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The fate of sulfate in chronic heart failure

Anne M. Koning[1,2], Wouter C. Meijers[3], Isidor Minović[4], Adrian Post[4], Martin Feelisch[5], Andreas Pasch[6], Henri G.D. Leuvenink[2], Rudolf A. de Boer[3], Stephan J.L. Bakker[4], Harry van Goor[1]

[1]University Medical Center Groningen, University of Groningen, Department of Pathology and Medical Biology, Groningen, the Netherlands

[2]University Medical Center Groningen, University of Groningen, Department of Surgery, Groningen, the Netherlands

[3]University Medical Center Groningen, University of Groningen, Department of Cardiology, Groningen, The Netherlands

[4]University Medical Center Groningen, University of Groningen, Department of Internal Medicine, Division of Nephrology, Groningen, the Netherlands

[5]University of Southampton and University Hospital Southampton NHS Foundation Trust, Clinical and Experimental Sciences, Faculty of Medicine, and NIHR Biomedical Research Centre, Southampton, United Kingdom

[6]University of Bern, Department of Clinical Research, Bern, Switzerland

AMK and HvG designed the study; RAdB and SJLB shared samples and data; IM and AP[4] performed plasma sulfate measurements; AMK and WCM analysed data; AMK wrote the manuscript; all authors critically reviewed and advised on the manuscript; AMK, WCM, IM and AP[4] were supervised by MF, AP[6], HGDL, RAdB, SJLB and HvG

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Corresponding author

Prof. dr. Harry van Goor

Department of Pathology and Medical Biology

University Medical Center Groningen

Hanzeplein 1, 9713 GZ, HP EA10, Groningen, the Netherlands

Phone: +31 50 3619529

Fax: +31 50 3619107

Email: h.van.goor@umcg.nl

Abstract

New leads to advance our understanding of heart failure (HF) pathophysiology are urgently needed. Previous studies have linked urinary sulfate excretion to a favourable cardiovascular risk profile. Sulfate is not only the end-product of hydrogen sulfide metabolism, but is also directly involved in various (patho)physiological processes, provoking scientific interest in its renal handling. This study investigates sulfate clearance in chronic HF (CHF) patients and healthy individuals and considers its relationship with disease outcome.

Parameters related to renal sulfate handling were determined in and compared between 96 previously characterized CHF patients and sex-matched healthy individuals. Among patients, sulfate clearance was analysed for associations with clinical and outcome parameters.

In CHF patients, plasma sulfate concentrations are significantly higher, whereas 24-h urinary excretion, fractional excretion and clearance of sulfate are significantly lower, compared to healthy individuals. Among patients, sulfate clearance is independently associated with diuretics use, creatinine clearance and 24-h urinary sodium excretion. Sulfate clearance is associated with favourable disease outcome (HR per SD increase 0.38 (95% CI 0.23-0.63), $P<0.001$). Although significance was lost after adjustment for creatinine clearance, the decrease of sulfate clearance in patients is independent of this parameter, indicating that sulfate clearance is not merely a reflection of renal function.

This exploratory study reveals aberrant sulfate clearance as a potential contributor to CHF pathophysiology, with reduced levels in patients and a positive association with favourable disease outcome. Further research is needed to unravel the nature of its involvement and to determine its potential as a biomarker and target for therapy.

Keywords

chronic heart failure, sulfate; renal handling; hydrogen sulfide; disease outcome

New and noteworthy

Sulfate clearance is decreased in chronic heart failure patients compared to healthy individuals. Among patients, sulfate clearance is positively associated with favourable disease outcome, i.e. a decreased rehospitalisation rate and increased patient survival. Hence, decreased sulfate clearance may be involved in the pathophysiology of heart failure.

Introduction

Despite therapeutic advances, heart failure (HF) remains a leading cause of morbidity and mortality, in particular among the elderly.[25] In fact, as the world is ageing, the burden of HF is anticipated to increase in coming years.[22] Accordingly, there is a pressing need for new leads to further our understanding of the pathophysiology of HF.

In this context, renal handling of sulfate is of interest. Indeed, a study of renal transplant recipients has shown that urinary sulfate excretion is positively associated with a favourable cardiovascular risk profile.[2] This profile includes a lower serum level of N-terminal pro-B-type natriuretic peptide (NT-proBNP), which is an established marker of HF progression.[15]

Sulfate is the fourth most abundant anion in human plasma and essential to all cells.[16] It is derived from the diet, both directly and through oxidation of the sulfur-containing amino acids (SAAs), cysteine and methionine.[16,26] The latter links sulfate to the production of hydrogen sulfide (H_2S), which is a reactive metabolite formed in the so-called transsulfuration pathway. H_2S has been recognised as a cardiovascular signalling molecule with various protective properties.[24] Furthermore, by way of sulfate conjugation, or sulfation, sulfate itself is involved in the biotransformation and detoxification of many endogenous and exogenous substances. Sulfated proteoglycans, for example, are indispensible for the structural integrity of cells and tissues.[7,16] Despite its physiological importance, sulfate has also been connected to pathology through sulfation of toxic intermediates derived form dietary compounds, which in turn have been implicated in carcinogenesis, as well as heart failure events.[4,11,34] Even though sulfation is generally considered to increase hydrophilicity and thereby to promote excretion, the way in which sulfate conjugation relates to the toxicity of these compounds remains to be elucidated.[6]

Sulfate homeostasis is maintained by the kidneys, which freely filter sulfate and then reabsorb it in the proximal tubule. Under normal conditions up to 90% of the filtered sulfate is

reabsorbed.[17,21,30] This active reabsorption is capacity limited, but also dynamic as the expression of the sodium/sulfate co-transporter, NaS1, is up- or down-regulated in response to various stimuli. Known positive stimuli include pregnancy, thyroid hormone, growth hormone, and vitamin D, whereas negative stimuli include the diet sulfate content and medication, such as non-steroidal anti-inflammatory drugs.[17] Furthermore, in children with chronic kidney disease even net secretion of sulfate has been described, underlining the dynamic nature of renal sulfate handling.[20]

The present study aimed at exploring changes in sulfate clearance in CHF patients compared to healthy individuals and to assess its relation to disease outcome.

Materials and method

Study subjects

This study is a post-hoc analysis of an open-label, blinded end point, randomized prospective trial (VitD-CHF trial).[27] In the period of 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. These patients 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). Study participants have previously been described in more detail.[19,27] Patients were randomized to receive either a daily dose of 2000 IU of vitamin D_3 (vitD) or no extra medication for six weeks. For the current analysis baseline samples were used, which were taken before the start of vitD treatment. Due to unavailability of plasma or urine samples, 5 patients had to be excluded, leaving 96 eligible for analysis. For comparison a group of 96 sex-matched healthy subjects was included, consisting of individuals who were

121 approved for living kidney donation in our center. None had a history of cardiovascular
122 events. The study was conducted in accordance with the Declaration of Helsinki and the local
123 Institutional Review Board approved the study protocol. All CHF patients and healthy
124 subjects included in this analysis provided written informed consent.
125
126 *Baseline characteristics*
127 Data on participants' disease state, medical history and medication were extracted from
128 patient records. Systolic and diastolic blood pressure and heart rate were measured according
129 to protocol. The body mass index (BMI) was calculated by dividing body weight by height
130 squared. Participants were instructed to collect 24-h urine the day before visiting the
131 outpatient clinic. On the day of the visit, after an overnight fast, serum and plasma samples
132 were obtained and routine laboratory measurements, including N-terminal pro-B-type
133 natriuretic peptide (NT-proBNP), albumin, total protein, creatinine, urinary albumin,
134 creatinine and sodium, HbA1c, cholesterol, high density lipoprotein (HDL), low density
135 lipoprotein (LDL), calcium, and parathyroid hormone (PTH) were performed. Aliquots of
136 blood and urine samples were stored at -80 °C for future analysis. Measurements of plasma
137 renin concentration (PRC), plasma renin activity (PRA), and aldosterone have been described
138 before.[27] The estimated glomerular filtration rate (eGFR) was determined using the 4-
139 variable Modification of Diet in Renal Disease (MDRD) formula. Creatinine clearance was
140 calculated as follows: (urine concentration [mM] * (volume [mL] of 24-h urine / urine
141 collection time [min])) / plasma concentration [mM].
142
143 *Sulfate clearance* Plasma and urinary sulfate concentrations were measured by means of a
144 validated ion-exchange chromatography assay (type 861; Metrohm, Herisau, Switzerland).
145 The intra- and inter-assay coefficients of variation were 3.4% and 6.1% for plasma and 2.0%

and 4.3% for urine analysis, respectively. Fractional excretion of sulfate was calculated using the following formula: (urinary sulfate [mM] * plasma creatinine [mM]) / (plasma sulfate [mM] * urinary creatinine [mM]) * 100. Sulfate clearance was calculated as follows: (urine concentration [mM] * (volume [mL] of 24-h urine / urine collection time [min])) / plasma concentration [mM].

Outcome parameter

The outcome parameter of this study is composed of HF related rehospitalisation and all-cause mortality. The mean follow-up period was 5.1 ± 0.5 years. No patients were lost to follow-up.

Statistical analysis

Healthy subjects were matched to CHF patients based on sex using point-and-click case-control matching in SPSS (version 22, IBM Corp, Armonk, NY, USA). Statistical analysis was performed with STATA software (version 14.0, Stata Corp, College Station, Texas, USA). Graphs were drawn in GraphPad Prism (version 5.0, GraphPad Software, La Jolla, California, USA).

The distribution of all variables was examined using histograms and probability plots. 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* (%).

The mean age of CHF patients and healthy subjects were compared by means of the Student's t-test. Linear regression analysis was applied to assess the association between sulfate clearance and age among healthy individuals. Differences in plasma and urinary sulfate concentrations, creatinine clearance, fractional excretion of sulfate and sulfate

clearance between CHF patients and healthy subjects were also studied using linear regression analysis, which allowed us to correct for age and, when testing the difference in sulfate clearance, for creatinine clearance.

Univariable and multivariable linear regression analyses were performed on data from CHF patients to identify variables that are independently associated with sulfate clearance. Next, these variables were included in a Cox proportional hazard model. Associations are shown with sulfate clearance as a continuous variable. Hazard ratios are shown per SD increase for normally distributed data and per doubling for logarithmically transformed skewed data.

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

Results

Patient characteristics

Baseline characteristics of the 96 stable CHF patients are presented in table 1. The mean age of the study subjects was 63 ± 10.1 years and 89 (93%) were male. The median duration of HF was 61 (29-106) months. Most patients were categorized in New York Heart Association (NYHA) class II (*n*=85, 89%) and their mean LVEF was 34.8 ± 8.3%. All patients were treated with medication according to the current European Society of Cardiology guidelines, including ACEi/ ARBs (*n*=96, 100%), β-blockers (*n*=93, 97%), MRAs (*n*=28, 29%), and diuretics (mainly furosemide, *n*=49, 49%).

193 **Table 1. Baseline characteristics CHF patients**

Stable CHF patients	
Characteristics	***n*=96**
Age, y	63.4 ± 10.1
Male, *n* (%)	89 (93)
Current smoker, *n* (%)	22 (23)
BMI, kg/m^2	28 ± 4.4
Systolic blood pressure, mmHg	116 ± 16.9
Diastolic blood pressure, mmHg	70.9 ± 10.3
Heart rate, bpm	67.7 ± 9.3
Heart failure history	
Duration HF, m*	61 (29-106)
Ischemic etiology, *n* (%)	68 (71)
NYHA class II/III, *n* (%)	85/11 (89/11)
LVEF (%)	34.8 ± 8.3
Medication	
ACEi/ARB, *n* (%)	96 (100)
β-blocker, *n* (%)	93 (97)
MRA, *n* (%)	28 (29)
Diuretic, *n* (%)	47 (49)
Laboratory measurements	
NT-proBNP, ng/l*	381 (200-904)
Serum albumin, g/l	44.4 ± 2.4
Total serum protein, g/l	72.2 ± 3.9
eGFR, ml/min/1.73m^2	80.6 ± 16.3
Creatinine clearance, ml/min	97.6 ± 31.3
24-h urinary albumin, mg/24 h	41.7 ± 207

24-h urinary sodium, mmol/24 h	166 ± 75.7
HbA1C, %	6.1 ± 0.6
Cholesterol, mmol/l	4.5 ± 1.1
HDL, mmol/l	1.2 ± 0.4
LDL, mmol/l	2.7 ± 0.9
Calcium, mmol/l	2.3 ± 0.1
PTH, pmol/l	7.6 ± 4.0
PRC, ng/l*	58.7 (17.1-195)
PRA, ng/ml/h*	5.1 (1.4-20.6)
Aldosterone, pmol/l*	0.3 ± 0.4
VitD supplementation†	48 (50)
1,25(OH)$_2$D, pmol/l	143.9 ± 44.91

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

†2000 IU of VitD daily for 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, HDL; high density lipoprotein, LDL; low density lipoprotein, PTH; parathyroid hormone, PRC; plasma renin concentration, PRA; plasma renin activity, VitD; vitamin D_3 (cholecalciferol), SD; standard deviation, IQR; inter quartile range

Renal sulfate handling in CHF patients and healthy individuals

Table 2 shows the plasma sulfate concentration, 24-h urinary excretion of sulfate, creatinine clearance, fractional excretion of sulfate and sulfate clearance in CHF patients and healthy subjects. Because of the association between sulfate clearance and age in CHF patients (table 3, coefficient: -0.545, P<0.001), as well as in healthy individuals (coefficient: -0.378, P<0.001) and patients being significantly older compared to healthy subjects (63.4 ± 10.1 vs.

51.9 ± 10.7 year, P<0.001), a correction for age was applied when comparing these groups. Linear regression analysis demonstrated that 24-h urinary sulfate excretion (15.5 ± 6.2 vs. 19.4 ± 6.8, P=0.007), fractional excretion of sulfate (33.1 (25.5-41.9) vs. 35.9 (31.6-42.3), P=0.005) and sulfate clearance (33.7 ± 15.7 vs. 51.0 ± 18.2, P<0.001) are significantly lower in patients, whereas their plasma sulfate concentration (0.34 (0.29-0.37) vs. 0.27 (0.24-0.30), P<0.001) is significantly higher. These differences are illustrated in Figure 1. Even though creatinine clearance (97.6 ± 31.3 vs. 132.6 ± 34.0, P<0.001) is also lower in patients compared to healthy individuals, the difference in sulfate clearance between these groups remained significant after additional adjustment for this parameter (P=0.005).

Table 2. Parameters related to renal sulfate handling in CHF patients and healthy individuals

Parameters	**CHF patients (*n*=96)**	**Healthy subjects (*n*=96)**	**P-value**[†]
Plasma sulfate, mM[*]	0.34 (0.29-0.37)	0.27 (0.24-0.30)	**<0.001**
24-h urinary sulfate, mmol/24 h	15.5 ± 6.2	19.4 ± 6.8	**0.007**
Creatinine clearance, ml/min	97.6 ± 31.3	133 ± 34.0	**<0.001**
Fractional sulfate excretion, %[*]	33.1 (25.5-41.9)	35.9 (31.6-42.3)	**0.005**
Sulfate clearance, ml/min	33.7 ± 15.7	51.0 ± 18.2	**0.001**

Normally distributed continuous data are presented as mean ± SD [*]Skewed data are presented as median (IQR) and were normalized by logarithmic transformation for analysis [†]Based on linear regression analysis, corrected for age

CHF; chronic heart failure, SD; standard deviation, IQR; inter quartile range

Factors associated with sulfate clearance in CHF patients

Univariable and multivariable linear regression analyses showed that in CHF patients use of diuretics, creatinine clearance and 24-h urinary sodium are independently associated with sulfate clearance (table 3).

Table 3. Univariable and multivariable linear regression analyses of sulfate clearance and clinical parameters in CHF

Sulfate clearance				
	Univariable regression		**Multivariable regression**	
Characteristics	**Coefficient**	**P-value**	**Coefficient**	**P-value**
Age	-0.545	**<0.001**		
Male	-11.354	0.066		
Current smoker	-0.975	0.710		
BMI	0.141	0.703		
Systolic blood pressure	0.035	0.713		
Diastolic blood pressure	0.275	0.079		
Heart rate	0.188	0.281		
Heart failure history				
Duration HF*	-0.793	0.649		
Ischemic etiology	-2.542	0.475		
NYHA class II/III	-14.112	**0.005**		
LVEF	-0.040	0.838		
Treatment				
β-blocker	10.838	0.243		
MRA	-5.285	0.136		
Diuretic	-9.389	**0.003**	-4.322	**0.048**
Laboratory measurements				
NT-proBNP*	-6.689	**<0.001**		

Serum albumin	1.877	**0.004**		
Total serum protein	-0.651	0.117		
Creatinine clearance	0.369	**<0.001**	0.331	**<0.001**
24-h urinary albumin	0.046	0.098		
24-h urinary sodium	0.177	**<0.001**	0.077	**0.023**
HbA1C	2.422	0.356		
Cholesterol	1.547	0.315		
HDL	-7.268	0.088		
LDL	1.930	0.271		
Calcium	-21.940	0.257		
PTH	-0.446	0.270		
PRC*	-1.640	0.086		
PRA*	-1.623	0.060		
Aldosterone*	-4.281	**0.032**		
$1,25(OH)_2D$	0.046	0.207		

*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, HDL; high density lipoprotein, LDL; low density lipoprotein, PTH; parathyroid hormone, PRC; plasma renin concentration, PRA; plasma renin activity

Renal sulfate handling and outcome in CHF patients

During follow-up for 5.1 ± 0.5 years, 12 patients (13%) were rehospitalised and 21 patients (22%) died. The composite outcome was recorded 29 times. Sulfate clearance was significantly higher in patients with favourable disease outcome, compared to those who were rehospitalised or died (37.7 ± 15.4 vs. 24.4 ± 12.2, P<0.001). Rehospitalisation and/or death occurred in 8 patients (17%) with above-average sulfate clearance compared to 21 patients

(44%) with below-average levels (log-rank test, P=0.004). The corresponding Kaplan-Meier plot is shown as Figure 2.

As presented in table 4, crude Cox regression analyses showed that 24-h urinary sulfate excretion (HR per SD increase 0.58 (95% confidence interval (CI) 0.39-0.89), P=0.012) and sulfate clearance (HR per SD increase 0.38 (95% CI 0.23-0.63), P<0.001) are positively associated with favourable disease outcome, i.e. a decreased rehospitalisation rate and increased patient survival. Accordingly, the plasma sulfate concentration was found to be negatively associated with disease outcome (HR per doubling 4.45 (95% CI 1.94-10.2), P<0.001).

Table 4. Cox proportional hazards models of the association of the plasma sulfate concentration, 24-h urinary excretion of sulfate, fractional excretion of sulfate and sulfate clearance with disease outcome in CHF

Model	HR (95% CI)	P-value
1: plasma sulfate concentration*	4.45 (1.94-10.2)	**<0.001**
2: 24-h urinary sulfate excretion	0.58 (0.39-0.89)	**0.012**
3: fractional excretion of sulfate*	0.55 (0.26-1.19)	0.130
4: sulfate clearance	0.38 (0.23-0.63)	**<0.001**

For normally distributed data the HR is presented per SD increase *For logarithmically transformed skewed data the HR is presented per doubling

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

While adjustment for use of diuretics and 24-h urinary sodium excretion only marginally affected the association of sulfate clearance with favourable disease outcome, adjustment for creatinine clearance caused significance to be lost (table 5, model 6, HR per SD increase 0.59 (0.30-1.18), P=0.140).

Table 5. Cox proportional hazards model of the association of sulfate clearance with disease outcome in CHF, adjusted for associated clinical parameter separately and in conjunction

Sulfate clearance		
Model	**HR* (95% CI)**	**P-value**
1: crude	0.38 (0.23-0.63)	**<0.001**
2: adjusted for diuretics use	0.42 (0.25-0.71)	**0.001**
3: adjusted for diuretics use and creatinine clearance	0.71 (0.35-1.44)	0.344
4: adjusted for diuretics use and 24-h urinary sodium	0.43 (0.25-0.75)	**0.003**
5: adjusted for diuretics use, creatinine clearance and 24-h urinary sodium	0.72 (0.35-1.45)	0.354
6: adjusted for creatinine clearance	0.59 (0.30-1.18)	0.140
7: adjusted for creatinine clearance and 24-h urinary sodium	0.60 (0.30-1.19)	0.149
8: adjusted for 24-h urinary sodium	0.39 (0.23-0.67)	**0.001**

HR per SD increase

CHF; chronic heart failure, HRs; hazard ratio, CI; confidence interval, SD; standard deviation

Table 6 shows that the association of sulfate clearance with favourable disease outcome is independent of established prognostic factors in HF, age, eGFR and NT-proBNP (model 4, HR 0.55 (95% CI 0.31-0.98), P=0.042).

Table 6. Cox proportional hazards model of the association of sulfate clearance and established prognostic factors with disease outcome in CHF

Sulfate clearance		
Model	**HR* (95% CI)**	**P-value**
1: crude	0.38 (0.23-0.63)	**<0.001**
2: adjusted for age	0.46 (0.27-0.79)	**0.005**
3: adjusted for age and eGFR	0.48 (0.28-0.83)	**0.009**
4: adjusted for age, eGFR and NT-proBNP	0.55 (0.31-0.98)	**0.042**

*HR per SD increase

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

Discussion

This exploratory study indicates that aberrant sulfate clearance is a potential contributor to the pathophysiology of CHF. Specifically, our data show that, compared to healthy individuals, CHF patients exhibit decreased sulfate clearance. Furthermore, among patients we have found sulfate clearance to be positively associated with favourable disease outcome, i.e. a decreased rehospitalisation rate and increased patient survival. After adjustment for creatinine clearance this association was no longer significant. Sequentially, one might assume that the association of sulfate clearance with disease outcome is explained by its relationship with creatinine clearance, i.e. renal function. In this respect, it is important to note that creatinine clearance itself is very strongly associated with disease outcome in HF and thus leaves limited room for other variables to improve the proportional hazards model. Combined with limited statistical power, this likely explains the non-significant P-value for the association of sulfate clearance with disease outcome when creatinine clearance is corrected for. Accordingly, in a larger cohort the association may well have remained significant. This assumption is substantiated by the fact that the decrease of sulfate clearance in patients compared to healthy individuals is

299 independent of creatinine clearance, indicating that sulfate clearance is not merely a reflection
300 of renal function and potentially provides new insight into the pathophysiology of HF.
301 While creatinine clearance caused significance to be lost, the association between sulfate
302 clearance and disease outcome did retain its significance after adjustment for established
303 prognostic factors in HF, age, eGFR, and NT-proBNP. This not only incites interest in the
304 renal handling of sulfate, but also underscores the need for cautiousness when eGFR is used
305 to represent renal function. The latter applies to disease cohorts in particular, as in patients
306 plasma creatinine levels may deviate, for example due to lower muscle mass or increased
307 catabolism.
308 The potential relevance of renal sulfate handling to CHF pathology lies in the connection of
309 sulfate to H_2S metabolism and the physiological importance of sulfation on the one hand and
310 the apparent need for regulation of sulfate homeostasis and the implication of sulfated toxic
311 intermediates in HF on the other.
312 To the best of our knowledge, we are the first to assess sulfate clearance in CHF patients. In
313 general, very few studies have related renal handling of sulfate to outcome of disease. As
314 mentioned above, a study of renal transplant recipients has reported urinary sulfate excretion
315 to be associated with a favourable cardiovascular risk profile.[2] Furthermore, in patients with
316 type 1 and type 2 diabetes a high urinary sulfate concentration has been found to be associated
317 with a reduced risk of renal disease progression.[1,3]
318 It is unclear whether urinary sulfate excretion primarily reflects intake and production of
319 sulfate or rather the rate of sulfate clearance by the kidneys. Our study unfortunately lacks
320 information on dietary intake. However, in renal transplant recipients and patients with
321 diabetes mellitus type 1 nephropathy the association of sulfate excretion with all-cause
322 mortality or renal disease progression was shown to be independent of dietary protein
323 intake.[1,2] Perhaps sulfate production through oxidation of SAAs is a stronger determinant,

especially because of the intermediate enzymatic production of H_2S as part of this process that could explain the benefit of high urinary sulfate excretion.[13] In relation to this, degradation of non-enzymatically produced H_2S, through reduction of bound sulfur and by sulfate reducing bacteria in the gut, represents another source.[12,29] H_2S is a gaseous signalling molecule involved in numerous (patho)physiological processes, featuring vasodilatory, angiogenic, anti-apoptotic, anti-inflammatory and antioxidant properties.[23,28,33] Accordingly, various preclinical studies have convincingly shown H_2S to be protective in HF.[24] Unfortunately, reliable methods to capture with confidence the entire pool of H_2S in biological samples are lacking, which impedes determination of the exact relationship between sulfate and H_2S production. The only way to accomplish this experimentally would involve the use of radio- or stable-isotope tracer methodology, which to the best of our knowledge has not been carried out to quantify H_2S metabolism to sulfate in humans. Besides, sulfate itself is an essential anion. The physiological importance of sulfation, pointed out above, is emphasized by the necessary increase of circulating sulfate concentrations in pregnancy.[8] As the foetus is unable to generate sulfate, it relies completely on the maternal sulfate supply. Hyposulfatemia and consequent inadequate sulfation capacity has been linked to foetal growth abnormalities and even intrauterine death.[8] The maternal increase of the serum sulfate concentration is mediated through up-regulation of sulfate reabsorption in the kidneys, demonstrating the importance of renal handling to the regulation of sulfate homeostasis.[5] The implication of sulfated toxic intermediates, such as indoxyl sulfate and p-cresyl sulfate, in HF led us to suppose that in fact sulfate clearance would be of interest in this context. As sulfation is generally considered to increase hydrophilicity and thereby to promote excretion of its targets, decreased sulfate clearance in HF may reflect a need for sulfate-mediated detoxification.[6] However, the way in which sulfate conjugation relates to the toxicity of these specific compounds has not been shown. Furthermore, the benefit of

increased sulfate clearance is substantiated by the finding that decreased sulfate reabsorption by knockout of the sodium/sulfate co-transporter, NaS1, in mice resulted in an increase of their lifespan by approximately 25%.[18] This was shown to be associated with significant up-regulation of anti-ageing genes, including *Sirt1*, *Hdac3* and *Cat*, which other studies have linked to HF. Indeed, *Sirt1* has been reported to be down-regulated in cardiomyocytes of patients with advanced HF.[14] Also, aberrant expression of the enzymes encoded by *Hdac3* and *Cat* has been shown to affect myocardial lipid metabolism and antioxidant defence, respectively.[9,31,32]

NaS1 has a major role in regulating the reabsorption of sulfate in the proximal tubule.[17] As it facilitates sodium/sulfate co-transport, the association we have found between 24-h urinary sodium excretion and sulfate clearance is unsurprising. While one could argue that in HF sodium excretion is beneficial, as opposed to sodium retention, this could only in part explain the association of sulfate clearance with favourable disease outcome. Hence, as is the case for creatinine clearance, sulfate clearance is not just a reflection of sodium excretion either, and presents an independent benefit in CHF patients.

To our knowledge, a relationship between use of diuretics and renal handling of sulfate has not been described. While the need to use a diuretic may simply reflect severity of disease, the association may also be related to the tubular sodium/sulfate co-transport. In this regard, sulfate clearance, through sodium excretion, may positively affect volume status, thereby decreasing the need to use a diuretic. Conversely, decreased sodium reabsorption by specific transporters, induced by a diuretic, perhaps results in a compensatory increase of sodium/sulfate co-transport, decreasing sulfate clearance.

Whereas a study in vitamin D deficient rats has shown supplementation to up-regulate NaS1 expression and, consequently, sulfate reabsorption, in our study vitamin D and sulfate clearance were not found to be associated. In contrast to the rats, CHF patients had relatively

high vitamin D levels at baseline, suggesting that vitamin D is relevant to sulfate reabsorption only in the case of a deficiency.[10]

Besides NaS1, two anion exchangers, Sat1 and CFEX, are also known to be involved in tubular sulfate reabsorption.[17] Changes in the expression of these transporters have been linked to disease states, including chronic renal failure and hypothyroidism.[16] The extent to which aberrant sulfate clearance in HF can be attributed to altered expression of sulfate transporters in the kidney remains to be elucidated. However, as sulfate reabsorption is capacity limited, without up-regulation of tubular transport, one would expect the filtered load to start exceeding the reabsorption capacity with increasing plasma sulfate levels, resulting in increased fractional excretion.[17] Interestingly, we have found fractional excretion to be lower in patients compared to healthy individuals in spite of higher plasma sulfate concentrations, suggesting that tubular reabsorption is up-regulated in CHF.

Regardless of the involvement of specific transporters in the renal handling of sulfate, the exact mechanism through which a higher sulfate clearance leads to an improved outcome in HF is yet unclear. Hence, further research is warranted to substantiate the role of sulfate clearance in HF pathology.

In addition to the lack of dietary information, our study has other limitations. First of all, statistical power is limited by the small size of the CHF cohort and number of times the composite outcome was recorded. Furthermore, it is a single center study of Caucasian subjects, confining the validity of extrapolating our results to other ethnicities. Also, our study population mainly consists of males, precluding analysis of gender-related differences. Finally, because of its cross-sectional design, possible causality of the relationship between sulfate clearance and disease outcome could not be examined. Strengths of this study include the homogenous and extensive characterization of the CHF patients and the relatively long follow-up period of on average 5.1 years.

In conclusion, sulfate clearance is reduced in CHF patients compared to healthy subjects and positively associated with favourable disease outcome among patients. Aberrant sulfate clearance may thus serve as a new lead to advance our understanding of the pathophysiology of HF. Further research is needed to unravel the nature of its involvement and to determine its potential as a biomarker and target for therapy.

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Disclosures

Conflicts of interest: none declared.

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Figure legends

Figure 1. Parameters related to renal sulfate handling in CHF patients and healthy individuals

In CHF patients, plasma sulfate concentration (A) is significantly higher, whereas 24-h urinary sulfate excretion, fractional excretion of sulfate and sulfate clearance (B-D) are significantly lower compared to healthy individuals.

Normally distributed data are presented as mean ± SD (D), Skewed data are presented as median (IQR) (A-C)

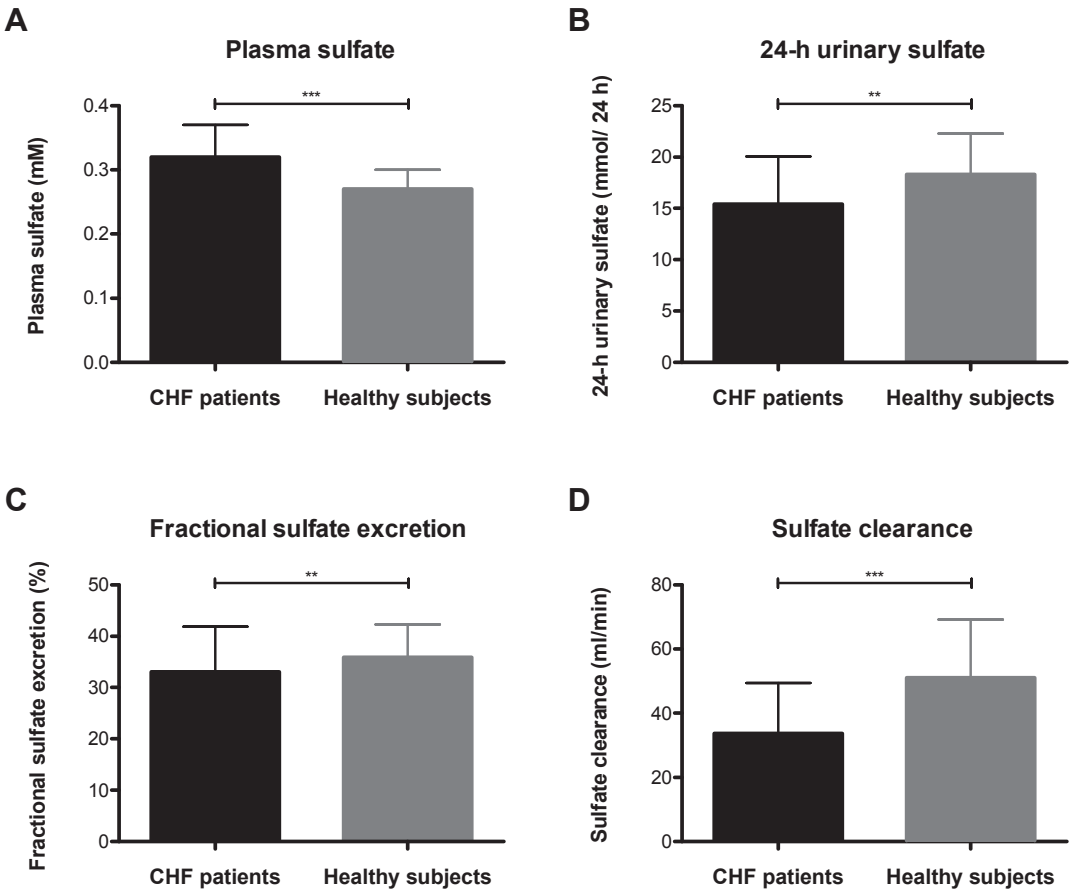
P-values are based on linear regression analysis, corrected for age, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

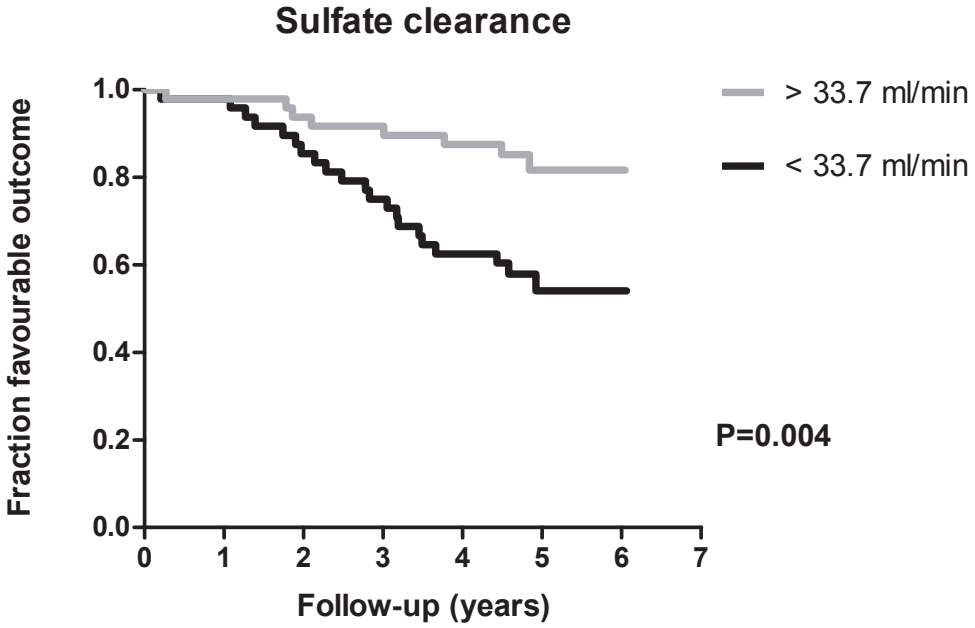
CHF; chronic heart failure

Figure 2. Kaplan-Meier analysis of the association of sulfate clearance 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). Sulfate clearance above the mean is significantly associated with favourable outcome in stable CHF patients (P=0.004).

CHF; chronic heart failure


A
Plasma sulfate
Plasma sulfate (mM)
0.4
0.3
0.2
0.1
0.0
***
CHF patients
Healthy subjects
B
24-h urinary sulfate
24-h urinary sulfate (mmol/ 24 h)
25
20
15
10
5
0
**
CHF patients
Healthy subjects
C
Fractional sulfate excretion
Fractional sulfate excretion (%)
50
40
30
20
10
0
**
CHF patients
Healthy subjects
D
Sulfate clearance
Sulfate clearance (ml/min)
80
60
40
20
0
***
CHF patients
Healthy subjects


Sulfate clearance
Fraction favourable outcome
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
7
Follow-up (years)
> 33.7 ml/min
< 33.7 ml/min
P=0.004