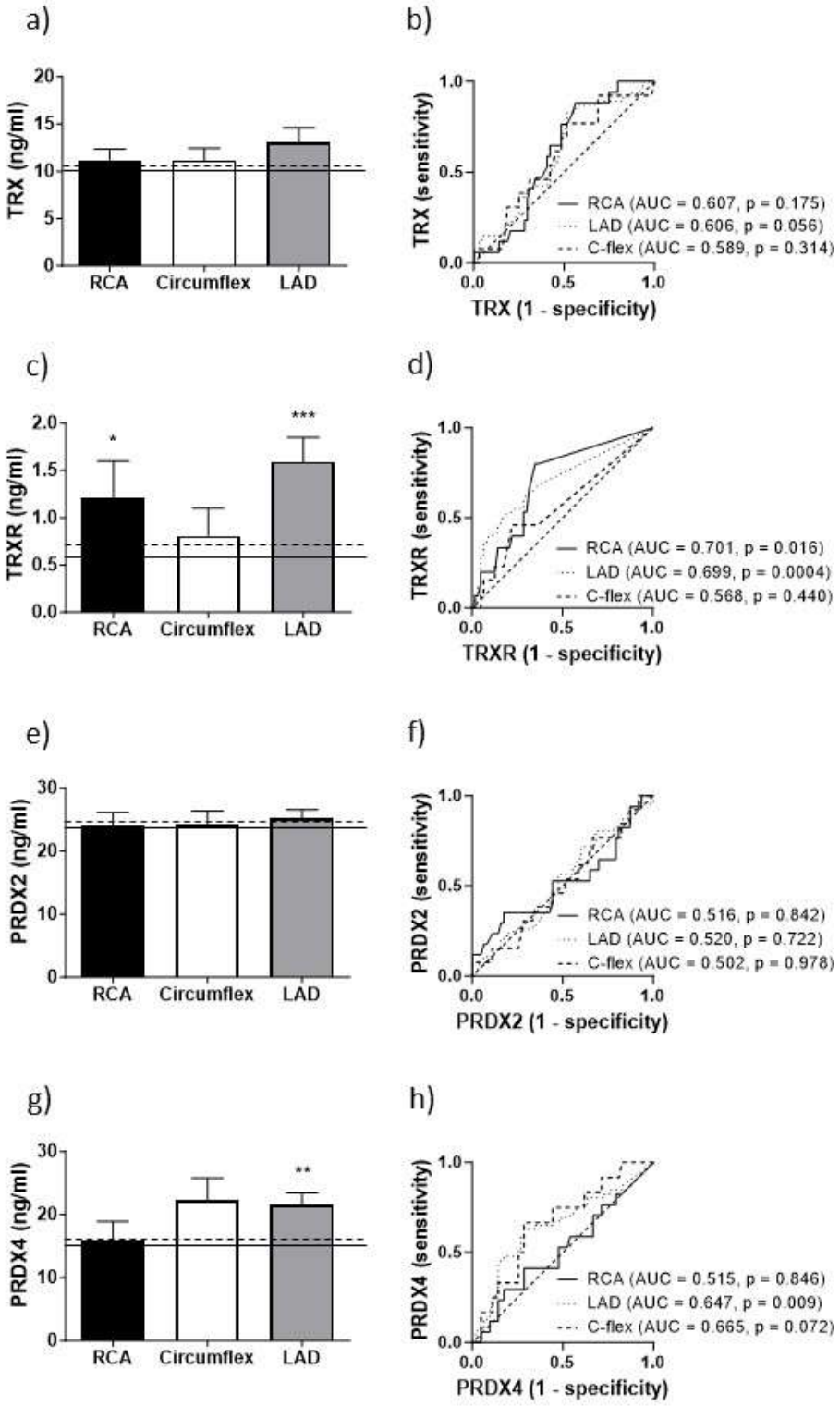
678 Fig 4



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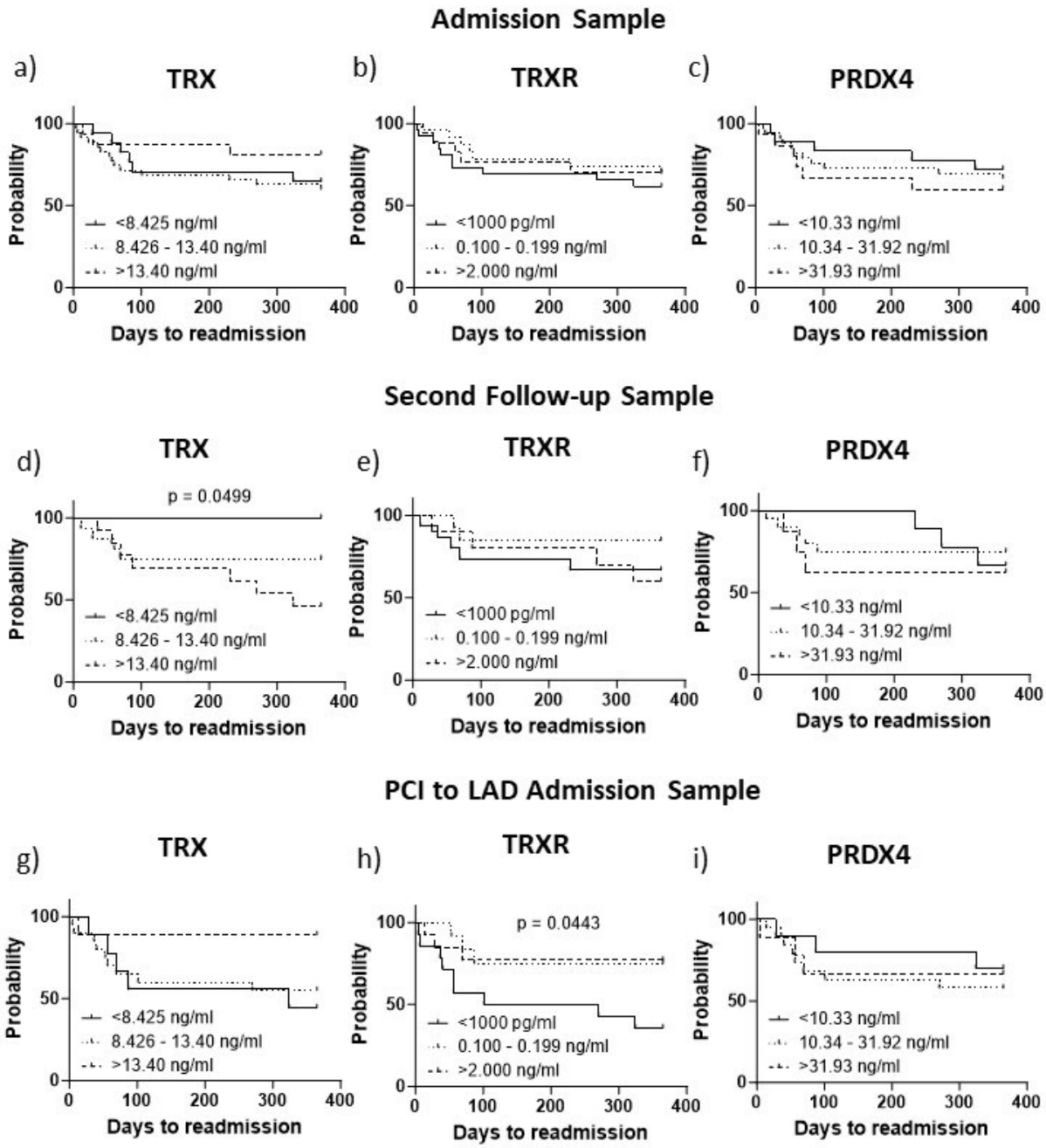
681 Fig 5


Admission Sample
a)
TRX
b)
TRXR
c)
PRDX4
Probability
Days to readmission
<8.425 ng/ml
8.426 - 13.40 ng/ml
>13.40 ng/ml
<1000 pg/ml
0.100 - 0.199 ng/ml
>2.000 ng/ml
<10.33 ng/ml
10.34 - 31.92 ng/ml
>31.93 ng/ml
Second Follow-up Sample
d)
TRX
p = 0.0499
e)
TRXR
f)
PRDX4
PCI to LAD Admission Sample
g)
TRX
h)
TRXR
p = 0.0443
i)
PRDX4

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