

Renal Nitrate Clearance in Chronic Kidney Disease

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Manuscript including abstract references, tables and figures: 3476

Abstract: 259 words

Number of tables and figures: 3

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Abstract:

Background: Nitric oxide (NO) is rapidly oxidised in humans to nitrite and nitrate, with nitrate being present in much greater abundance. These oxidation products can be recycled back into nitric oxide via a complex entero-salivary pathway, thus preserving NO activity. Approximately 65% of circulating nitrate is excreted in the urine in 48 hours, with the excretory pathway of the remainder unknown. The effect of declining renal function on nitrate clearance is unknown

Methods: Forty five subjects, 21M, 24F, median age 69 (range 27-75 years) with renal function assessed by CKD-EPI eGFR between 9 and 89 ml/min/1.73m² completed the study. Following a 24 h low nitrate diet a microplate spectrophotometric method was employed to measure plasma nitrate concentration and 24 h urinary nitrate excretion were measured to determine renal nitrate clearance.

Results: There was a strong positive correlation between urinary nitrate clearance and eGFR, (Spearman R =0.7665, p<0.0001) with a moderate negative correlation between plasma nitrate concentration and CKD-EPI eGFR, (Spearman's R =-0.37, p= 0.012). There was a trend between fractional excretion of nitrate and CKD-EPI eGFR (ml/min/1.73m²) Spearman's R 0.27, p=0.07 though this did not reach statistical significance. Plasma nitrate concentration and serum creatinine concentration were positively correlated, Spearman's R =0.39, p=0.008.

Conclusions: We have observed a strong positive association between renal nitrate clearance and renal function such that plasma nitrate rises as renal function falls. Fractional excretion of nitrate appears to decline as renal function falls. As such, urinary nitrate excretion is unlikely to be a reliable marker of endogenous NO synthesis in settings where renal function is altered.

Background:

Nitric oxide (NO) is a pluripotent free radical synthesised by the nitric oxide synthase (NOS) family of enzymes. The three principle isoforms endothelial, neuronal, and inducible, generate NO for the regulation of vascular tone, signalling, and host defence respectively [1]. Newly synthesised NO is rapidly oxidised to nitrite and nitrate, with nitrate being present in much greater abundance. These oxidation products can be recycled back into nitric oxide via a complex entero-salivary pathway [2], thus preserving NO activity, notably in ischemic and hypoxic conditions where it may prolong cell survival[3; 4]. This circulating nitrate is viewed as a store of NO by multiple researchers [5].

Little is known about nitrate clearance in man. Nitrate readily passes through the glomerulus and is mostly reabsorbed by the proximal renal tubules. In anaesthetised dogs with normal renal function, the volume of blood cleared of nitrate by the kidney is approximately 26 ml/min [6] [7] (for comparison the volume of blood cleared of creatinine in healthy humans is around 100ml/min), with a linear relationship between urinary nitrate excretion rate and plasma nitrate concentration [7] being demonstrated. Wagner et al. [6] showed that a dose of 15N isotopically-labelled nitrate is completely eliminated from humans in 48 hours, but only 65% is excreted in the urine; the fate of the other 35% remains unknown.

Urinary nitrate excretion has been used as a marker of endogenous NO synthesis in multiple disease states [8; 9; 10; 11; 12]. The validity of this depends on the, as yet unproven, assumption that with changes in renal function there is no alteration in nitrate excretion by the kidneys or by non-renal excretory pathways. If urinary nitrate excretion was altered by renal impairment this would make it an unreliable marker of NO synthesis in this setting.

As it is unknown whether renal impairment affects the rate of urinary nitrate excretion, we tested the hypothesis that declining kidney function in man will lead to a change in urinary nitrate excretion.

METHODS

Ethical approval was obtained from the NRES Committee South West - Exeter Research Ethics Committee (14/SW/0041). We sought to recruit fifty participants with varying degrees of renal function, associated with chronic kidney disease (CKD). To enable us to test the hypothesis that declining renal function leads to a fall in the amount of nitrate lost in the urine we recruited balanced groups of healthy volunteers and patients with a breadth of renal function from the normal range to very severe renal impairment. Recruitment was stratified into 5 groups of 10 participants initially identified based on their eGFR (estimated Glomerular Filtration Rate)(ml/min/1.73m²) as calculated using the CKD-EPI equation [13].

Group 1	Normal renal function – eGFR >60
Group 2	Mild renal impairment - eGFR 45-59
Group 3	Moderate renal impairment - eGFR 30-44
Group 4	Severe renal impairment - eGFR 15-29
Group 5	Very severe renal impairment – eGFR <15 (excluding dialysis patients).

Subjects were invited to participate if they were between the ages of 18 and 75 and able to give informed consent. Subjects were excluded if they had a body mass index (BMI) <20 or >30, were receiving regular therapy with organic nitrates, nicorandil, or phosphodiesterase inhibitors, had received antibiotic therapy within the preceding two weeks, had suffered a myocardial infarction or cerebro-vascular event within the preceding three months, or were current smokers (any smoking event in last 3 months) or were unable to give consent. Patients were also excluded from the study if they were on renal replacement therapy (including transplant recipients), had uncontrolled thyroid disease or the presence of active infection/inflammation.

Patients were recruited from the renal unit at the Royal Devon and Exeter NHS Foundation Trust . After obtaining written informed consent the patients were studied in the NIHR Exeter Clinical Research Facility. Healthy volunteers were recruited from databases of volunteers who had consented to being contacted for research studies which are held by the NIHR Exeter Clinical Research Facility.

For two days before the study and for the twenty four hours of the urine collection participants were asked to follow a low nitrate diet. Dietary advice sheets were provided. All prescribed medications were continued as usual. Participants were given instructions and materials for a twenty four hour urine collection at the end of their screening visit. Participants completed a 24 hour urine collection in the widely accepted way, i.e., discarding the first void of the day and then collect all subsequent urine including the first void of the following day. At 09:00 hours, on the morning of the study day, they attended the study centre for a fasting blood sample and blood pressure measurement. Blood pressure was taken from the mean of three supine brachial measurements using an automated blood pressure device (Omron M6, Omron Healthcare Europe B.V. Hoofddorp, The Netherlands). Completed urine collections were returned to the study centre the following day.

Assays

Urine and plasma nitrate concentrations were measured using the VCl₃/Greiss reagent based spectrophotometric plate method described by Miranda et al [14] and validated by our group for use with urine [15].

Renal nitrate clearance was calculated using the classic clearance equation:

$$C_x = U_x V / P_x$$

wherein the clearance of a substance (x) is the product of the urine concentration of x (U_x) and the volume of urine produced in a given time (V), divided by the plasma concentration of x (P_x).

The fractional excretion of a solute by the kidney is the amount of that solute which is filtered by the kidney and subsequently excreted in the urine (rather than being reabsorbed in the renal tubules). Fractional excretion of a solute is calculated as below.

$$FE_{\text{nitrate}} = 100(\text{nitrate}_{\text{urine}} \times \text{creatinine}_{\text{plasma}}) / (\text{nitrate}_{\text{plasma}} \times \text{creatinine}_{\text{urine}})$$

Creatinine was measured in the clinical biochemistry laboratory at the Royal Devon and Exeter Hospital.

Statistical Analysis

For the clearance study we sought to recruit 50 participants to enable us to detect a correlation coefficient of 0.4 or greater with 90% power, $\alpha=0.05$. Data were analysed using Graphpad Prism 5. Data were analysed using non-parametric statistics

Results

Fifty six patients were recruited. Eleven were withdrawn for the following reasons; intervening illness 2 (transient ischaemic attack, recurrent hypoglycaemia); hyponatraemia; anthropometric measurements outside inclusion criteria 3 (high BMI, high blood pressure, CKD-EPI eGFR higher at consent visit therefore falling into group already complete); excluded medications 2 (antibiotics, chemotherapy); unable to make time commitment 3. Data from 45 participants were included in the final analysis.

Table 1. Baseline characteristics of clearance study patients

Age (years)#	69 (59-72) range 27-75
BMI kg/m ² *	25.6 ± 2.6
Sex female (%)	24/45 (53%)
Systolic BP (mm Hg)#	136 (127-144)
Diastolic BP (mm Hg)*	78 (71-85)
CRP (mg/l) (n=43)	35/43 = ≤2 range 1-10
Diabetes (%)	5/45 (11%)

Median and IQR

* mean and SD

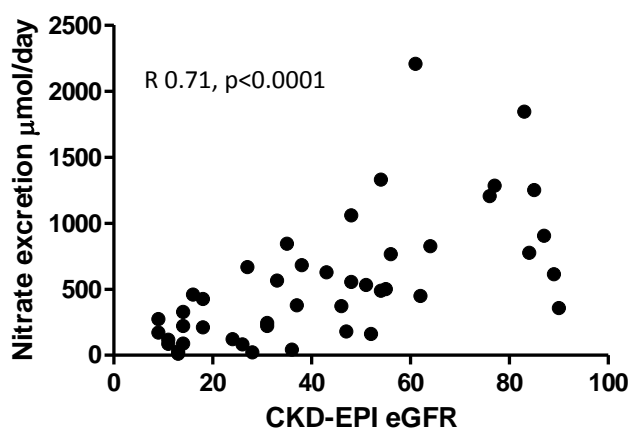


Figure 1. Relationship between total nitrate excretion $\mu\text{mol/day}$ and CKD-EPI eGFR ($\text{ml/min}/1.73\text{m}^2$),

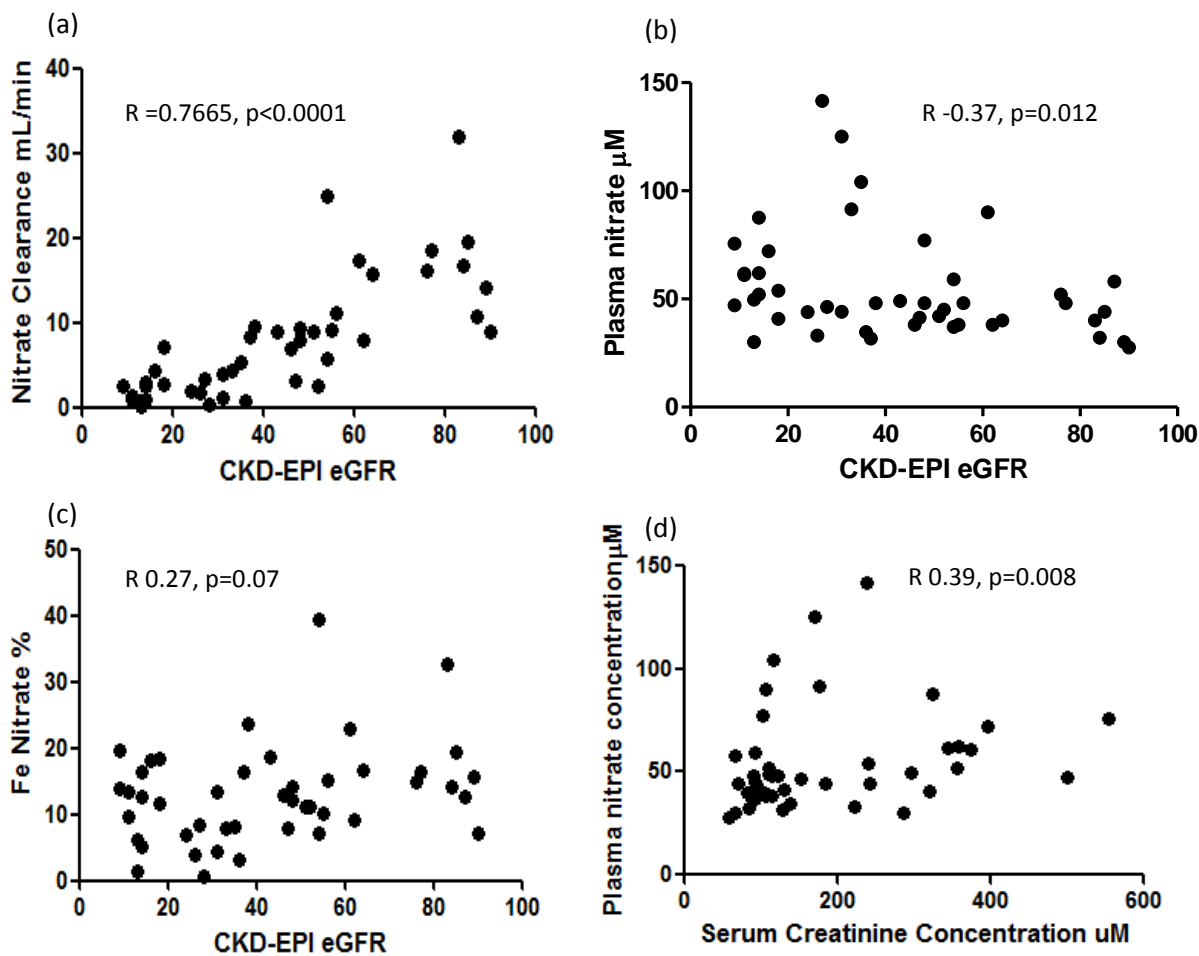


Figure 2: Relationships between (a) CKD-EPI eGFR (ml/min/1.73m²) and renal nitrate clearance (ml/min/1.73m²), Spearman's R =0.7665, p<0.0001, (b) plasma nitrate concentration and CKD-EPI eGFR (ml/min/1.73m²), Spearman's R =-0.37, p=0.012, (c) Fractional excretion of nitrate and CKD-EPI eGFR (ml/min/1.73m²) Spearman's R 0.27, p=0.07, (d) plasma nitrate concentration and serum creatinine concentration Spearman's R of 0.39, p=0.008.

There was a strong correlation between renal nitrate clearance and eGFR, Spearman R =0.7665, p<0.0001 with a moderate correlation between plasma nitrate concentration and eGFR, Spearman R =-0.37, p= 0.012. There appeared to be a trend between fractional excretion of nitrate and CKD-EPI eGFR (ml/min) though this did not reach statistical significance, Spearman's R 0.27, p=0.07. When plasma nitrate concentration and serum creatinine concentration were examined an association was apparent with a Spearman's R of 0.39, p=0.008.

In the present cohort there was no evidence of a correlation between blood pressure and 24 hour urinary nitrate excretion (data not shown). There was no relationship between plasma nitrate concentration and CRP (data not shown). Given that 35/43 participants had a CRP of ≤2 mg/l with a

maximum of 10 mg/l this would be expected. Within our cohort there was no relationship between age and CKD-EPI eGFR, though we acknowledge this is not reflective of the general population, nor was there a relationship between age and nitrate clearance (see data supplement).

A post-hoc analysis showed that across the cohort the fractional excretion rate for nitrate was $13.0 \pm 7.4\%$. When divided across the median (CKD-EPI eGFR 38ml/min), FE nitrate was 14.22(10.97, 17.14)% for those with the higher CKD-EPI eGFR compared with 9.78(5.16,16.39)%, $p = 0.04$ Mann-Whitney test (see data supplement).

Discussion

We have shown that as kidney function falls, so does renal nitrate clearance. In keeping with previous work we show that those with poorer kidney function have a lower total nitrate excretion across the 24 hour period. Although this would conventionally be accepted as evidence for diminished NO synthesis in groups with pathology, our data suggest this measure in isolation will provide an inaccurate estimate of NO synthesis at lower eGFRs as demonstrated by more detailed studies of renal nitrate handling. There was an apparent trend to reduction in fractional excretion of nitrate at lower GFRs though this did not reach statistical significance.

Data from the Framingham cohort [16] suggested a weak inverse association between plasma nitrate concentration and eGFR. Our data confirm that relationship and extend it to individuals with very low CKD-EPI eGFR's.

Confirming that plasma nitrate concentration rises, albeit modestly, as CKD-EPI eGFR falls lends further credence to the idea that the weak positive association between mortality and plasma nitrate concentration in the Framingham cohort is largely driven renal function. Together, our study and the work by Maas et al [16] is at odds with the absence of a detectable difference in plasma nitrate concentrations from subjects on haemodialysis compared with healthy controls[17; 18]. It is likely that this is artefactual due to small sample sizes in the dialysis studies, though it remains possible that being in receipt of renal replacement therapy may alter plasma nitrate concentrations.

One of the key determinants of renal excretion of a solute is the degree of tubular reabsorption of that solute. Our data suggests that as eGFR falls, the proportion of nitrate reclaimed by the tubules rises though we did not observe a statistically significant correlation. There are abundant data to suggest that endothelial NO synthesis is impaired in CKD by multiple mechanisms[19; 20]. It is possible that this more avid retention of nitrate at lower levels of renal function described in our study is an attempt to increase the bioavailability of NO in a setting where NO synthesis is likely to be impaired. Our post-hoc analysis of the fractional excretion of nitrate supports this hypothesis.

We have previously shown a relationship between blood pressure and 24 hour urinary nitrate excretion in 919 older adults with well-preserved renal function (eGFR $>30\text{ml/min}/1.73\text{m}^2$) from the InChianti cohort[15]. No such relationship was evident in the smaller sample size studied here. The most likely explanation is that for the present cohort, the impact of renal impairment on blood pressure is likely to be far larger than that of nitrate exposure in the InChianti cohort, where only those with an eGFR $>30\text{ml/min}/1.73\text{m}^2$ were included. Furthermore, for the purposes of the

present study dietary intake of nitrate was restricted which reduces the variation in nitrate excretion.

Whilst it is known that around 65% of circulating nitrate is lost in the urine in health, the excretory pathway(s) for the remainder is unknown. It is plausible that such pathways are responsible for a greater proportion of nitrate clearance as renal function declines. There is evidence of intracellular storage of nitrate in both human and animal models with concentrations of nitrate being up to four times higher in muscle than in plasma[21; 22]. It is unknown whether intracellular concentrations are altered by differing levels of renal function. It is also plausible that in other disease states a greater proportion of NO is lost to the generation of reactive nitrogen species such as peroxynitrite or to other reaction products such as S-nitrosothiols[23].

Urinary nitrate excretion has been used to estimate NO synthesis rates in CKD[20; 24], hypertension[25], and congestive heart failure[26]. From the data presented here, it is clear that 24 hour urinary nitrate excretion cannot be considered a reliable measure of total body nitric oxide synthesis in CKD. This is likely to be the case in multiple other disease states. In rheumatoid arthritis for example, it has previously been shown that total urinary nitrate excretion was not different compared with controls. In contrast serum NOx was elevated, while renal nitrate clearance rate and fractional excretion of NOx were lower [27]. This clearly demonstrates variation in the renal handling of nitrate in pathological states with a complexity that is not appreciated from the measurement of total nitrate excretion alone. A small case control study of patients with primary pulmonary hypertension from Demonocheaux et al [28], who used an isotope enrichment technique, demonstrated a substantial reduction in NO synthesis but unaltered urinary nitrate excretion. Taken together with our data this would suggest that a reliance on 24 hour NOx excretion as a measure for overall NO synthesis may be misleading.

CONCLUSION

We have shown a strong positive association between renal nitrate clearance and renal function such that plasma nitrate rises as renal function falls. Fractional excretion of nitrate appears to decline as renal function falls though this would need to be confirmed in a larger study. As such, urinary nitrate excretion is unlikely to be a reliable marker of endogenous NO synthesis in settings where renal function is altered.

Acknowledgements

The study was funded by an NHS Small Grant and supported by the NIHR Exeter Clinical Research Facility. The funders had no input in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. The views and opinions shown within this paper are those of the authors and do not necessarily represent those of the National Institute for Health Research, National Health Service or the Department of Health. We would like to thank the technicians involved in the study and all the volunteers.

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