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Serum free sulfhydryl status is associated with patient and graft survival in renal transplant recipients

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ABSTRACT

Oxidative stress contributes significantly to graft failure, morbidity and mortality in renal transplant recipients (RTR). In cells, free sulfhydryl groups (reduced thiols, R-SH) are the transducers of redox-regulated events; their oxidation status is modulated by interaction with reactive oxygen and nitrogen oxide species and thought to be in equilibrium with the circulating pool. We hypothesized that high levels of serum free thiols are a reflection of a favorable redox status and therefore positively associated with cardiovascular risk parameters, patient and graft survival in RTR.

To test this, reactive free thiol groups (R-SH; corrected for total protein) were quantified in serum of 695 RTR (57% male, 53 ± 13 yr, functioning graft ≥ 1 yr) using Ellman's Reagent, and R-SH determinants were evaluated with multivariable linear regression models. Associations between R-SH and mortality or graft failure were assessed using multivariable Cox regression analyses.

In multivariable models, male gender, estimated glomerular filtration rate and serum thiosulfate positively associated with R-SH while BMI, HbA1c, corrected calcium and NT-pro-BNP inversely associated with R-SH (model $R^2 = 0.26$). During follow-up (3.1 [2.7–3.9] yrs), 79 (11%) patients died and 45 (7%) patients developed graft failure. R-SH correlated inversely with all-cause mortality (HR 0.58 [95% CI 0.45–0.75] per SD increase) and graft failure (HR 0.42 [0.30–0.59]; both $P < 0.001$), independent of parameters with which R-SH significantly associated in the multivariable regression analyses, except for NT-pro-BNP.

Serum R-SH are associated with a beneficial cardiovascular risk profile and better patient and graft survival in RTR, suggesting potential usefulness as low-cost, high-throughput screening tool for whole-body redox status in translational studies. Whether R-SH modification improves long-term outcome of RTR warrants further exploration.

1. Introduction

Renal transplantation is the preferred treatment for most patients with end-stage renal disease (ESRD). Although both quality of life and prognosis of renal transplant recipients (RTR) have significantly improved [1], morbidity and mortality rates remain high [2].

Aberrant production of reactive oxygen species (ROS) causes oxidative stress and is assumed to be one of the hazardous players that contribute to the development of graft failure and patient mortality in RTR [3]. Via the induction of an increased inflammatory response, oxidative stress leads to the onset and progression of many diseases, including cardiovascular disease (CVD) [4]. High levels of free sulfhydryl groups (R-SH) may be a reflection of a favorable redox status since they avidly interact with ROS and other reactive species [5]. In healthy individuals, the concentrations of serum protein thiols are highly regulated [6] and form part of an intricate redox network that underpins human adaptation to nutritional, metabolic and environmental challenges [7]. Conditions promoting oxidative stress result in the oxidation of free sulfhydryl groups to their corresponding disulfides, which is associated with risk for CVD [6]. The extracellular redox status

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influences CVD through pro-inflammatory signaling, which involves mitochondrial oxidation, nuclear factor-κB activation and elevated expression of genes for monocyte recruitment to endothelial cells [6]. While the thiol/disulfide status of glutathione and cysteine, for example, are acknowledged readouts of oxidative stress their analytical determination at the prevailing low concentrations in plasma or serum can be challenging and requires specific detection techniques such as high-pressure liquid chromatography or mass spectrometry not available to all laboratories. Moreover, at present there is no consensus about the significance of specific low-molecular-weight or protein thiols for disease protection and many low-abundant thiols are prone to rapid artificial oxidation. Less time-consuming, more robust yet affordable alternatives requiring only a spectrophotometer or plate reader would therefore be of value for potential use in translational medicine, but examples in the literature are few and many studies are underpowered. Based on the associations of free sulfhydryl groups with oxidative stress, we hypothesized that total free sulfhydryl groups might have merit as a gross read-out of cardiovascular health in RTR. In the present study we therefore investigated whether reactive sulfhydryl groups in serum are associated with a beneficial cardiovascular risk profile (a.o. NT-proBNP) and improved patient and graft survival in stable RTR. To this end, we measured total free sulfhydryl status using Ellman's Reagent in serum of 695 stable RTR and analyzed its relationship with cardiovascular risk parameters, graft failure and all-cause mortality.

2. Subjects and methods

2.1. Study design and population

The study protocol was approved by the Institutional Review Board (METc 2008/186) and was in adherence to the Declaration of Helsinki. From November 2008 till June 2011, we invited all stable RTR ($\geq$18 years, n=817) with a functioning graft for over one year, which were treated in the outpatient clinic of the University Medical Center Groningen (UMCG), the Netherlands. After giving written informed consent, a total of 707 (87%) participated in the present study. R-SH was measured in serum samples of 695 RTR (98%). Further details of the study population have been published previously [8,9].

2.2. Outcome parameters

The primary outcome measures of this study were all-cause mortality and the death-censored graft failure. The latter was defined as restart of dialysis or retransplantation. Outcome measures were recorded until the end of May 2013; median follow-up was 3.1 [2.7–3.9] years, with no participants lost to follow-up.

2.3. Clinical parameters

Information on participants' health status, medical history and medication use was extracted from patient records. To categorize smoking behavior into current, former or never smoked, a self-report questionnaire was used. Relevant transplant information was extracted from the UMCG renal transplant database. Blood pressure and heart rate were measured using a semi-automatic device (Dinamap® 1846, Critikon, Tampa, FL, USA) every minute for the duration of fifteen minutes, following a strict protocol [10]. An average of the last three values was taken as a final value. Body weight and height were measured and body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). As described previously [8], all participants were instructed to collect 24 h-urine sample at the day prior to their visit to the outpatient clinic. In the morning after an overnight fasting period and completing the 24 h-urine collection, blood was drawn and subsequently venous blood gas analyses were performed photometrically. Electrolytes, phosphate, albumin, urea and creatinine in plasma and urine were measured using routine laboratory methods, which was also the case for serum cholesterol, HbA1c and hs-CRP. Urinary sulfate and thiosulfate concentrations were measured as previously described [8]. Serum thiosulfate concentrations were determined by HPLC following delipidation by 2 chloroform-methanol (2:1) extraction steps and derivatization of thiosulfate with 18.4 mM mono-bromobimane [11]. Samples were measured using a Waters Alliance 2695 HPLC, LiChrospher 60 RP select B (Merck Milipore) columns and a RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan). Mobile phase was a 0.25% acetic acid in deionized H_2O / methanol gradient (88:12 to 0:100, both Merck Milipore).

Renal function was assessed by calculating the estimated glomerular filtration rate (eGFR) using the CKD Epidemiology Collaboration (CKD-EPI) equation [12]. Serum calcium was corrected for hypoalbuminemia ($<$40 g/L) using the following formula: corrected calcium=serum calcium (mmol/L)+0.02 $\times$ (40 – serum albumin [g/L]).

To determine protein carbonylation, serum protein was precipitated with 20% trichloroacetic acid before derivatization with 2,4 dinitrophenylhydrazine (both Sigma Aldrich, Buchs, Switzerland, DNPH CAS-number 119-26-6) and spectrophotometric measurement of the resultant (orange) reaction products at 400 nm (reference 650 nm), as described by Levine and colleagues [13].

2.4. R-SH measurement; colorimetric detection of free thiol groups

Serum samples were stored at −80 °C until R-SH measurement. Free thiol groups were detected as described previously [14,15], with slight modifications. In short, serum samples were diluted 1:4 with 0.1 M Tris buffer (pH 8.2). After measuring background absorption at 412 nm using a Sunrise microplate reader (Tecan Group AG, Männedorf, Switzerland), with a reference measurement at 630 nm, 10 μL 3.8 mM 5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB; Ellman's Reagent, CAS-number 69–78-3, Sigma Aldrich Corporation, St. Louis, MO, USA) in 0.1 M phosphate buffer (pH 7) was added to the samples; after an incubation period of 20 min at room temperature sample absorbances were measured again. Concentrations of free thiol groups were determined by parallel measurements of a L-Cysteine (CAS-number 52-90-4, Fluka Biochemika, Buchs, Switzerland) standard curve [15.625 μM to 1000 μM] in 0.1 M Tris/10 M EDTA (pH 8.2). The intra- and interday variability of the -SH determinations in the transplant cohort both have a CV $<$10%. Finally, to determine the R-SH status per RTR, free thiol groups were corrected for total serum protein, by calculating the free thiol groups/total serum protein ratio. This correction was performed since proteins largely determine the amount of potentially measurable free thiol groups (the total pool of free low-molecular weight thiols is typically below 10 μM whereas the total protein RSH pool of healthy individuals is in the hundreds of μM).

2.5. Statistical analysis

To test the distribution of all parameters, histograms and probability plots were displayed followed by the Kolmogorov-Smirnov test. Skewed data were normalized for analyses by logarithmic transformation (hsCRP, triglycerides, albuminuria, serum and urinary thiosulfate). The study population was subdivided into tertiles of R-SH to visualize linear and non-linear associations with R-SH. To establish *P*-values for differences in R-SH tertiles an