Exploring Redox Biology in physiology and disease

Koning, Anne

Chapter 11

Understanding the renal handling of nitrite and nitrate in health and disease

Anne M. Koning*
Bernadette O. Fernandez*
Michelle A. Miller
Clive Osmond
Wouter C. Meijers
Chen Ji
Pasquale Strazzullo
Leonie H. Venema
Magdalena Minnion
Melanie Madhani
Michael P. Frenneaux
Malte Kelm
Gerjan Navis
Rudolf de Boer
Francesco P. Cappuccio
Harry van Goor#
Martin Feelisch#

Manuscript in preparation

**These authors contributed equally #These authors share senior authorship*

Abstract

Background and Aims

Circulating nitrite and nitrate concentrations are frequently used as markers of endogenous nitric oxide (NO) formation. Although excretion is as important as production in determining the steady-state concentration of a biomarker, surprisingly little is known about the handling of either anion by the kidneys. The current assumption that both anions are handled in a similar fashion lacks experimental evidence. In the present study we therefore sought to elucidate the renal handling of nitrite and nitrate in humans and to explore its potential relevance for clinical outcome.

Methods

Nitrite and nitrate plasma concentrations, and 24-h urinary excretion, fractional excretion and clearance rates were determined in three separate study populations. The first, a multi-ethnic population-based cohort (n=457) provided data on gender- and ethnicity-related variations. In the second cohort, consisting of living kidney donors (n=101), the influence of changes in renal function was assessed. Finally, a cohort comprising stable chronic heart failure (CHF) patients (n=95) was used to evaluate the clinical relevance of varying nitrite and nitrate handling by the kidneys.

Results

Renal nitrite and nitrate handling varied with gender and ethnicity, independent of renal function. In general, only ~1% of filtered nitrite and ~40% of filtered nitrate was excreted in the urine. Median clearance was 0.89 (IQR 0.10-5.12) ml/min for nitrite and 31.4 (IQR 22.3-44.7) ml/min for nitrate. Nitrate, but not nitrite, clearance was found to be positively associated with disease outcome in CHF patients.

Conclusion

Nitrite and nitrate are handled by the kidneys in separate ways. The positive association of nitrate, but not nitrite, clearance with outcome of disease in CHF patients shows the clinical relevance of this distinction. Further research is warranted to assess the cause-effect relationship of this association and to unravel the mechanisms involved in renal nitrite and nitrate handling.

1 Introduction

Cardiovascular disease (CVD) remains a leading cause of death and disability worldwide, and early identification of individuals at risk is a public health priority(1). Endothelial dysfunction has been identified as a crucial risk factor, but currently used procedures to assess vascular function are unsuitable for routine population screening. Blood or urine-based diagnostic tests would be desirable, yet approaches using contemporary biomarkers of cardiovascular (CV) risk have met with limited success. This is due, in part, to an earlier emphasis on indicators of organ/tissue damage rather than mechanism-based markers. Reduced availability of nitric oxide (NO) appears to be a key factor not only in vascular dysfunction but also in the perturbations in endocrine and mitochondrial signalling, underpinning the clustering of risk factors of metabolic origin defined as the "metabolic syndrome".(2–4) Several pathologies are associated with impaired or enhanced NO formation, and circulating levels of nitrite (NO_2^-) and nitrate (NO_3^-) have been used, often in combination (NOx), as markers of NO formation in the clinical setting. While nitrate is an abundant constituent of a healthy diet containing green leafy vegetables, its level in drinking water and certain foods is strictly regulated for fear of haemoglobin oxidation and cancer development. However, recent experimental observations of organ protective effects of nitrite and blood pressure-lowering effects of nitrate suggest that these anions benefit health.(5,6) In particular, the discovery of mammalian nitrite and nitrate reductase activities has given rise to the hope that therapeutic nitrite or nitrate administration followed by reductive bio-activation may enhance NO availability. Due to analytical challenges, however, our knowledge about biological effects in response to changes in nitrite and nitrate availability around the physiological level remains limited.

Now that the analytical pitfalls that challenged earlier studies have been addressed, the time has come to assess the prognostic utility of nitrite and nitrate in the cardiovascular arena. This, however, clearly requires a better understanding of the transport and elimination of nitrite and nitrate *in vivo*.(7) Both anions are assumed to be handled in an identical fashion by the kidneys, but this has never been formally investigated. Moreover, gender differences in endothelial function and CV risk are usually attributed to hormonal differences affecting NO production, rather than variations in elimination of its oxidative breakdown products.(8) Although excretion is as important as production in determining the steady-state concentration of a biomarker, and circulating NOx levels are elevated in renal insufficiency, very few studies have taken renal clearances into account, and virtually nothing is known about inter-individual variability in nitrite and nitrate clearances or differences between ethnic groups.(9,10) We therefore sought to determine renal clearance of nitrite and nitrate using paired blood and urine samples from a multi-ethnic, population-based, epidemiological study. In addition, renal handling of nitrite and nitrate was evaluated in healthy kidney donors, providing the unique opportunity to monitor the consequences of a decreased renal function in the same, healthy individual. Finally, in a cohort of chronic heart failure (CHF) patients, the clinical relevance of nitrite and nitrate handling by the kidneys was explored.

Our results challenge the current notion that renal nitrite and nitrate handling are identical, and opposing associations of nitrite and nitrate clearance with outcome of disease underscore the clinical relevance of their distinction.

2 Methods

2.1 Study populations

This study describes three study populations; the first is derived from a multi-ethnic, population-based survey, the Wandsworth Heart and Stroke Study (*n*=457), the second consists of healthy kidney donors (*n*=101), and the third is a cohort of stable CHF patients from the VitD-CHF trial (*n*=95). These populations have all been described in detail elsewhere.(11–14)

The Wandsworth study was carried out between 1994 and 1996 in South-West London to estimate the prevalence of cardiovascular risk and included men and women between 40 and 59 years of age belonging to three distinct ethnic groups: Caucasians, Blacks (West Africans or Carribeans), and South Asians (South Asians/Indians).(11,12) All participants of ethnic minority groups were first-generation immigrants. The entire study population consisted of ~250 people of each gender and ethnic group. Of a total of 1577 participants, 664 individuals were without pre-existing CVD and not taking any medication, and 457 of those provided fasted blood and timed urine samples on the same day. The latter were used for analysis in the present study.(15) An age > 50 years in women was used to define post-menopausal status without individual verification.

The second study cohort comprised healthy human individuals approved for living kidney donation in the University Medical Center Groningen (UMCG), the Netherlands. None had a history of kidney disease, CV events or diabetes. In the case of hypertension, this was treated with a maximum of one antihypertensive drug.(13)

The third study involved a cohort belonging to an open-label, blinded end point, randomized prospective VitD-CHF trial.(14) In the period between March 2010 and November 2011, 101 stable CHF patients presenting at the outpatient clinic of the UMCG, the Netherlands were included in this trial. 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). 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 matched plasma or urine samples, 6 patients had to be excluded, leaving 95 eligible for analysis.

All three studies were conducted in accordance with the Declaration of Helsinki. The study protocols were approved by the local ethics committees, and each subject provided written informed consent to participate. Data on participant's disease state, medical history and medication were extracted from patient records. For detailed descriptions on the recordings

of anthropometric and hemodynamic variables and routine laboratory measurements, the reader is referred to earlier descriptions of these study populations.(11–14)

2.2 Nitrite and nitrate measurements

Nitrite and nitrate concentrations in plasma and urine were determined by high-performance liquid chromatography (HPLC) using a dedicated analysis system comprising of an ion chromatograph with on-line reduction and post-column derivatization (ENO-20, EiCom), an automated sample injector (234, Gilson), and a computerized data acquisition and analysis system (PowerChrom, ADInstruments). Aliquots of EDTA plasma samples were supplemented with N-ethylmaleimide (NEM, in PBS; 10 mM final concentration), vortexed, and deproteinised with ice-cold methanol (1:1 v/v), cleared by centrifugation and immediately subjected to analysis; urine was analysed following methanol-precipitation without dilution, for nitrite, and after 1:50 dilution with PBS, for nitrate. Sample processing was performed in a staggered fashion to ensure reproducible processing times. Repeated daily calibrations were carried out. The lower limits of detection and quantitation of this method were 0.005 and 0.015 µM, respectively, at an injection volume of 20 µl. Intra- and inter-day coefficients of variation corresponded to 2.1 and 3.9% for nitrite and 6.3 and 15.2% for nitrate, respectively (as determined over 5-7 days across two separate analysis systems running in parallel).

2.3 Calculations and statistical analysis

Creatinine, as well as nitrite and nitrate clearances were calculated as follows: (urine concentration (mM) * (volume (ml) / urine collection time (min))) / plasma concentration (mM). Fractional excretion of nitrite and nitrate were determined by dividing their clearances by the corresponding creatinine clearance, and multiplying it by 100. For kidney donors and CHF patients 24-h urine samples were used. For the Wandsworth Heart and Stroke Study subjects' 24-h urine samples were not available, and 24-h urinary excretion was estimated on the basis of timed urinary excretion obtained at the time of blood collection.

Statistical analyses were performed using SPSS (version 22, IBM Corp, Armonk, NY, 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 prior to analysis. Nominal data are presented as *n* (%).

Parameters related to the renal handling of nitrite and nitrate were compared between sexes and ethnicities by means of the Mann-Witney U test, the Kruskal-Wallis test, One-way ANOVA, or the Student's t-test, when appropriate. Univariable linear regression analysis was applied to assess associations of nitrite and nitrate clearance and baseline characteristics in the Wandsworth Study subjects. Differences between CHF patients and healthy kidney donors were also studied using linear regression analysis, which allowed correction for sex, age and creatine clearance. Cox proportional hazards analyses were performed to assess the associations of parameters related to the renal handling of nitrite and nitrate with outcome of disease. A Kaplan Meier plot and log rank test were used to illustrate the association of nitrate

clearance above and below the median with outcome of disease. Univariable and multivariable linear regression analyses were performed on data from CHF patients to identify variables that are independently associated with nitrate clearance. Next, these variables were included in a Cox proportional hazard model. Associations are shown with nitrate clearance above and below the median. All reported P-values are two-tailed, and values of $P<0.05$ were considered statistically significant.

3 Results

3.1 Baseline characteristics of study populations

Table 1 summarizes the baseline characteristics of the three populations described in this study. Whereas the cohorts of kidney donors and CHF patients consist only of Caucasian subjects, the Wandsworth study provides data on Caucasian, African and South Asian participants. Additional baseline characteristics of CHF patients related to their disease are provided in Table 4.

Table 1: Baseline characteristics of study populations

Characteristics	**Wandsworth Study** *n*=457	**Kidney donors** *n*=101	**CHF patients** *n*=95
Ethnicity, *n* (%)			
Caucasian	174 (38)	101 (100)	95 (100)
African	137 (30)	0 (0)	0 (0)
South Asian	146 (32)	0 (0)	0 (0)
Male, *n* (%)	238 (52)	49 (49)	88 (93)
Age, y	49.0 ± 5.9	52.8 ± 9.5	63.5 ± 10.2
Current smoker, *n* (%)	94 (21)	21 (21)	21 (22)
BMI, kg/m^2	25.7 ± 4.3	26.0 ± 3.5	28.1 ± 4.4
SBP, mmHg	123 ± 17.3	121 ± 14.1	116 ± 17.1
DBP, mmHg	81.5 ± 9.64	75.0 ± 8.79	71.1 ± 10.7
Hypertension, *n* (%)	44 (10)	33 (32.7)	nd#
Diabetes, *n* (%)	0 (0)	0 (0)	14 (15)
Glucose, mmol/l	5.0 ± 0.6	5.4 ± 0.5	nd
Cholesterol, mmol/l	5.8 ± 1.1	5.2 ± 1.1	4.4 ± 1.0
HDL, mmol/l	1.3 ± 0.4	nd	1.2 ± 0.4

Normally distributed data is presented as mean ± SD ˚Skewed data are presented as median (IQR) #could not be determined as all CHF patients are prescribed blood pressure lowering medication irrespective of hypertension CHF; chronic heart failure, BMI; body mass index, SBP; systolic blood pressure, DBP; diastolic blood pressure, HDL; high-density lipoprotein, nd; not determined

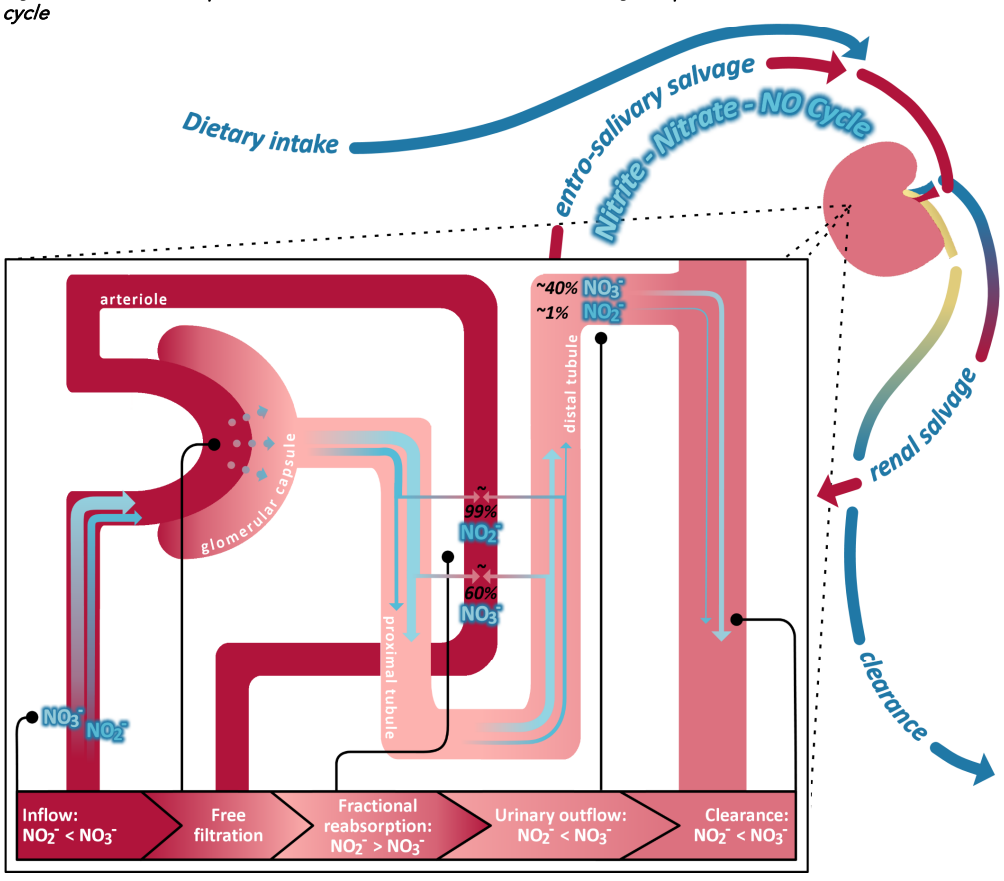
4 Renal handling of nitrite and nitrate in the general population: the Wandsworth study

4.1 Nitrite and nitrate concentrations in blood and urine

Fasting plasma concentrations of nitrite and nitrate were in the sub-micromolar and tens of micromolar range, respectively. The median plasma nitrite concentration was 0.73 (0.37-1.62) µmol/l, compared to a median plasma nitrate concentration of 25.6 (20.2-34.5) mmol/l.

All analysed urine samples contained low, but constitutive levels of nitrite. Median 24-h urinary nitrite excretion was 0.74 (0.14-6.15) µmol/24 h, while the excretion of nitrate was much more pronounced (1.12 (0.81-1.76) mmol/24 h).

Figure 1: Schematic representation of renal nitrite and nitrate salvage as part of the nitrite-nitrate-NO cycle



Schematic representation of the differential renal handling of nitrite and nitrate. A median of 98.9% of filtered nitrite and 60.4% of filtered nitrate undergoes tubular reabsorption, suggesting that both anions are required for a yet undefined but physiologically meaningful process. We now show that, next to the well-known entero-salivary pathway, tubular reabsorption in the kidney represents another important route of nitrite and nitrate salvage.

4.2 Differential renal handling of nitrite and nitrate and associated subject characteristics

Fractional excretion and clearance rates of nitrite and nitrate differ considerably. Whereas only 1.1 (0.1-7.0)% of filtered nitrite is excreted, 39.6 (27.6-51.3)% of filtered nitrate is found in urine. Correspondingly, a median of 98.9% of nitrite and 60.4% of nitrate is salvaged by tubular reabsorption (Fig. 1). Filtered load and fractional reabsorption result in a median clearance rate of 0.89 (0.10-5.12) ml/min for nitrite and 31.4 (22.3-44.7) ml/min for nitrate.

Sex adjusted linear regression analysis showed that nitrite and nitrate clearance are associated with distinct subject characteristics (Tab. 2). Only nitrite clearance was found to be inversely associated with hypertension and fasting glucose levels. While both nitrite and nitrate clearance showed a relationship with BMI, this was inverse for nitrite and positive for nitrate.

Table 2: Univariable linear regression analyses of nitrite and nitrate clearance in and baseline characteristics of subjects from the Wandsworth study

	Univariable regression			
	Nitrite clearance*		**Nitrate clearance***	
Characteristics	**Coefficient**	**P-value†**	**Coefficient**	**P-value†**
Age	-0.018	0.698	-0.069	0.141
Current smoker	-0.084	0.076	0.023	0.638
BMI	-0.107	**0.026**	0.142	**0.003**
SBP	0.064	0.168	-0.007	0.883
DBP	0.062	0.187	0.044	0.357
Hypertension	-0.118	**0.011**	-0.059	0.211
Glucose	-0.127	**0.006**	0.074	0.117
Cholesterol	0.001	0.980	-0.033	0.481
HDL	0.079	0.115	0.081	0.111

**Skewed data were normalized by logarithmic transformation for analysis †Based on linear regression analysis, corrected for sex, BMI; body mass index, SBP; systolic blood pressure, DBP; diastolic blood pressure, HDL; high-density lipoprotein*

4.3 Difference in the renal handling of nitrite and nitrate by gender and ethnicity

Gender- and ethnicity-related differences in the renal handling of nitrite and nitrate are described in Table 3 and illustrated in Figure 2.

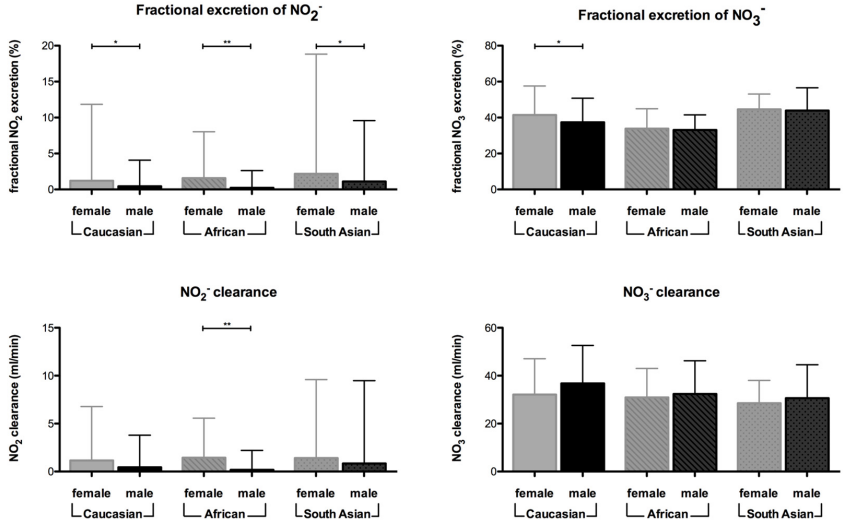
While plasma nitrite concentrations were similar for men and woman from all ethnic groups, 24-h urinary excretion of nitrite tended to be lower in males compared to females. Even though this difference only reached statistical significance in Africans, it is in line with the lower fractional excretion of nitrite in males compared to females, which was found to be statistically significant across ethnicities. Similarly, nitrite clearance tended to be lower in males, with a significant difference between African male and female subjects. Fractional excretion rates of nitrite did not differ between pre- and post-menopausal women (1.1 (0.2-11.0) vs. 2.0 (0.3-4.2)%, respectively, P=0.189), suggesting that the observed difference between sexes was unrelated to hormonal status.

Regarding the renal handling of nitrate, the only gender-related difference was a lower fractional excretion in males compared to females among Caucasians. Despite similar plasma levels, the relatively low fractional excretion was not accompanied by a lower clearance rate for nitrate, probably because it was compensated for by the coinciding greater renal function in Caucasian males compared to females. Fractional excretion of nitrate was the same for Caucasian pre- and post-menopausal women (40.5 (32.2-55.6) vs. 42.1 (28.9-65.5)%, respectively, P=0.918).

Plasma nitrite concentrations varied between ethnic groups for females only (P=0.012), with the highest levels in Caucasian and the lowest levels in South Asian subjects, which could not be explained by differences in renal function or fractional excretion of nitrite. Among males, fractional nitrite excretion was found to be highest in South Asian and lowest in African subjects.

Plasma nitrate concentrations differed between ethnic groups for both females and males (P=0.046 and P=0.049, respectively). While the lowest levels were seen in Africans of both sexes, the highest levels were found in South Asian females and Caucasian males. In females, the variation in plasma nitrate concentrations may be connected to ethnicity-related differences in fractional excretion of nitrate, which showed a similar distribution. In males, higher plasma concentrations were accompanied by higher 24-h urinary excretion rates, suggesting differences in the metabolic turnover of nitrate between males of different ethnic groups. Finally, fractional excretion of nitrate was highest in males of South Asian and lowest in males of African origin.

Figure 2: Gender and ethnicity-related differences in the fractional excretion and clearance of nitrite and nitrate in the Wandsworth study



*Gender- and ethnicity related differences in the renal handling of nitrite (left panel) and nitrate (right panel). Data are presented as median (IQR). P-values are shown for differences between sexes, *P<0.05, **P<0.01. For P-values regarding ethnicity-related differences, the reader is referred to Table 3.*
NO_2^-; nitrite, NO_3^-; nitrate

Table 3: Parameters related to the renal handling of nitrite and nitrate in the Wandsworth study categorized by gender and ethnicity

	Caucasian *n*=174			**African** *n*=137			**South Asian** *n*=146				
Parameters	**female** *n*=77	**male** *n*=97	**P-value**	**female** *n*=76	**male** *n*=61	**P-value**	**female** *n*=66	**male** *n*=80	**P-value**	**P-value all females**	**P-value all males**
Plasma nitrite, µmol/l*	0.85 (0.43-1.80)	0.94 (0.45-1.74)	0.679	0.65 (0.34-1.42)	0.97 (0.38-1.55)	0.311	0.55 (0.29-1.01)	0.67 (0.29-1.74)	0.095	**0.012**	0.254
Plasma nitrate, µmol/l*	26.4 (21.0-35.1)	27.1 (21.8-37.4)	0.415	22.7 (17.0-32.4)	24.1 (17.4-32.1)	0.668	27.3 (21.6-35.8)	25.9 (20.2-35.4)	0.534	**0.046**	**0.049**
24-h urinary nitrite, µmol/24 h#*	1.22 (0.21-7.47)	0.60 (0.13-5.97)	0.106	0.71 (0.15-6.45)	0.27 (0.05-2.08)	**0.024**	1.56 (0.23-9.44)	0.76 (0.09-6.90)	0.232	0.781	0.183
24-h urinary nitrate, mmol/24 h#*	1.12 (0.82-2.00)	1.47 (1.03-2.00)	0.059	0.94 (0.76-1.32)	1.04 (0.77-1.75)	0.408	1.00 (0.77-1.53)	1.24 (0.71-1.76)	0.479	0.156	**0.005**
Creatinine clearance, ml/min	81.9 ± 26.6	107.5 ±32.5	**<0.001**	92.7 ± 32.3	103.0 ± 33.7	0.076	67.9 ± 24.5	76.4 ± 27.2	0.055	**<0.001**	**<0.001**
Fractional nitrite excretion, %*	1.21 (0.24-11.8)	0.44 (0.07-4.08)	**0.016**	1.58 (0.13-8.03)	0.22 (0.06-2.63)	**0.005**	2.18 (0.41-18.8)	1.11 (0.12-9.58)	**0.047**	0.206	**0.045**
Fractional nitrate excretion, %*	41.4 (30.2-57.6)	37.4 (24.5-50.8)	**0.034**	33.8 (27.1-44.9)	33.1 (25.3-41.5)	0.350	44.6 (32.7-53.1)	43.9 (32.0-56.6)	0.877	**0.010**	**0.001**
Nitrite clearance, ml/min*	1.16 (0.20-6.78)	0.45 (0.08-3.80)	0.076	1.44 (0.11-5.57)	0.18 (0.06-2.20)	**0.008**	1.41 (0.23-9.60)	0.85 (0.07-9.47)	0.087	0.498	0.204
Nitrate clearance, ml/min*	32.1 (23.6-47.1)	36.7 23.6-52.6)	0.208	30.9 (21.7-43.0)	32.4 (23.0-46.2)	0.703	28.5 (19.4-38.0)	30.6 (19.7-44.6)	0.203	0.168	0.136

*Normally distributed data are presented as mean ± SD *Skewed data are presented as median (IQR) #estimated on the basis of timed urinary excretion*

5 Renal handling of nitrite and nitrate; lessons from living kidney donors

5.1 Nitrite and nitrate concentrations and renal handling in kidney donors before organ donation

Table 4 summarizes the data on parameters related to the renal handling of nitrite and nitrate in healthy kidney donors pre and post donation. Pre donation, all nitrate-associated values are similar to those found in the Wandsworth study, whereas median plasma concentration as well as 24-h urinary excretion of nitrite was about 10 times lower in samples from the kidney donors compared to the Wandsworth study subjects. The reasons behind this finding are currently unclear, but likely relate to differences in blood processing rather than reflect real differences in steady-state levels (nitrite is subject to rapid oxidation unless separated from blood cells within a few minutes after blood collection). The gender-related differences in the fractional excretion of nitrite and nitrate found in Caucasians from the Wandsworth study could not be confirmed, although similar trends were observed.

5.2 Changes in the renal handling of nitrite and nitrate following the removal of one kidney

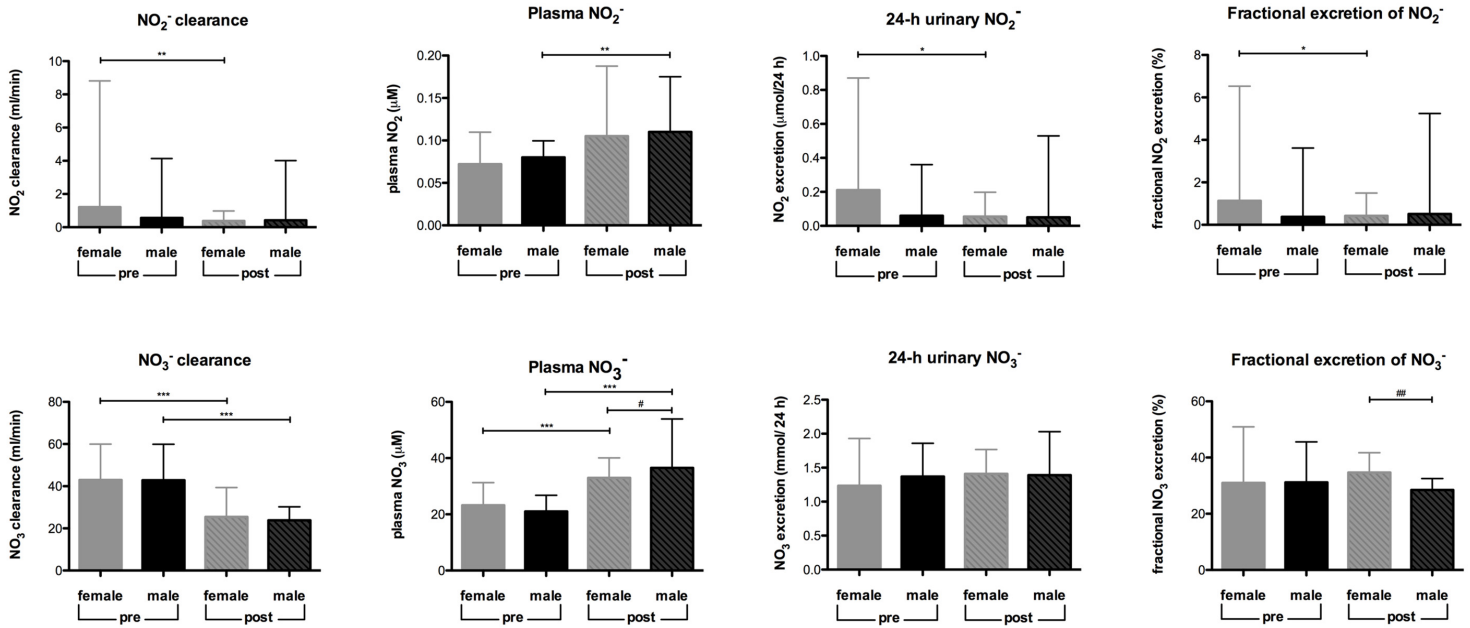
Changes in the renal handling of nitrite and nitrate in kidney donors following the removal of one kidney are described in Table 4 and depicted in Figure 3. These changes are remarkably similar to the adaptations of the renal handling of creatinine following kidney donation, depicted in Figure 4. The data illustrate that the clearance of nitrite and nitrate are equal to the glomerular filtration rate (GFR) minus the fractional tubular reabsorption. Together, these mechanisms maintain a steady-state in which urinary excretion matches the nitrite and nitrate turnover, resulting in relatively constant plasma concentrations. Following donation, a new steady state is reached in which urinary excretion is preserved by means of increased plasma levels which ensure increased filtered loads, thereby compensating for the decreased GFR as a consequence of the removal of one of the two kidneys.

Table 4: Parameters related to the renal handling of nitrite and nitrate in female and male kidney donors pre and post donation

	Pre kidney donation			**Post kidney donation**				
Parameters	**female *n*=52**	**male *n*=49**	**P-value**	**female *n*=52**	**male *n*=49**	**P-value**	**P-value all females**	**P-value all males**
Plasma nitrite, µmol/l*	0.07 (0.06-0.11)	0.08 (0.05-0.10)	0.427	0.11 (0.07-0.19)	0.11 (0.06-0.18)	0.522	0.118	**0.005**
Plasma nitrate, µmol/l*	23.2 (15.3-31.3)	21.0 (16.4-26.8)	0.664	32.9 (27.0-40.0)	36.5 (31.7-53.9)	**0.018**	**<0.001**	**<0.001**
24-h urinary nitrite, µmol/24 h*	0.21 (0.01-0.87)	0.06 (0.01-0.36)	0.226	0.06 (0.02-0.20)	0.05 (0.02-0.53)	0.788	**0.010**	0.546
24-h urinary nitrate, mmol/24 h*	1.24 (0.80-1.93)	1.37 (0.94-1.86)	0.140	1.41 (1.00-1.78)	1.39 (1.11-2.03)	0.451	0.665	0.964
Creatinine clearance, ml/min	125 ± 43.9	139 ± 36.6	0.072	79.0 ± 18.7	88.9 ± 22.7	**0.018**	**<0.001**	**<0.001**
Fractional nitrite excretion, %*	1.13 (0.06-6.53)	0.38 (0.09-3.62)	0.244	0.43 (0.20-1.50)	0.51 (0.11-5.25)	0.976	**0.024**	0.340
Fractional nitrate excretion, %*	31.0 (21.8-50.9)	31.2 (22.4-45.6)	0.935	34.7 (26.9-41.7)	28.5 (22.8-32.6)	**0.007**	0.747	0.085
Nitrite clearance, ml/min*	1.21 (0.07-8.82)	0.56 (0.11-4.13)	0.324	0.38 (0.14-0.98)	0.42 (0.11-4.02)	0.778	**0.002**	0.988
Nitrate clearance, ml/min*	43.0 (24.0-60.0)	42.8 (34.2-59.9)	0.330	25.4 (19.4-39.4)	23.8 (19.9-30.2)	0.425	**<0.001**	**<0.001**

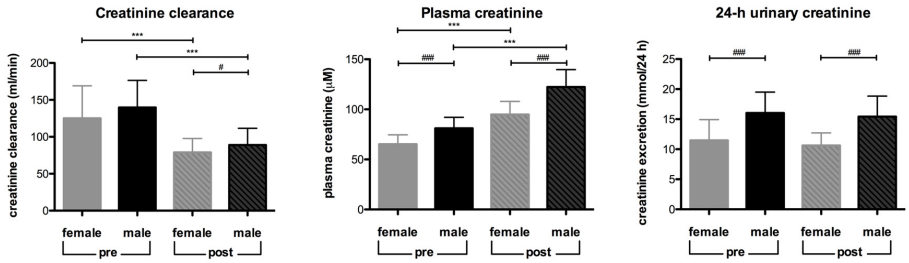
*Normally distributed data are presented as mean ± SD *Skewed data are presented as median (IQR)*

Figure 3: Renal handling of nitrite and nitrate in females and males pre and post kidney donation



*Renal handling of nitrite (upper panel) and nitrate (lower panel) in kidney donors pre and post donation, categorized by gender. Pre donation glomerular filtration and fractional reabsorption (1-fractional excretion) maintain a state in which urinary excretion matches the turnover of NOx. Following donation, a new steady-state is reached in which urinary excretion is preserved by means of increased plasma levels which compensate for the decreased glomerular filtration rate due to the removal of one of the two kidneys. Data are presented as median (IQR). P-values are shown for differences between sexes (#P<0.05, ##P<0.01) and pre and post donation values (*P<0.05, **P<0.01, ***P<0.001). NO_2^-; nitrite, NO_3^-; nitrate, NOx; nitrite and nitrate*

Figure 4: Renal handling of creatinine in females and males pre and post kidney donation



*Adaptations of the renal handling of creatinine in kidney donors following the removal of one kidney for donation. Pre donation glomerular filtration and fractional reabsorption (1-fractional excretion) maintain a state in which urinary excretion matches the turnover of creatinine. Following donation, a new steady state is reached in which urinary excretion is preserved by means of increased plasma levels which compensate for the decreased glomerular filtration rate as a consequence of the removal of one of the two kidneys. Data are presented as mean ± SD. P-values are shown for differences between sexes (#P<0.05, ###P<0.001) and pre and post donation values (***P<0.001).*

6 Clinical relevance of renal handling of nitrite and nitrate in CHF

6.1 Renal handling of nitrite and nitrate in CHF patients and healthy kidney donors

Table 5 shows data on parameters related to the renal handling of nitrite and nitrate in CHF patients and healthy kidney donors pre donation. Whereas in the Wandsworth study nitrite and nitrate clearance were not found to be associated with age, in CHF patients, as well as kidney donors nitrate clearance and age did show a significant relation (coefficient -0.311, P=0.002 and coefficient -0.267, P=0.007, respectively). Because CHF patients were significantly older compared to kidney donors (63.5 ±10.2 vs. 52.8 ± 9.5, P<0001), a correction for age was applied when comparing these groups. Also, since the majority of CHF patients were males (*n*=88, 93%), compared to 49 (49%) of kidney donors, associations were additionally adjusted for gender.

Plasma nitrite and nitrate levels, 24-h urinary nitrite excretion and fractional excretion of nitrite are significantly higher, whereas 24-h urinary nitrate excretion, fractional excretion of nitrate and nitrate clearance are significantly lower in patients compared to controls, independent of sex and age (Tab. 5). With the exception of 24-h urinary excretion of nitrate, these differences remained significant after additional adjustment for creatinine clearance (all P<0.001), indicating that, in CHF patients, nitrite and nitrate handling by the kidney are not merely a reflection of renal function.

6.2 Renal handling of nitrite and nitrate and disease outcome in CHF

During follow-up for 5.1 ± 0.5 years, 11 patients (12%) were rehospitalised and 20 patients (21%) died. The composite outcome was recorded 28 times.

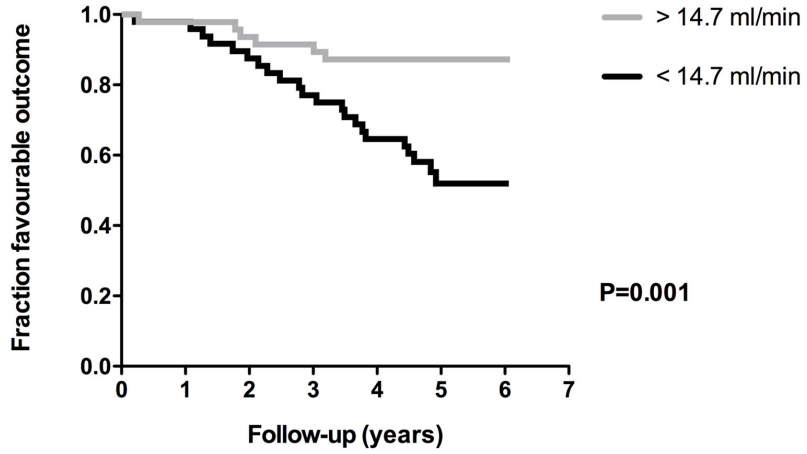
Crude Cox regression analysis showed no association of plasma levels of either nitrite or nitrate with outcome of disease. However, 24-h urinary excretion, fractional excretion and clearance of both nitrite and nitrate did show significant associations with disease outcome in CHF. Interestingly, nitrite-related parameters were all associated with increased hazard ratios,

Table 5: Parameters related to the renal handling of nitrite and nitrate in CHF patients and healthy kidney donors at baseline

Parameters	CHF patients (*n*=95)	Kidney donors (*n*=101)	P-value†
Plasma nitrite, µmol/l*	0.35 (0.27-0.49)	0.08 (0.06-0.10)	**<0.001**
Plasma nitrate, µmol/l*	34.4 (23.5-51.3)	21.5 (15.9-29.5)	**<0.001**
24-h urinary nitrite, µmol/24 h	0.46 (0.33-0.72)	0.06 (0.01-0.76)	**<0.001**
24-h urinary nitrate, mmol/24 h	0.73 (0.52-1.27)	1.24 (0.86-1.89)	**0.015**
Creatinine clearance, ml/min	98.1 ± 31.9	132 ± 41.0	**0.001**
Fractional nitrite excretion, %*	0.97 (0.54-1.69)	0.52 (0.73-5.81)	0.103
Fractional nitrate excretion, %*	15.8 (10.2-23.2)	31.1 (22.0-49.2)	**<0.001**
Nitrite clearance, ml/min*	0.84 (0.60-1.61)	0.65 (0.08-7.12)	0.235
Nitrate clearance, ml/min*	14.7 (8.8-24.8)	42.8 (30.8-59.4)	**<0.001**

*Normally distributed continuous data are presented as mean ± SD *Skewed data are presented as median (IQR)*
†Based on linear regression analysis, corrected for age and sex
CHF; chronic heart failure

Figure 5: Kaplan Meier analysis of the association of nitrate clearance above and below the median with disease outcome in CHF patients



Kaplan-Meier plot with log-rank test for outcome (a composite of HF-related rehospitalisation and all-cause mortality), showing that renal nitrate clearance above the median is significantly associated with favourable outcome in stable CHF patients (P=0.001).

whereas nitrate-related parameters consistently showed associations with favourable outcome of disease, i.e. a decreased rehospitalisation rate and increased patients survival. Of all of these parameters nitrate clearance showed the strongest association (Tab. 7, model 1, hazard ratio (HR) for nitrate clearance above the median 0.27 (95% confidence interval (CI) 0.11-0.68), P=0.005).

Rehospitalisation and/or death occurred in 6 patients (13%) with nitrate clearance above the median, compared to 22 patients (45%) with a below median clearance rate (log-rank test, P=0.001). The corresponding Kaplan-Meier plot is shown as Figure 5.

Table 6 provides extra baseline characteristics of CHF patients. Univariable and multivariable linear regression analyses showed that in, CHF patients, BMI, NT-proBNP and creatinine clearance are independently associated with nitrate clearance (Tab. 6).

Table 6: Univariable and multivariable linear regression analyses of nitrate clearance and baseline characteristics of CHF patients

		Nitrate clearance*			
		Univariable regression		Multivariable regression	
Characteristics	Descriptive statistics	Coefficient	P-value	Coefficient	P-value
Male, *n* (%)	88 (93)	-0.224	0.629		
Age, y	63.5 ± 10.2	-0.036	**0.002**		
Current smoker, *n* (%)	21 (22)	0.080	0.685		
BMI, kg/m^2	28.1 ± 4.4	-0.061	**0.027**	-0.086	**<0.001**
SBP, mmHg	116 ± 17.1	-0.008	0.258		
DBP, mmHg	71.1 ± 10.7	-0.019	0.099		
Hypertension, *n* (%)	32 (34)	-0.574	**0.023**		
Diabetes, *n* (%)	14 (15)	-0.466	0.171		
Duration HF, m*	62.0 (33.0-108)	0.076	0.566		
Ischemic etiology, *n* (%)	68 (72)	-0.194	0.470		
NYHA class II/III, *n* (%)	83 (87)/12 (13)	-0.442	0.224		
LVEF (%)	34.6 ± 8.3	-0.009	0.543		
NT-proBNP, ng/l*	376 (200-728)	-0.492	**<0.001**	-0.313	**0.007**
Creatinine clearance, ml/min	98.1 ± 31.9	0.017	**<0.001**	0.014	**<0.001**
Cholesterol, mmol/l	4.4 ± 1.0	0.019	0.873		
HDL, mmol/l	1.2 ± 0.4	-0.115	0.720		

*Normally distributed continuous data are presented as mean ± SD *Skewed data were normalized by logarithmic transformation for analysis. CHF; chronic heart failure, BMI; body mass index, SBP; systolic blood pressure, DBP; diastolic blood pressure, HF; heart failure, NYHA; New York Heart Association, LVEF; left ventricular ejection fraction, NT-proBNP; N-terminal pro-B-type natriuretic peptide, HDL; high-density lipoprotein*

As shown in Table 7, the association between nitrate clearance *above* the median and favourable disease outcome remained significant after adjustment for associated factors, BMI and NT-proBNP. Adjustment for creatinine clearance, on the other hand, caused significance to be lost (Tab. 7, model 4, HR 0.47 (95% CI 0.17-1.29), P=0.144).

Table 7: Cox regression analysis of nitrate clearance and outcome of disease in CHF patients, adjusted for associated variables

Nitrate clearance above and below the median		
Model	**HR (95% CI)**	**P-value**
1: crude	0.27 (0.11-0.68)	**0.005**
2: model 1, adjusted for BMI	0.26 (0.11-0.65)	**0.004**
3: model 1, adjusted for NT-proBNP	0.37 (0.14-0.94)	**0.037**
4: model 1, adjusted for creatinine clearance	0.47 (0.17-1.29)	0.144

CHF; chronic heart failure, BMI; body mass index, NT-proBNP; N-terminal pro-B-type natriuretic peptide

7 Discussion

Using combined blood and urine analysis we here provide novel insight into the renal handling of the ubiquitous anions nitrite and nitrate. The key findings of our study are that nitrite and nitrate are discretely handled by the kidneys, with considerable gender- and ethnicity-related differences, which are not explained by variations in renal function. Furthermore, the opposing associations of nitrite and nitrate clearance with disease outcome in CHF demonstrate the clinical relevance of their distinction. Moreover, our study is the first to provide reference values for nitrite and nitrate clearances in healthy, middle-aged men and women of different ethnic origins.

Nitrogen oxides (NOx) are normal constituents of human tissues and biological fluids originating from dietary intake and NO metabolism. Nitrate accounts for the majority of the circulating NOx pool, which varies with age, physical activity and immune status. Whole body NO production in healthy human adults ranges from 0.5 to 1.5 mmol per day.(16) Nitrate is the final NO oxidation product, and measurements of (^{15}N-enriched) urinary nitrate following (^{15}N-labelled) L-arginine administration forms the basis of most current techniques to assess bodily NO formation by NO synthase (NOS).(17) Dietary NOx intake varies with lifestyle, food preference and cooking habits. Mean daily intake from food in the UK is estimated to amount to 0.03 mmol of nitrite and 1.5 mmol of nitrate, with cured meats accounting for 65% of nitrite and vegetables contributing > 90% of nitrate.(18) Thus, the amount of nitrate ingested with food roughly compares to that produced by NOS. Nitrate is subject to entero-salivary recirculation, in which oral commensal bacteria reduce part of this nitrate to nitrite, which upon swallowing ends up in the stomach, contributing to first-line antibacterial defence and gastric mucosal protection.(5) Plasma nitrite, but not nitrate, concentrations sensitively reflect constitutive NOS activity, but concentrations are much lower than those of nitrate.(19) In healthy humans, levels rarely exceed 1 µM, which is below the detection limit of most standard techniques of quantification. Most of the earlier studies, therefore, reported only the sum of nitrite and nitrate (NOx), rather than individual concentrations. Moreover, inspection of the methodology section of many papers referring to nitrite reveals that, in most cases, what had been measured was in fact nitrate, contributing to the confusion that surrounds the quantification of these anions.

Steady-state concentrations of biomarkers are determined by their rates of production *and* their elimination, as is illustrated by the increased plasma levels in healthy kidney donors following the removal of one of the two kidneys for donation. Several factors other than endogenous NO production are known to affect circulating nitrite and nitrate concentrations, rendering conclusions about *de novo* NO production from simple measurements of plasma concentrations problematic. Impaired renal function increases circulating NOx concentrations. Therefore, measured nitrite and nitrate concentrations are often normalized for circulating creatinine.(9) However, this does not necessarily represent an adjustment for differences in GFR, but rather for differences in muscle mass. Moreover, nitrite and nitrate are not eliminated by simple filtration alone but subject to tubular reabsorption at different sites of the nephron.(20,21) Although the kidneys are known to play a central role in NOx elimination, the process of renal handling remains poorly characterized. Interestingly, high doses of nitrate were once used as diuretics, but their precise mechanism of action has not been established.(22) Similarly, little is known about cellular uptake and transport of nitrite and nitrate across cell membranes in other parts of the body, and most of the information available was obtained using high, supra-physiological concentrations. While it can be assumed that nitrite and nitrate are freely filtered by the glomeruli and that a large percentage of the filtered load is reabsorbed in the proximal and possibly other segments of the renal tubule, almost nothing is known about the nature of these uptake processes.(20,21) A study in two healthy volunteers seems to support the current assumption that the handling of nitrite by the kidney is identical to that of nitrate, and further suggests the involvement of carbonic anhydrase in the absorption process, by showing an increase of the urinary excretion of both nitrite and nitrate after administration of the carbonic anhydrase inhibitor, acetazolamide.(23) While we cannot exclude the possibility that a fraction of the filtered nitrite and nitrate is handled identically at a proximal site of the nephron, data from the current study indicate that, at some stage, nitrite is handled differently from nitrate such that only ~1% of the filtered nitrite and ~40% of the filtered nitrate are excreted in the urine. Accordingly, our data show that, next to the well-known entero-salivary recirculation pathway, tubular reabsorption in the kidney represents another important route of nitrite and nitrate salvage. The high reabsorption rates suggest that either anion is required for some yet undefined but physiologically important process.

While nitrate was found to be a constituent of human urine almost a century ago, nitrite is generally believed not to be present in urine under normal conditions.(24) Due to the rather low concentrations of nitrite, information about the physiological concentration range of this anion in healthy human urine is lacking. Elevated urinary nitrite is generally attributed to bacterial reduction of nitrate (which forms the basis of rapid screening for urinary tract infections), although it can also be due to other causes.(25) We here show that nitrite is constitutively present at sub-micromolar concentrations in urine of healthy people suggesting continuous excretion, albeit at a rather low rate. Although population data on urinary nitrate excretion are available, no reference values for clearances exist, and the few values for renal nitrate clearance in the literature are all from rather small studies.(26) To the best of our knowledge, no information on nitrite clearance in healthy adults has been reported, nor have

renal clearances of nitrite and nitrate been determined side-by-side in a larger population sample.

Our observation that nitrite, but not nitrate, clearance tended to be lower in males is intriguing. First, it clearly shows that nitrite and nitrate handling is not simply a function of GFR, letting attempts to normalize for creatinine appear even more inappropriate. Moreover, it underscores that both anions are handled differently, at least to some extent, along the nephron. This is further substantiated by the finding that nitrite and nitrate clearance are associated with distinct subject characteristics such as anthropometric and metabolic variables. Given that reabsorption is likely to be an active, regulated process, one possible interpretation of our data is that males possess a mechanism twice as effective in holding on to circulating nitrite compared to females. Conversely, as whole body NO production has been shown to be higher in women compared to men, higher nitrite clearance may serve to limit circulating nitrite levels in females.(8) Alternatively, this sexual dimorphism may reflect differences in nitrite utilization. The reasons behind these variations will remain unclear until we know what physiological role(s) nitrite and nitrate play.

The positive association between nitrate clearance and favourable outcome of disease in CHF patients observed in the present study suggests that individuals with a higher nitrate clearance are at lower risk of adverse events. Alternatively, individuals with a lower risk profile may have a higher nitrate clearance. Future prospective studies are required to assess the cause-effect relationship of these associations, and additional investigations into the involved mechanisms are warranted to shed further light onto the renal handling of NOx.

To generate reference values for nitrite and nitrate clearances, our study utilised a random sample of the general population resident in an inner city borough with a high proportion of ethnic minorities. The fact that participants lived within the same geographical area may have mitigated some potential effects of differences in environmental background, including differences in socio-economic status. The selection of individuals excluded diabetics, treated individuals and women on oral contraceptives or hormone-replacement therapy. This has led to exclusions that varied by ethnic group. Although the original population and subsequent selection criteria limit the generalizability of our findings to a rather healthy population, it does provide a valid assessment of nitrite and nitrate clearances in male and female individuals from different ethnic groups. The study examined first-generation immigrants of ethnic minority groups with both parents born in the country of origin and belonging to the same ethnic background, thus markedly reducing the possible impact arising from an unknown degree of admixture.(11) Blacks were first-generation immigrants of West African and Caribbean descent, and results may therefore not directly be extrapolated to African-Americans. Kidney donors and CHF patients were from Caucasian origin only and therefore the data generated on these cohorts may not be representative of other ethnicities.

We used a sensitive and extensively validated analytical technique for simultaneous nitrite and nitrate determination, providing robust data across the physiological concentration range down to the very low concentrations of nitrite prevailing in human urine. In all samples NOx concentrations were determined in the same laboratory, albeit over a rather long period of time.

In summary, nitrite and nitrate are discretely handled by the kidneys, with considerable variations between sexes and ethnicities. Besides differences in blood and urine concentrations related to ethnicity we find that the renal handling of nitrite, but less so that of nitrate, differs between sexes, with consistently higher clearances for nitrite in women compared to men. Our data suggest the involvement of distinct uptake or transport mechanisms for nitrite and nitrate in the human kidneys. The opposing associations of nitrite and nitrate clearance with outcome of disease in CHF patients underscore the clinical relevance of their distinction. Whether these clearance differences are genetically determined or vary with lifestyle and disease warrants further investigation.

Funding

The WHSS has received support from the former Wandsworth (Health Gain Project 94/5) and South Thames Regional Health Authorities (PGS001), NHS Research and Development Directorate (SE4), British Heart Foundation (PG/93140 and PG/2001023), the former British Diabetic Association (RD95/0984), and The Stroke Association (all to FPC). The present analysis was supported by a Strategic Grant from the University of Warwick Research Development Fund (to FPC, MAM, and MF), funds from the UK Medical Research Council (Strategic Appointment Scheme to MF; G1001536 to MM, MPF and MF), and grants from the Dutch Kidney Foundation (NSN C08-2254 and IP13-114, both to HvG), the Innovational Research Incentives Scheme program of the Netherlands Organization of Scientific Research (VIDI grant 917.13.350), the Netherlands Foundation for Cardiovascular Excellence (both to RAdB), the Netherlands Heart Foundation (Dekker grant 2015 T034, to WCM), and the Groningen University Institute for Drug Exploration (to AMK).

Acknowledgements

The authors would like to express their gratitude to Marian Bulthuis for her excellent technical assistance and to Else Koning for the illustrations she has created for this article.

Conflicts of interest

None declared.

References

1. Organisation Mondiale de la Santé. Global status report on noncommunicable diseases 2014: attaining the nine global noncommunicable diseases targets; a shared responsibility.Geneva: World Health Organization; 2014.
2. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res. 2000 Nov 10;87(10):840–4.
3. Duplain H, Burcelin R, Sartori C, Cook S, Egli M, Lepori M, et al. Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase. Circulation. 2001 Jul 17;104(3):342–5.
4. Huang PL. eNOS, metabolic syndrome and cardiovascular disease. Trends Endocrinol Metab TEM. 2009 Aug;20(6):295–302.
5. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov. 2008 Feb;7(2):156–67.
6. Lundberg JO, Gladwin MT, Ahluwalia A, Benjamin N, Bryan NS, Butler A, et al. Nitrate and nitrite in biology, nutrition and therapeutics. Nat Chem Biol. 2009 Dec;5(12):865–9.
7. Baylis C, Vallance P. Measurement of nitrite and nitrate levels in plasma and urine--what does this measure tell us about the activity of the endogenous nitric oxide system? Curr Opin Nephrol Hypertens. 1998 Jan;7(1):59–62.
8. Forte P, Kneale BJ, Milne E, Chowienczyk PJ, Johnston A, Benjamin N, et al. Evidence for a difference in nitric oxide biosynthesis between healthy women and men. Hypertens Dallas Tex 1979. 1998 Oct;32(4):730–4.
9. Mackenzie IM, Ekangaki A, Young JD, Garrard CS. Effect of renal function on serum nitrogen oxide concentrations. Clin Chem. 1996 Mar;42(3):440–4.
10. Weinberg JB, Lang T, Wilkinson WE, Pisetsky DS, St Clair EW. Serum, urinary, and salivary nitric oxide in rheumatoid arthritis: complexities of interpreting nitric oxide measures. Arthritis Res Ther. 2006;8(5):R140.
11. Cappuccio FP, Cook DG, Atkinson RW, Strazzullo P. Prevalence, detection, and management of cardiovascular risk factors in different ethnic groups in south London. Heart Br Card Soc. 1997 Dec;78(6):555–63.
12. Cappuccio FP, Cook DG, Atkinson RW, Wicks PD. The Wandsworth Heart and Stroke Study. A population-based survey of cardiovascular risk factors in different ethnic groups. Methods and baseline findings. Nutr Metab Cardiovasc Dis. 1998;8:371–85.
13. van den Berg E, Pasch A, Westendorp WH, et al. Urinary Sulfur Metabolites Associate with a Favorable Cardiovascular Risk Profile and Survival Benefit in Renal Transplant Recipients. J. Am. Soc. Nephrol. JASN. 2014; 25(6):1303-12.
14. Schroten NF, Ruifrok WPT, Kleijn L, Dokter MM, Silljé HH, Lambers Heerspink HJ, et al. Short-term vitamin D3 supplementation lowers plasma renin activity in patients with stable chronic heart failure: An open-label, blinded end point, randomized prospective trial (VitD-CHF trial). Am Heart J. 2013 Aug;166(2):357–364.e2.
15. Miller MA, Sagnella GA, Kerry SM, Strazzullo P, Cook DG, Cappuccio FP. Ethnic differences in circulating soluble adhesion molecules: the Wandsworth Heart and Stroke Study. Clin Sci Lond Engl 1979. 2003 Jun;104(6):591–8.
16. Castillo L, Beaumier L, Ajami AM, Young VR. Whole body nitric oxide synthesis in healthy men determined from (15N) arginine-to-(15N)citrulline labeling. Proc Natl Acad Sci U S A. 1996 Oct 15;93(21):11460–5.
17. Wennmalm A, Benthin G, Edlund A, Jungersten L, Kieler-Jensen N, Lundin S, et al. Metabolism and excretion of nitric oxide in humans. An experimental and clinical study. Circ Res. 1993 Dec;73(6):1121–7.
18. Knight TM, Forman D, Al-Dabbagh SA, Doll R. Estimation of dietary intake of nitrate and nitrite in Great Britain. Food Chem Toxicol Int J Publ Br Ind Biol Res Assoc. 1987 Apr;25(4):277–85.
19. Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, et al. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. Free Radic Biol Med. 2003 Oct 1;35(7):790–6.
20. Godfrey M, Majid DS. Renal handling of circulating nitrates in anesthetized dogs. Am J Physiol. 1998 Jul;275(1 Pt 2):F68-73.

21. Rahma M, Kimura S, Yoneyama H, Kosaka H, Nishiyama A, Fukui T, et al. Effects of furosemide on the tubular reabsorption of nitrates in anesthetized dogs. Eur J Pharmacol. 2001 Sep 28;428(1):113–9.
22. Butler AR, Feelisch M. Therapeutic uses of inorganic nitrite and nitrate: from the past to the future. Circulation. 2008 Apr 22;117(16):2151–9.
23. Tsikas D, Schwarz A, Stichtenoth DO. Simultaneous measurement of (15N)nitrate and (15N)nitrite enrichment and concentration in urine by gas chromatography mass spectrometry as pentafluorobenzyl derivatives. Anal Chem. 2010 Mar 15;82(6):2585–7.
24. Mitchell H, Shonle H, Grindley H. The origin of nitrates in urine. J Biol Chem. 1916;(24):461–90.
25. Watts S, Bryan D, Marill K. Is there a link between hyperbilirubinemia and elevated urine nitrite. Am J Emerg Med. 2007 Jan;25(1):10–4. 26. Hill MJ, Elliott P, Joossens JV, Packer PJ, Kesteloot H, Nichols R, et al. Twenty-four hour urinary nitrate excretion in 48 populations from 30 countries: an ECP-INTERSALT collaborative study. Int J Epidemiol. 1996 Jun;25(3):505–12.