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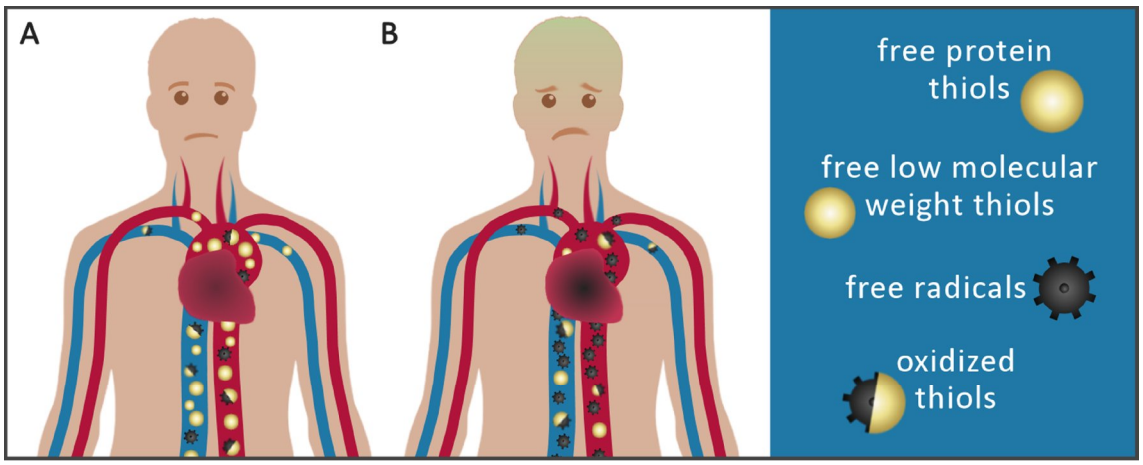
**Graphical abstract**



# Serum free thiols in chronic heart failure

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**Abstract**

Oxidative stress is a key element of the pathophysiology of heart failure (HF). As free thiols are readily oxidized by reactive oxygen and sulfur species, their circulating level may directly reflect the systemic redox status. This study addresses the role of serum free thiols in chronic HF, which is of particular interest as free thiols are amenable to therapeutic modulation and thus are a potential target for therapy.

Free thiols were measured in serum of 101 previously characterized stable chronic HF patients (93% male, age 63.7 ± 10.0 y, left ventricular ejection fraction 34.6 ± 8.2%), adjusted for total serum protein, and subsequently analysed for associations with clinical and outcome parameters.

The mean serum free thiol concentration was 3.6 ± 0.5 μM/g protein. Patients with above-average levels were younger, had better renal function, lower levels of NT-proBNP and PTH, and higher levels of cholesterol. Furthermore, above-average levels were associated with favourable disease outcome, i.e. a decreased rehospitalisation rate and increased patient survival (HR 0.27 (95% CI 0.11-0.62), P=0.002) independent of associated clinical parameters, age and PTH. After adjustment for cholesterol or established prognostic factors in HF, eGFR and NT-proBNP the association was no longer significant, suggesting involvement of these variables in a common pathophysiological pathway.

This exploratory study demonstrates favourable associations of serum free thiols with markers of HF severity and prognosis as well as disease outcome, which should be further investigated in larger prospective studies. Restoring redox status by therapeutic modulation of free thiols may be a promising strategy to improve disease outcome in CHF.

**Keywords**

chronic heart failure; thiols; redox status; oxidative stress; rehospitalisation; survival

## 1 Introduction

Heart failure (HF) remains a leading cause of morbidity and mortality, especially among the growing population of the elderly.(1,2) A recent systematic review reported a median prevalence of HF of 11.8% in people over 60 years of age. In contrast to HF with a preserved ejection fraction, HF with a reduced ejection fraction is more common in men than in women and affects 3.3% of the older population.(2) In general, the prognosis is poor and half of all patients diagnosed with HF die within 5 years.(1)

HF is a complex clinical syndrome that results from abnormal cardiac structure and/or function.(3) These abnormalities cause failure of the heart to deliver oxygen and other nutrients at a rate that meets the body's metabolic demands.(4) In an attempt to resolve the imbalance, the body responds with adaptive mechanisms, including activation of the sympathetic nervous system and the renin-angiotensin-aldosterone system. Paradoxically, these adaptive mechanisms are associated with increased production of reactive oxygen species (ROS).(5) Unaccompanied by adequate upregulation of antioxidative defence mechanisms, excess ROS production leads to oxidative stress, which in turn contributes to the development of myocardial and vascular dysfunction.(5) Under oxidative stress conditions, various cellular and tissue components are known to become targets for oxidation through reactions in which free thiols and membrane lipids play prominent roles. Typically, a shift in redox status with reductions in reduced and increases in oxidized thiols, along with rises in the concentration of lipid oxidation products is observed.(6,7) These processes are accompanied by perturbations of cardiac physiology due to progressive changes in redox signalling at multiple levels.(8)

Systemic oxidative stress can be measured as the depletion of the free thiol pool in serum.(9) In contrast to the intracellular pool, which mainly consists of low molecular weight (LMW) thiols, in serum LMW thiols have a small share and protein thiols predominate.(10)

Since reduced thiols are readily oxidized by ROS and other reactive species, their level may be interpreted as a direct reflection of the overall redox status.(9,11) Once oxidized, the thiols in serum are less readily reduced compared to their intracellular counterparts, and may therefore provide a relatively stable reflection of the systemic redox status. More importantly, free thiols are active components of the antioxidant machinery, which are known to be receptive to therapeutic modulation, for example by cysteine derivatives such as N-actylcysteine (NAC).(10,12,13) Hence, they form a potential target for therapy.

Serum free thiol depletion has been reported in patients with cardiovascular disease (CVD), including acute myocardial infarction, when compared to controls.(9,14) Also, thiol oxidation has been linked to risk factors of CVD, including aging, smoking, and obesity.(15) A large body of evidence supports the role of oxidative stress in the pathogenesis of HF. Bearing in mind the relationship between free thiols and oxidative stress, in the present study we aimed to address the role of free thiols in chronic heart failure (CHF). This is of particular interest as free thiols form a potential target for therapy.(12,13)

## 2 Methods

### *2.1 Patient population*

This study is a post-hoc analysis of an open-label, blinded end point, randomized prospective trial (VitD-CHF trial).(16) From March 2010 to November 2011 101 stable CHF patients presenting at the outpatient clinic of the University Medical Center Groningen, in Groningen, the Netherlands were included in this trial. Patients included in this trial were ≥18 years of age, had a left ventricular ejection fraction (LVEF) <45% and were treated with optimal HF medication (i.e. angiotensin-converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARBs), β-blockers, and mineralocorticoid-receptor antagonists (MRAs) when indicated). These patients were randomized to receive either 2000 IU of vitamin $D_3$

(vitD) daily or no extra medication for six weeks. The cohort has previously been described in more detail.(16,17) The study was conducted in accordance with the Declaration of Helsinki. The local Institutional Review Board approved the study protocol and all study subjects provided written informed consent.

*2.2Baseline characteristics*

Data on participants' disease state, medical history and medication were extracted from patient records. Systolic and diastolic blood pressure and heart rate were measured according to protocol. The body mass index (BMI) was calculated by dividing body weight by height squared. Participants were instructed to collect 24-hour urine the day before visiting the outpatient clinic. On the day of the visit, after an overnight fast, serum and plasma samples were obtained and routine laboratory measurements, including N-terminal pro-B-type natriuretic peptide (NT-proBNP), albumin, total protein, creatinine, urinary albumin and sodium, HbA1c, cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), calcium, and parathyroid hormone (PTH) were performed. Aliquots of blood and urine samples were stored at -80 °C for future analysis. Measurements of plasma renin concentration (PRC), plasma renin activity (PRA), and aldosterone have been described before.(16) The estimated glomerular filtration rate (eGFR) was calculated using the 4-variable Modification of Diet in Renal Disease (MDRD) formula.

*2.3 Detection of free thiols*

Serum samples were stored at -80 °C until free thiol measurement. Thiol groups were detected as previously described, with minor modifications.(18,19) In short, 75 µl serum samples were diluted 1:4 with a 0.1 M Tris buffer (pH 8.2) and then transferred to a microplate. Using a Sunrise microplate reader (Tecan Trading AG, Männedorf, Switzerland),

background absorption was measured at 412 nm with a reference filter at 630 nm. Subsequently, 10 µl 3.8 mM 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB, CAS-number 69-78-3, Sigma Aldrich Corporation, Saint Louis, MO, USA) in a 0.1 M phosphate buffer (pH 7) was added to the samples. Following 20 minutes of incubation at room temperature, absorption was measured again. The concentration of free thiols in the samples was determined by comparing their absorbance reading to that of an L-cysteine (CAS-number 52-90-4, Fluka Biochemika, Buchs, Switzerland) standard in the concentration range of 15.6-1000 µM in 0.1 M Tris and 10 mM EDTA (pH 8.2). Since proteins are by far the predominant source of thiols in serum, free thiol concentrations are expressed per gram of total serum protein.(10)

*2.4 Outcome parameter*

The outcome parameter of this study is a composite of HF related rehospitalisation and all-cause mortality. The mean follow-up period was 4.6 ± 0.5 years. No patients were lost to follow-up.

*2.5 Statistical analysis*

Statistical analysis was performed with STATA software (version 13.0, Stata Corp, College Station, Texas, USA). Graphs were drawn in GraphPad Prism 5.0.

The distribution of all variables was examined using histograms, probability plots and the Kolmogorov–Smirnov test. Normally distributed continuous data are presented as mean ± standard deviation (SD). Skewed data are presented as median (interquartile range (IQR)) and were normalized by logarithmic transformation for analysis. Nominal data are presented as *n* (%).

Associations are shown with protein-adjusted free thiols as a continuous variable (for clinical parameters) or above and below the mean (for the outcome parameter). Differences in baseline characteristics between groups were determined using the Student's t-test for normally distributed continuous data, the Wilcoxon rank-sum test for skewed data and the Chi-square test for nominal data.

A Kaplan-Meier plot and log-rank test were used to test the association between free thiols per gram of protein above and below the mean and NT-proBNP with the outcome parameter. Univariable and multivariable linear regression analyses were applied to identify variables that are independently associated with free thiols per gram of protein. Subsequently, these variables were included in a Cox proportional hazard model. Additionally, the association of free thiols per gram of protein above and below the mean with the outcome parameter was adjusted for established prognostic factors in HF, eGFR and NT-proBNP in a second Cox proportional hazard model.(20,21) The discriminative power of these models was compared by means of logistic regression analyses followed by calculation of the areas under the curve.

All reported P-values are two-tailed and values of $P<0.05$ were considered statistically significant.

## 3 Results

### *3.1 Patient characteristics*

Baseline characteristics of the 101 stable CHF patients are presented in table 1. The mean age of the study subjects was 64 ± 10 years and 94 (93%) of them were male. The mean duration of HF at the time of baseline measurements was 80 ± 65 months. Most patients were categorized in New York Heart Association (NYHA) class II (*n*=89, 88%) and the mean LVEF was 35 ± 8%. All patients were treated with medication according to the current

European Society of Cardiology guidelines, including ACEi/ ARBs (*n*=101, 100%), β-blockers (*n*=98, 97%), MRAs (*n*=29, 29%), and diuretics (mainly furosemide, *n*=49, 49%).

Total free thiol concentrations were normally distributed, with a mean of 257.8 ± 40.3 μM or 3.6 ± 0.5 μM per gram of protein (figure 1).

**Table 1. Baseline characteristics**

| **Stable CHF patients**<br>**Serum free thiols per gram of protein above and below the mean** | | | | |
|---|---|---|---|---|
| **Characteristics** | **Overall**<br>***n*=101** | **Below mean**<br>***n*=51** | **Above mean**<br>***n*=50** | **P-value** |
| Free thiols/ total serum protein, μM/g | 3.6 ± 0.5 | 3.1 ± 0.3 | 4.0 ± 0.3 | **<0.001** |
| Age, y | 63.7 ± 10.0 | 67.1 ± 10.1 | 60.3 ± 8.8 | **<0.001** |
| Male, *n* (%) | 94 (93) | 47 (92) | 47 (94) | 0.72 |
| Current smoker, *n* (%) | 22 (22) | 8 (16) | 14 (28) | 0.13 |
| BMI, kg/m$^2$ | 28.0 ± 4.4 | 28.3 ± 4.2 | 27.6 ± 4.5 | 0.44 |
| Systolic blood pressure, mmHg | 116.4 ± 16.9 | 114.7 ± 16.0 | 118.0 ± 17.7 | 0.33 |
| Diastolic blood pressure, mmHg | 71.0 ± 10.6 | 69.7 ± 11.0 | 72.2 ± 10.0 | 0.24 |
| Heart rate, bpm | 67.6 ± 9.3 | 65.7 ± 7.4 | 69.4 ± 10.7 | **0.046** |
| Heart failure history | | | | |
| Duration HF, m | 80.2 ± 65.4 | 84.2 ± 62.6 | 76.2 ± 68.6 | 0.54 |
| Ischemic etiology, *n* (%) | 73 (72) | 38 (75) | 35 (70) | 0.61 |
| NYHA class II/III, *n* (%) | 89/12 (88/12) | 42/9 (82/18) | 47/3 (94/6) | 0.071 |
| LVEF (%) | 34.6 ± 8.2 | 33.4 ± 8.3 | 35.9 ± 7.9 | 0.11 |
| Treatment | | | | |
| ACEi/ARB, *n* (%) | 101 (100) | 51 (100) | 50 (100) | |
| β-blocker, *n* (%) | 98 (97) | 50 (98) | 48 (96) | 0.55 |
| MRA, *n* (%) | 29 (29) | 18 (35) | 11 (22) | 0.14 |
| Diuretic, *n* (%) | 49 (49) | 31 (61) | 18 (36) | **0.013** |

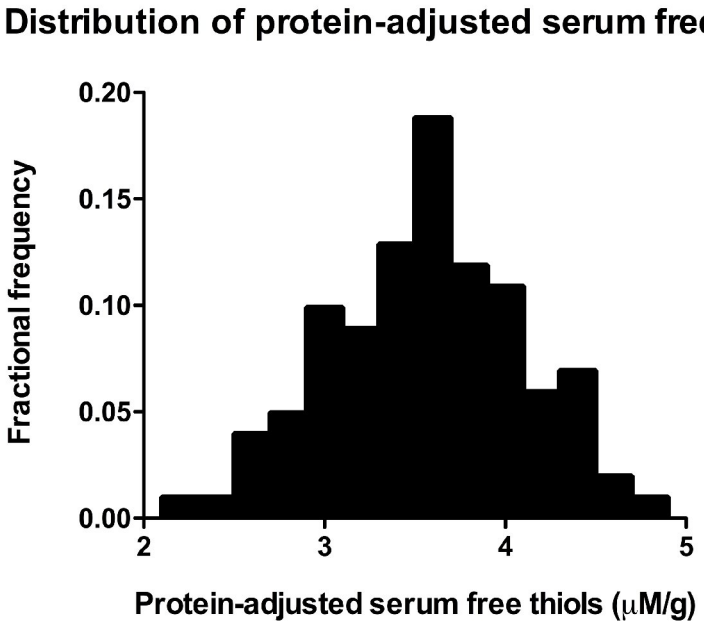
| Laboratory measurements | | | | |
|---|---|---|---|---|
| NT-proBNP, ng/L* | 375.5 (203.0-781.5) | 513.0 (237.0-1335.0) | 238.0 (195.0-566.0) | **0.016** |
| Serum albumin, g/L | 44.5 ± 2.4 | 43.8 ± 2.2 | 45.1 ± 2.4 | **0.004** |
| Total serum protein, g/L | 72.3 ± 4.0 | 72.5 ± 4.4 | 72.2 ± 3.6 | 0.75 |
| eGFR, ml/min/1.73m$^2$ | 80.4 ± 16.5 | 76.7 ± 17.5 | 84.1 ± 14.6 | **0.023** |
| 24-h urinary albumin, mg/24 h | 16.6 ± 57.1 | 12.1 ± 26.4 | 21.2 ± 76.9 | 0.43 |
| 24-h urinary sodium, mmol/24 h | 165.7 ± 74.7 | 147.2 ± 68.4 | 184.5 ± 76.8 | **0.012** |
| HbA1C, % | 6.1 ± 0.6 | 6.1 ± 0.6 | 6.0 ± 0.7 | 0.68 |
| Cholesterol, mmol/L | 4.4 ± 1.1 | 4.1 ± 0.9 | 4.8 ± 1.1 | **<0.001** |
| HDL, mmol/L | 1.2 ± 0.4 | 1.2 ± 0.4 | 1.2 ± 0.4 | 0.87 |
| LDL, mmol/L | 2.6 ± 0.9 | 2.4 ± 0.7 | 2.9 ± 1.0 | **0.001** |
| Calcium, mmol/L | 2.3 ± 0.1 | 2.3 ± 0.1 | 2.3 ± 0.1 | 0.17 |
| PTH, pmol/L | 7.7 ± 4.0 | 8.8 ± 4.5 | 6.5 ± 3.1 | **0.003** |
| PRC, ng/L* | 60.6 (17.5-193.0) | 66.6 (20.7-209.0) | 56.6 (17.1-156.0) | 0.37 |
| PRA, ng/mL/h* | 5.2 (1.5-19.7) | 6.0 (2.0-33.8) | 4.5 (1.2-16.3) | 0.24 |
| Aldosterone, pmol/L* | 0.2 (0.1-0.4) | 0.3 (0.1-0.5) | 0.2 (0.1-0.3) | 0.047 |
| VitD supplementation# | 50 (50) | 24 (47) | 26 (52) | 0.62 |
| 1,25(OH)$_2$D, pmol/L | 144.5 ± 44.5 | 141.8 ± 43.6 | 147.0 ± 45.6 | 0.56 |
| 1,25(OH)$_2$D, 6 w, pmol/L## | 169.5 ± 62.1 | 166.3 ± 62.0 | 173.0 ± 62.7 | 0.59 |

Normally distributed continuous data are presented as mean ± SD *Skewed data are presented as median (IQR)

#2000 IU of VitD daily for six weeks ##1,25(OH)$_2$D after six weeks

CHF; chronic heart failure, BMI; body mass index, HF; heart failure, NYHA; New York Heart Association, LVEF; left ventricular ejection fraction, ACEi; angiotensin-converting enzyme inhibitor, ARB; angiotensin receptor blocker, MRA; mineralocorticoid-receptor antagonists, NT-proBNP; N-terminal pro-B-type natriuretic peptide, eGFR; estimated glomerular filtration rate, HDL; high density lipoprotein, LDL; low density lipoprotein, PTH; parathyroid hormone, PRC; plasma renin concentration, PRA; plasma renin activity, VitD; vitamin $D_3$ (cholecalciferol)

**Figure 1. Histogram of protein-adjusted serum free thiols in CHF patients**




The protein-adjusted serum free thiol concentrations measured in 101 CHF patients (3.6 ± 0.5 μM/g) were normally distributed.

On comparison of patients with free thiols per gram of protein above and below the mean, age, heart rate, use of diuretics, NT-proBNP, serum albumin, eGFR, 24-h urinary sodium, cholesterol, LDL, and PTH were significantly different between groups (table 1). Participants with free thiols per gram of protein above the mean were significantly younger (60 ± 9 vs. 67 ± 10 y, P<0.001), had a higher heart rate (69 ± 11 vs. 66 ± 7.4 bpm, P=0.046), and were less often treated with diuretics (*n*=18 (36%) vs. *n*=31 (61%), P=0.013). These patients also had lower levels of NT-proBNP (238 (195-566) vs. 513 (237-1335) ng/L, P=0.016) and PTH (7 ± 3 vs. 9 ± 5 pmol/L, P=0.003), whereas their levels of serum albumin (45 ± 2 vs. 44 ± 2, P=0.004), eGFR (84 ± 15 vs. 77 ± 18, P=0.023), 24-h urinary sodium (185 ± 77 vs. 147 ± 68, P=0.012), cholesterol (5 ± 1 vs. 4 ± 1, P<0.001) and LDL (3 ± 1 vs. 2 ± 1, P=0.001) were higher. Patients who were randomized to receive 2000 IU of vitD daily for six weeks were

spread evenly across groups. Also, active vitD levels at baseline and after six weeks of supplementation were comparable for patients with free thiols per gram of protein above and below the mean.

Univariable and multivariable linear regression analyses showed that age, cholesterol and PTH are independently associated with protein-adjusted free thiols (table 2). Multivariable regression analysis did not identify an association with NT-proBNP due to interaction with cholesterol.

**Table 2. Univariable and multivariable linear regression analyses of protein-adjusted serum free thiols and clinical parameters in CHF**

| **Serum free thiols per gram of protein** | | | | |
|---|---|---|---|---|
| | **Univariable regression** | | **Multivariable regression** | |
| **Characteristics** | **Coefficient** | **P-value** | **Coefficient** | **P-value** |
| Age | -0.025 | **<0.001** | -0.023 | **<0.001** |
| Male | -0.071 | 0.737 | | |
| Current smoker | 0.061 | 0.492 | | |
| BMI | -0.008 | 0.495 | | |
| Systolic blood pressure | 0.005 | 0.119 | | |
| Diastolic blood pressure | 0.009 | 0.091 | | |
| Heart rate | 0.136 | **0.017** | | |
| Heart failure history | | | | |
| Duration HF | -0.001 | 0.240 | | |
| Ischemic etiology | 0.002 | 0.990 | | |
| NYHA class II/III | -0.318 | 0.053 | | |
| LVEF | 0.011 | 0.086 | | |
| Treatment | | | | |
| β-blocker | -0.172 | 0.585 | | |
| MRA | -0.115 | 0.331 | | |

| | | | | |
|---|---|---|---|---|
| Diuretic | -0.279 | **0.008** | | |
| Laboratory measurements | | | | |
| NT-proBNP* | -0.214 | **<0.001** | | |
| Serum albumin | 0.082 | **<0.001** | | |
| eGFR | 0.009 | **0.003** | | |
| 24-h urinary albumin | 0.001 | 0.746 | | |
| 24-h urinary sodium | 0.002 | 0.122 | | |
| HbA1C | -0.052 | 0.544 | | |
| Cholesterol | 0.153 | **0.002** | 0.095 | **0.026** |
| HDL | 0.010 | 0.943 | | |
| LDL | 0.145 | **0.012** | | |
| Calcium | 0.809 | 0.198 | | |
| PTH | -0.052 | **<0.001** | -0.040 | **<0.001** |
| PRC* | -0.44 | 0.181 | | |
| PRA* | -0.045 | 0.126 | | |
| Aldosterone* | -0.171 | **0.010** | | |

*Skewed data, normalized by logarithmic transformation

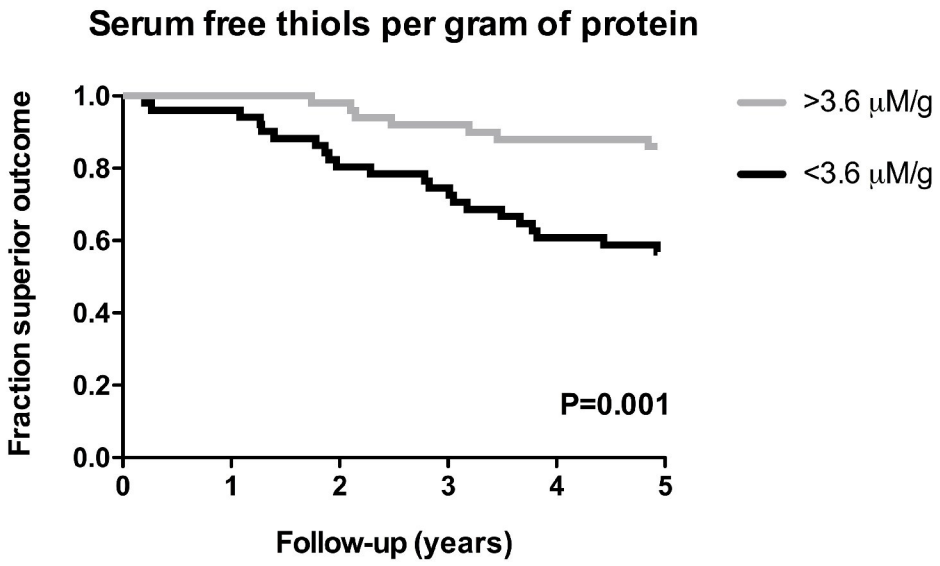
CHF; chronic heart failure, BMI; body mass index, HF; heart failure, NYHA; New York Heart Association, LVEF; left ventricular ejection fraction, ACEi; angiotensin-converting enzyme inhibitor, ARB; angiotensin receptor blocker, MRA; mineralocorticoid-receptor antagonists, NT-proBNP; N-terminal pro-B-type natriuretic peptide, eGFR; estimated glomerular filtration rate, HDL; high density lipoprotein, LDL; low density lipoprotein, PTH; parathyroid hormone, PRC; plasma renin concentration, PRA; plasma renin activity

### *3.2 Free thiols and outcome*

The mean follow-up period was 4.6 ± 0.5 year. During this period, 13 patients (13%) were rehospitalised and 20 patients (20%) died. The composite outcome was recorded 29 times. Rehospitalisation and/or death occurred in 7 patients (14%) with *above*-average free thiols per gram of protein compared to 22 patients (43%) with below-average levels (log-rank test,

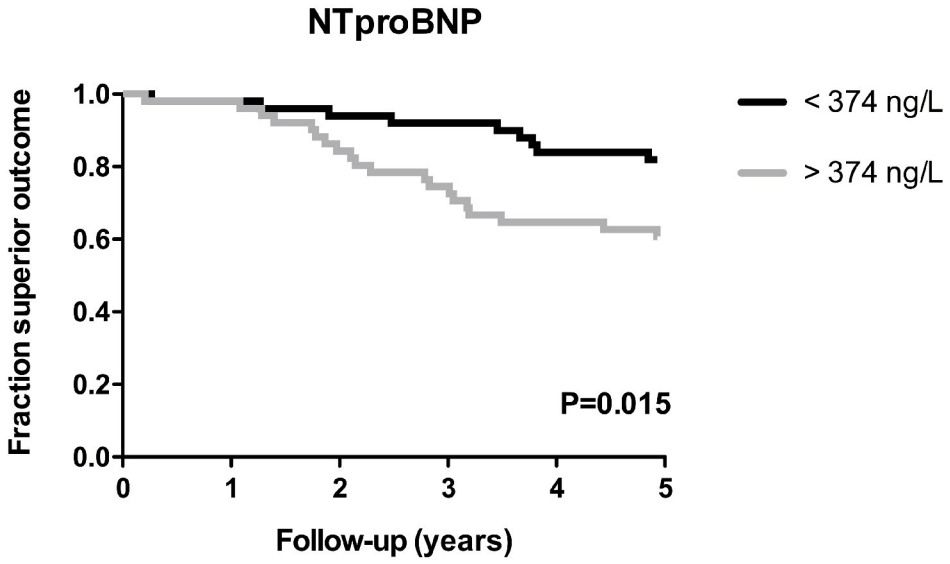
P=0.001). The corresponding Kaplan-Meier plot is shown in figure 2. Figure 3 shows a Kaplan-Meier plot of the association of NT-proBNP above and below the median with disease outcome. Interestingly, on comparison of figures 2 and 3 a similar albeit naturally inverse trend is observed.

**Figure 2. Kaplan-Meier analysis of the association of protein-adjusted serum free thiols above and below the mean with outcome in CHF**




Kaplan-Meier plot with log-rank test for outcome (a composite of HF-related rehospitalisation and all-cause mortality). Serum free thiols per gram of protein *above* the mean are significantly associated with favourable outcome in stable CHF patients (P=0.001).

**Figure 3. Kaplan-Meier analysis of the association of NT-proBNP above and below the median with outcome in CHF**




Kaplan-Meier plot with log-rank test for outcome (a composite of HF related rehospitalisation and all-cause mortality). NT-proBNP *below* the median is significantly associated with favourable outcome in stable CHF patients (P=0.015).

As depicted in table 3, crude Cox regression analysis shows that above-average levels of free thiols per gram of protein are significantly associated with favourable outcome, i.e. a decreased rehospitalisation rate and increased patient survival (crude model, hazard ratio (HR) 0.27 (95% confidence interval (CI) 0.11-0.62), P=0.002). After adjustment for age and PTH (model 3) the association with outcome remained significant (HR 0.35 (95% CI 0.14-0.87), P=0.024, table 3). Further adjustment for cholesterol (model 4) resulted in a non-significant HR for adverse events (HR 0.43 (95% CI 0.17-1.09), P=0.076, table 3). This may indicate that a shared mechanism underlies the associations of protein-adjusted free thiols and cholesterol with outcome. Further information on the relative contribution of age, PTH and cholesterol to the Cox proportional hazard model is provided in supplemental table S1.

**Table 3. Cox proportional hazard model of the association of protein-adjusted serum free thiols and associated clinical parameters with disease outcome in CHF**

| Serum free thiols per gram of protein above and below the mean | | |
|---|---|---|
| **Model** | **HR (95% CI)** | **P-value** |
| 1: crude | 0.27 (0.11-0.62) | **0.002** |
| 2: adjusted for age | 0.38 (0.16-0.94) | **0.035** |
| 3: adjusted for age and PTH | 0.35 (0.14-0.87) | **0.024** |
| 4: adjusted for age, PTH and cholesterol | 0.43 (0.17-1.09) | 0.076 |

CHF; chronic heart failure, HR; hazard ratio, CI; confidence interval, PTH; parathyroid hormone

Table 4 shows that, in this cohort, the association of free thiols per gram of protein above and below the mean with outcome is no longer significant when it is adjusted for established prognostic factors in HF, eGFR and NT-proBNP (model 4, HR 0.43 (95% CI 0.17-1.06), P=0.068).

**Table 4. Cox proportional hazard model of the association of protein-adjusted serum free thiols and established prognostic factors with disease outcome in CHF**

| Serum free thiols per gram of protein above and below the mean | | |
|---|---|---|
| **Model** | **HR (95% CI)** | **P-value** |
| 1: crude | 0.27 (0.11-0.62) | **0.002** |
| 2: adjusted for age | 0.38 (0.16-0.94) | **0.035** |
| 3: adjusted for age and eGFR | 0.40 (0.16-1.00) | **0.050** |
| 4: adjusted for age, eGFR and NT-proBNP | 0.43 (0.17-1.06) | 0.068 |

HF; heart failure, CHF; chronic heart failure, HR; hazard ratio, CI; confidence interval, eGFR; estimated glomerular filtration rate, NT-proBNP; N-terminal pro-B-type natriuretic peptide

Calculation of the area under the curve for the models displayed in tables 3 and 4 revealed that their performance is comparable (table 5). In both cases, adding serum free thiols to the

model increased discriminative probability by 2%. These increases were not significant (AUC=0.788/0.806, P=0.399 and AUC=0.790/0.809, P=0.469 respectively).

**Table 5. Contribution of protein-adjusted serum free thiols above and below the mean to the area under the curve of models for disease outcome in CHF**

| Model | AUC | P-value |
|---|---|---|
| 1a: age, PTH and cholesterol | 0.788 | |
| 1b: 1a + serum free thiols* | 0.806 | 0.399 |
| 2a: eGFR and NT-proBNP | 0.790 | |
| 2b: 2a + serum free thiols * | 0.809 | 0.469 |

*Serum free thiols per gram of protein above and below the mean

CHF; chronic heart failure, AUC; area under the curve, eGFR; estimated glomerular filtration rate, NT-proBNP; N-terminal pro-B-type natriuretic peptide

## 4 Discussion

This study sheds light on the potential significance of serum free thiols in CHF. Our exploratory analyses suggest that higher levels of serum free thiols are associated with favourable disease outcome, i.e. a decreased rehospitalisation rate and increased patient survival, in stable CHF patients. The Kaplan Meier plot in figure 2 shows a significant distinction of disease outcome on the basis of protein-adjusted serum free thiols, despite the small size of the cohort and number of events. When taking into account that this study concerns patients with stable disease, this result is even more striking. After adjustment for associated factors and established prognostic factors in HF the association was no longer significant, which may indicate involvement of these variables in a common pathophysiological pathway. Furthermore, our data clearly indicate that serum free thiols are favourably associated with markers of HF severity and prognosis. Patients with above-

average serum free thiols were younger, had better renal function, lower levels of NT-proBNP and PTH, and higher levels of cholesterol.

Previous studies reported on marked reductions of serum protein thiols in active diseases, including CVD.(9,14) Also, the oxidation of thiols has been linked to risk factors of CVD, including ageing, smoking, and obesity.(15) However, to the best of our knowledge, we are the first to explore the association of total serum free thiols with disease outcome.

Our findings are relevant, not only from a diagnostic perspective, but also because free thiols are known to be receptive to therapeutic modulation, and thus are a potential target for therapy.(10,12,13) Indeed, in multiple clinical trials, the cysteine derivative NAC has been shown to both directly reduce disulfide bonds and act as a glutathione (GSH) precursor.(13) Other thiol compounds, with similar modes of action, have also been described.(12) Although therapeutic results have been inconsistent, these compounds may prove to be effective in selected patients, in particular those with low free thiol concentrations.

Serum free thiols are readily oxidized by ROS and other reactive species.(11) Therefore, their level forms a direct reflection of the balance between oxidants and antioxidant capacity and resultant oxidative stress.(9,11) This applies to physiological and pathological conditions alike. Since oxidative stress is an important element of the pathophysiology of CHF, contributing to the development of myocardial and vascular dysfunction, an inverse relationship between free thiols and disease progression is conceivable.(5)

Besides reflecting redox status, thiols are crucial active components of antioxidant capacity. The thiol groups measured in serum as part of this study include both protein thiols and LMW thiols. Most studies looking into the disulfide reducing machinery have focussed on LMW thiols, in particular GSH and cysteine. For example, a recent study has shown higher cystine and lower glutathione levels to be associated with an increased risk of mortality in patients with coronary artery disease.(22) However, in the extracellular compartment,

proteins are by far the largest source of free and oxidized thiols.(10,23) In fact, the majority of thiols in serum is accounted for by the single sulfhydryl group of albumin, which is the most abundant plasma protein.(10) Because of this, we present the amount of free thiols per gram of total serum protein. Total serum free thiols, adjusted for protein, are examined as a reflection of the overall redox status. Although beyond the scope of this study, there may be compositional variations in the thiol pool specific to chronic heart failure worth pinpointing in the future, especially in the context of drug development.

Both protein thiols and LMW thiols are important targets of reactive species.(10) Recent data suggest that these are not limited to ROS but include reactive sulfur species as well.(24) The exact nature of the reactions that account for the reduction in serum free thiol concentrations in the present study remains unknown and this warrants further investigation. However, the magnitude of the inter-individual differences observed suggests that this variability is not due to changes in the LMW thiol pool, the total of which is in the lower tens of micromolar range.(10) Reversible oxidative posttranslational modifications of protein thiols by several small molecules have been suggested to protect proteins from irreversible oxidative damage. These small molecules include homocysteine, cysteine, GSH, and cysteinylglycine, as well as the gasotransmitters nitric oxide and hydrogen sulfide ($H_2S$). The modifications these molecules induce, individually or in conjunction, are S-thiolation, S-nitrosylation, and S-sulfhydration.(11,23,25) Interestingly, whereas the concentration of unbound reduced cysteine, for example, is only about 10 μM in human serum, that of protein-bound cysteine is 150-175 μM.(23) S-sulfhydration is indirectly brought about by $H_2S$, either through reactions of oxidized thiols (e.g. mixed disulfides) with sulfide or through reactions of sulfide oxidation products (including polysulfides) with thiols, giving rise to persulfides.(26–28) These labile modifications are likely lost during sample collection and processing, regenerating free thiols. Regardless, persulfides are known to react with DTNB

similarly to thiols.(29) Thus, any remaining persulfide will have contributed to the serum free thiol levels determined in this study. Further research is warranted to unravel the composition of the DTNB-reactive pool of total free thiols in human serum. Potentially, pharmacological stimulation of the molecules inducing reversible protein modifications could be a useful strategy to influence the amount of free thiols. Moreover, certain thiols are involved in redox signalling by acting as redox-switches and oxidative posttranslational modification of these critical protein thiols can alter protein function.(11)

Redox signalling is an integral part of physiology. Whereas excessive levels of ROS can perturb redox status, physiological levels are involved in beneficial cellular processes, including immunity, cell growth and apoptosis.(30,31) Moreover, recurrent episodes of oxidative stress have been shown to up-regulate endogenous antioxidant mechanisms, which in turn may promote health and longevity.(31,32) Thus, hampering of the physiological functions of ROS may in part explain the contradicting results of studies focussing on antioxidant therapies. Another conceivable explanation lies in the fact that physiological regulatory processes tend to limit the concentrations of antioxidants that can be reached. For this reason, especially dietary antioxidants may not reach sufficient bioavailability to provide adequate antioxidant capacity, or may only do so in patients with a deficiency at baseline.(31,33) Finally, oxidation of an antioxidant substance before or during ingestion may not only abate its efficacy, but in fact induce oxidative stress.(31)

In this study, age, cholesterol and PTH were found to be independently associated with free thiols. The association of free thiols with disease outcome remained significant after adjustment for age and PTH. Free thiols and age are inversely related. On the one hand, this is thought to be caused by excess ROS production with age. On the other hand, decreased production of endogenous antioxidants, and thus thiol depletion itself may contribute to the imbalance between ROS production and antioxidant capacity, further aggravating oxidative

stress.(12) To our knowledge, a relationship between free thiols and PTH has not been described. This hormone is associated with cardiovascular events, even in the general population.(13) Moreover, it plays a causal role in the pathophysiology of HF, primarily by interacting with aldosterone.(34,35) Interestingly, on further adjustment for cholesterol, the association of free thiols with disease outcome was lost. This may indicate that a shared mechanism links both free thiols and cholesterol to disease outcome. Our data show a positive correlation between free thiols and cholesterol. Previous research has shown that plasma thiol concentrations are negatively correlated with serum LDL, whereas they are positively correlated with serum HDL in hyperlipidemic patients.(36) The cholesterol concentrations of the majority of patients in this study were within the normolipidemic range, which is probably attributable to the use of statins. Nevertheless, the positive correlation between free thiols and cholesterol suggests that, within the normal range, a higher cholesterol concentration is beneficial. This is in line with a former study stating that cholesterol itself can act as an antioxidant. On oxidation, oxysterols are formed, which in turn are rapidly degraded by the liver.(37) This assumption is further supported by the inverse interaction between cholesterol and NT-proBNP found in this study, as NT-proBNP is an established HF biomarker which is positively correlated with disease progression.(21)

Our study has several limitations. Most importantly, it concerns a small cohort of only 101 patients in which the composite outcome was recorded 29 times, limiting the study's statistical power. Furthermore, it is a single center study of Caucasian subjects, confining the possibility of extrapolating our results to other ethnicities. Finally, because of its cross-sectional design, causality of the relationship between free thiols and disease outcome could not be addressed. Strengths of this study include the homogenous and extensive characterization of the study subjects and the relatively long follow-up period of on average 4.6 years.

In conclusion, this exploratory study demonstrates an association of serum free thiols with favourable disease outcome in CHF. Substantiating this finding, serum free thiols were also found to be favourably associated with established markers of HF severity and prognosis in patients. These associations should be investigated further in larger, prospective studies. Restoring redox status by therapeutic modulation of free thiols may be a promising strategy to improve disease outcome in CHF.

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**Conflicts of Interest**

Conflicts of Interest: none declared.

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