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Renal handling of nitrate in women and men with elevated blood pressure

Michaela L. Sundqvist1,2 | Jon O. Lundberg1 | Eddie Weitzberg1,3 | Mattias Carlström1

1Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden
2Department of Sport and Health Sciences, Swedish School of Sport and Health Sciences, Stockholm, Sweden
3Department of Perioperative Medicine and Intensive Care, Karolinska University Hospital, Stockholm, Sweden

Correspondence
Michaela L. Sundqvist and Mattias Carlström, Department of Physiology and Pharmacology, Karolinska Institutet, Solnavägen 9, Biomedicum 5B, 171 65 Solna, Sweden.
Email: michaela.sundqvist@ki.se (M. L. S.) and mattias.carlstrom@ki.se (M. C.)

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Abstract
Aim: The inorganic anions nitrate and nitrite are oxidation products of nitric oxide (NO) that have often been used as an index of NO generation. More than just being surrogate markers of NO, nitrate/nitrite can recycle to bioactive NO again. Nitrate is predominantly eliminated via the kidneys; however, there is less knowledge regarding tubular handling. The aim of this study, as part of a large randomized controlled trial, was to explore potential sex differences in renal nitrate handling during low and high dietary nitrate intake. We hypothesized that renal clearance and excretion of nitrate are higher in men compared to women.

Methods: In prehypertensive and hypertensive individuals (n = 231), nitrate and nitrite were measured in plasma and urine at low dietary nitrate intake (baseline) and after 5 weeks supplementation with nitrate (300 mg potassium nitrate/day) or placebo (300 mg potassium chloride/day). Twenty-four hours ambulatory blood pressure recordings and urine collections were conducted.

Results: At baseline, plasma nitrate and nitrite, as well as the downstream marker of NO signalling cyclic guanosine monophosphate, were similar in women and men. Approximately 80% of filtered nitrate was spared by the kidneys. Urinary nitrate concentration, amount of nitrate excreted, renal nitrate clearance (C(nitrate)) and fractional excretion of nitrate (FE(nitrate)) were lower in women compared to men. No association was observed between plasma nitrate concentrations and glomerular filtration rate (GFR), nor between FE(nitrate) and GFR in either sex. After 5 weeks of nitrate supplementation plasma nitrate and nitrite increased significantly, but blood pressure remained unchanged. FE(nitrate) increased significantly and the sex difference observed at baseline disappeared.

Conclusion: Our findings demonstrate substantial nitrate sparing capacity of the kidneys, which is higher in women compared to men. This suggests higher tubular nitrate reabsorption in women but the underlying mechanism(s) warrants further investigation.

Keywords: excretion, hypertension, kidney, nitrate, nitric oxide, nitrite
1  |  INTRODUCTION

Inorganic nitrate (NO\textsubscript{3}\textsuperscript{−}) and nitrite (NO\textsubscript{2}\textsuperscript{−}) are oxidation products from endogenously generated nitric oxide (NO) as well as normal constituents in our diet. In mammals, these anions are also substrates for NO generation via a pathway involving the oral microflora and enzymatic and non-enzymatic reduction in blood and tissues.\textsuperscript{1} Circulating nitrate is actively taken up by the salivary glands and excreted into the oral cavity where oral bacteria efficiently reduce salivary nitrate to nitrite.\textsuperscript{2} After swallowing and intestinal absorption of nitrite it can be further reduced to bioactive NO and other reactive nitrogen oxide intermediates.\textsuperscript{3} The kidneys play an important role in the regulation of nitrate and nitrite, although the details regarding the tubular handling of these anions are not yet clear. A major part of ingested nitrate is excreted in the urine within 48 hours.\textsuperscript{4}

Since NO is crucially involved in the regulation of cardiovascular and metabolic functions great attention has been paid to the possibility of boosting NO generation by the dietary administration of inorganic nitrate or nitrite. Numerous experimental studies, using cardiovascular and metabolic disease models, have demonstrated beneficial effects of nitrate and nitrite treatment, including blood pressure reduction and improved glucose control.\textsuperscript{5,6} Studies in humans support preclinical findings, showing that the administration of these anions reduces blood pressure in both normotensive\textsuperscript{7} and hypertensive individuals,\textsuperscript{8,9} improves endothelial function,\textsuperscript{9} protects against myocardial ischemia/reperfusion injury\textsuperscript{10} and improves exercise performance.\textsuperscript{11} Larger population studies looking at leafy green vegetables, which contain high amounts of nitrate, in relation to outcomes such as coronary heart disease,\textsuperscript{12,13} type 2 diabetes\textsuperscript{14} and all-course cardiovascular mortality have suggested an inverse relationship.

It has been suggested that NO bioactivity, largely due to oxidative stress and scavenging of NO, is reduced with ageing and in cardiovascular disease.\textsuperscript{15,16} Being oxidation products of NO synthase (NOS)-derived NO, plasma nitrate and nitrite have been used extensively as surrogate markers of NO formation. In humans, Kleinbongard and colleagues showed that plasma nitrite was gradually decreased upon the addition of cardiovascular risk factors and correlated with endothelial function.\textsuperscript{18} Under fasting conditions another way to estimate NO generation is to measure urinary nitrate excretion, where increased daily excretion (indicative of increased NOS activity) has been coupled with lower blood pressure.\textsuperscript{19} However, measurements of nitrate and nitrite as markers of NO generation are hampered by the fact that our dietary habits significantly influence such measurements. Since the half-life of nitrate is approximately 6 hours, even overnight fasting before sampling might not be enough. Moreover, renal function may affect plasma levels of these anions even though little is known about the specific tubular handling of nitrate and nitrite.\textsuperscript{20,21}

As a part of a clinical trial investigating the effects of dietary nitrate on blood pressure in prehypertensive and hypertensive subjects (ClinicalTrials.gov Identifier: NCT02916615), we measured nitrate and nitrite in plasma, saliva and urine.\textsuperscript{22} In the run-in phase of this trial all subjects were avoiding nitrate-containing food for 2 weeks (ie, Baseline), after which one group received placebo (300 mg potassium chloride/day) and one group received nitrate (300 mg potassium nitrate/day) for another 5 weeks (ie, Intervention). Throughout the study, all individuals continued with a nitrate-restricted diet. In this randomized controlled trial, we aimed to explore potential sex differences in renal nitrate handling during low and high dietary nitrate intake. We hypothesized that renal clearance and excretion of nitrate is higher in men compared to women.

2  |  RESULTS

2.1  |  Characteristics of the study population at baseline

In this study, 231 pre-hypertensive and hypertensive subjects were included (122 women and 109 men). The women in this cohort were slightly older and had somewhat lower body mass index (BMI) compared to the men (Table 1). Glomerular filtration rate (GFR) was significantly lower in women than in men (99.6 ± 24 vs 125.3 ± 30 mL/min, \(P < .0001\)), but when corrected for body surface area this difference was largely abolished (Table 1). Mean ambulatory systolic blood pressure (ASBP) was 130 ± 11 in women and 133 ± 9 in men (\(P = .09\)). Mean ambulatory diastolic blood pressure (ADBP) was lower in women (77 ± 7 mmHg) compared to men (80 ± 7 mmHg, \(P = .0005\)). There was no significant difference in the use of anti-hypertensive medication(s) between women and men (Table 2).

2.2  |  Nitrate and nitrite handling at baseline

Baseline characteristics of the study population and data on nitrate and nitrite in plasma, saliva and urine as well as renal handling of nitrate in the 231 subjects are presented in Table 1 and Figure 1. Enterosalivary nitrate circulation is an important component of the nitrate-nitrite-NO pathway, where increased concentrating ability of nitrate in the salivary glands has been associated with greater formation of reactive NO species in the blood and more profound effects on blood pressure.\textsuperscript{23} In the current study, however, we did not observe any significant differences in salivary nitrate levels between sexes. Also, the plasma nitrate and nitrite levels were similar in men and women. However, women had lower concentrations of nitrate in urine and excreted less nitrate...
tional excretion of nitrate (FEnitrate) was significantly lower in women compared to men; this difference remained significant when adjusted for body weight. Finally, renal fractional excretion (26 ± 10 mL/min, P = .0036*; Figure 2C,D) in either sex. No statistically significant differences in 24h sodium and potassium excretion were observed between women and men (Table 1), although men tended to have slightly higher sodium excretion which indicates higher intake of salt.

### 2.3 Nitrate and nitrite handling following nitrate supplementation

After baseline measurements, two subgroups were followed for an additional 5 weeks with either placebo (n = 78) or nitrate pill (n = 77). In the low-nitrate diet group, blood pressure, plasma and salivary levels of nitrate and nitrite as well as all renal nitrate handling parameters were all unchanged compared to Baseline (Table S2). In the nitrate treatment group, plasma and saliva nitrate and nitrite increased, as expected, but blood pressure remained unchanged (Table S2). Regarding the renal parameters, urinary nitrate concentration, nitrate excretion, FE\textsubscript{nitrate} and C\textsubscript{nitrate} all increased significantly, but GFR was not significantly affected (Table S2).

We analysed the sex aspects in the groups above and found that following low dietary nitrate supplementation (Placebo), blood pressures were unchanged in both sexes (Table S3). There was still no difference in plasma or saliva nitrate and nitrite between sexes. Similar to the baseline period, all renal nitrate handling parameters (ie, urine nitrate concentration, renal excretion of nitrate and C\textsubscript{nitrate} as well as FE\textsubscript{nitrate}) were still lower in the men who received placebo (Table S3). In the nitrate-treated group, however, the difference in blood pressure between the sexes had disappeared (Table S3). Plasma nitrate became
higher in the women (124 ± 50 µM) compared to the men (85 ± 49 µM), while plasma nitrite reached similar levels in women (0.47 ± 0.35 µM) and in men (0.46 ± 0.42 µM). As expected, saliva levels of both nitrate and nitrite increased following nitrate supplementation. However, there was no sex difference in saliva nitrate and nitrite after 5 weeks of nitrate supplementation (Table 3). Interestingly, in the nitrate-treated group, the significant differences in renal nitrate handling parameters (same as listed above) that were seen at baseline were lost. Regarding the urinary excretion of nitrate,
TABLE 3  Blood pressure, nitrate and nitrite in plasma and urine amongst women and men after 5 weeks of placebo or nitrate supplementation

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n = 78)</th>
<th>Nitrate (n = 77)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Women (n = 42)</td>
<td>Men (n = 36)</td>
<td>P-value</td>
</tr>
<tr>
<td>ASBP (mm Hg)</td>
<td>130 ± 11</td>
<td>131 ± 9</td>
<td>P = .583</td>
</tr>
<tr>
<td>ADBP (mm Hg)</td>
<td>77 ± 7</td>
<td>81 ± 8</td>
<td>P = .022</td>
</tr>
<tr>
<td>GFR (mL/min/m²)</td>
<td>55.4 ± 13</td>
<td>58.3 ± 9</td>
<td>P = .759</td>
</tr>
<tr>
<td>Plasma Nitrate (µmol/L)</td>
<td>33 ± 12</td>
<td>32 ± 27</td>
<td>P = .055</td>
</tr>
<tr>
<td>Plasma Nitrite (µmol/L)</td>
<td>0.39 ± 0.33</td>
<td>0.25 ± 0.19</td>
<td>P = .035</td>
</tr>
<tr>
<td>Plasma cGMP (nmol/L)²</td>
<td>1.5 ± 2.3</td>
<td>2.8 ± 3.1</td>
<td>P = .003*</td>
</tr>
<tr>
<td>Saliva Nitrate (µmol/L)</td>
<td>271 ± 270</td>
<td>364 ± 827</td>
<td>P = .787</td>
</tr>
<tr>
<td>Saliva Nitrite (µmol/L)</td>
<td>144 ± 94</td>
<td>173 ± 211</td>
<td>P = .756</td>
</tr>
<tr>
<td>Urine Nitrate (µmol/L)</td>
<td>445 ± 284</td>
<td>621 ± 336</td>
<td>P = .014</td>
</tr>
<tr>
<td>Urine Volume 24 h (mL)</td>
<td>1712 ± 647</td>
<td>1906 ± 842</td>
<td>P = .254</td>
</tr>
<tr>
<td>Nitrate Excretion (mg/24 h)</td>
<td>41 ± 23</td>
<td>67 ± 56</td>
<td>P &lt; .0001*</td>
</tr>
<tr>
<td>Nitrate Clearance (mg/24h/kg)</td>
<td>0.61 ± 0.4</td>
<td>0.80 ± 0.6</td>
<td>P = .015</td>
</tr>
<tr>
<td>Nitrate Clearance (mL/min/kg)</td>
<td>15 ± 7</td>
<td>25 ± 10</td>
<td>P &lt; .0001*</td>
</tr>
<tr>
<td>Nitrate Excretion (mg/24h/kg)</td>
<td>0.22 ± 0.1</td>
<td>0.30 ± 0.1</td>
<td>P = .0012*</td>
</tr>
<tr>
<td>FEₙitrate (%)</td>
<td>16 ± 7</td>
<td>21 ± 7</td>
<td>P = .0006*</td>
</tr>
<tr>
<td>Na⁺ Excretion (mmol/kg/24 h)</td>
<td>1.78 ± 0.7</td>
<td>1.94 ± 0.6</td>
<td>P = .262</td>
</tr>
<tr>
<td>K⁺ Excretion (mmol/kg/24 h)</td>
<td>1.04 ± 0.3</td>
<td>1.05 ± 0.4</td>
<td>P = .877</td>
</tr>
</tbody>
</table>

Note: Normally distributed data are analysed with unpaired t tests and non-normally distributed data with Mann-Whitney tests. Values are presented as mean ± SDs. To adjust for multiple testing, Bonferroni correction was utilized and a P value less than .0125 was considered to be statistically significant (marked with *).

Abbreviations: ADBP, ambulatory diastolic blood pressure; ASBP, ambulatory systolic blood pressure; cGMP, cyclic guanosine monophosphate. Cₙitrate, renal clearance of nitrate; FEₙitrate, renal fractional excretion of nitrate; GFR, glomerular filtration rate.

²cGMP was measured in n = 38 women and n = 34 men in the placebo group and n = 34 women and n = 38 men in the nitrate group.

when adjusting for body weight, the previous difference between sexes was even reversed such that women excreted more nitrate than men. None of the interventions were associated with any significant changes in 24 hours sodium and potassium excretion for women and men (Table 3).

2.4 Plasma cyclic guanosine monophosphate levels

The downstream marker of NO signalling cyclic guanosine monophosphate (cGMP) was not different between sexes at baseline (Table 1). Following 5 weeks of intervention, no differences were observed in the placebo or in the nitrate group as a whole (Table S2). However, cGMP levels appeared to be lower in women than in men in the placebo group, whereas no such difference was observed between sexes in the nitrate group (Table 3).

3 DISCUSSION

In a relatively large cohort of prehypertensive and hypertensive men and women, extracted from a previous clinical trial, we have investigated the handling of inorganic nitrate and blood pressure under conditions of restricted dietary nitrate intake and compared this to a period of nitrate supplementation. Despite similar plasma levels of nitrate in men and women we found that the renal handling of nitrate was different between the sexes, where women had significantly lower renal clearance and fractional excretion of nitrate compared to men under basal conditions. This was associated with slightly lower diastolic blood pressure in women. Following nitrate supplementation, however, these differences in blood pressure and renal handling were lost. These data suggest that renal tubular reabsorption of nitrate is intrinsically higher in women than in men, but that tubular reabsorption of nitrate becomes saturated at higher intake of nitrate.

Circulating nitrate and nitrite have been widely used as surrogate markers of endogenous NO generation, since actual NO measurements in plasma and tissues are not feasible for technical reasons. However, measurements of nitrate/nitrite levels as an index of NOS-derived NO production are hampered by the strong influence of dietary intake of these anions. Plasma half-life of nitrate and nitrite is approximately 6 hours and 30 minutes, respectively, which means that even overnight fasting may not be sufficient to evaluate the endogenous generation and renal handling of these anions. In
this study, all individuals were on a very low nitrate diet for a prolonged period, which makes our measurements more representative of endogenous NO production via the NOS pathway. This is supported by the fact that the daily renal excretion of nitrate was approximately 1 mmol, which in humans is the suggested amount of NO generated by the NOS system per day. After two weeks of low-nitrate diet (ie, Baseline), saliva and plasma levels of nitrate and nitrite were similar between men and women, although plasma nitrite tended to be somewhat higher in women ($P = .085$) (Table 1). After additional five weeks of low dietary nitrate, women trended to have higher plasma nitrate and nitrite levels than men, whereas salivary levels of these anions were similar between sexes (Table 3). Previous studies have indicated sex differences regarding the handling of nitrate and nitrite. Kapil and coworkers showed that women (18-45 years of age) had higher nitrite levels in saliva, plasma and urine compared to men, despite similar nitrate levels in these matrices. The somewhat different results between our and the Kapil study may be explained by the different characteristics of the study population. Compared to the work by Kapil and colleagues, our study individuals were considerably older and with elevated blood pressure.

Increased age has been linked with reduced NO bioactivity, which mechanistically could be coupled with reduced eNOS activity, oxidative stress and scavenging of NO. To what extent reduced kidney function and altered the tubular renal handling of nitrate and nitrite occur with ageing is less clear. Moreover, potential differences in oral commensal bacteria (ie, abundance of nitrate-reducing bacteria) and salivary gland function may have contributed to the different results on salivary and plasma nitrite levels between our and Kapil’s study. Also, we cannot exclude that differences in nitrate excretion between women and men could be due to differences in dietary intake of nitrate from other sources than vegetables (eg, higher consumption of meat products). Baseline characteristics in our study were obtained following two weeks of dietary nitrate restriction whereas only 24 hours of nitrate restriction was used in the study by Kapil. Finally, in our study, there was a small yet significant difference in age (2 years) between sexes. However, we find it unlikely that this would have contributed to any of the observed differences regarding nitrate homeostasis and renal handling between women and men.

Several studies have indicated reduced NO bioactivity with increasing age and in cardiovascular disease. In our prehypertensive and hypertensive population, the nitrate and nitrite levels give no such indication despite the prolonged period with dietary nitrate restriction. When we relate these levels to our previous in-house measurement with the same method in young and healthy individuals, we do not find significantly lower levels in the present cohort. An explanation might be that a reduction in NO bioavailability to a large extent depends on NO reacting with other radicals, limiting the availability, but the end products of these reactions would still be nitrate. Hence, plasma nitrate and nitrite or 24h urinary excretion in the fasting state are probably better markers of NO generation rather than indices of actual NO bioavailability. Another reason for the seemingly normal plasma nitrate levels could be that our hypertensive subjects had a low-grade inflammation with iNOS induction, generating NO and subsequently nitrate and nitrite.

The most striking difference between the sexes, which has not been previously described, was the difference in renal handling of nitrate. Women had significantly lower clearance and fractional excretion of nitrate compared to men, and hence appeared (although not statistically significant) to excrete less nitrate in the urine over the 24h observation period. This difference cannot be explained by differences in plasma nitrate concentration, urine volume or GFR between sexes. The difference between women and men was sustained or even potentiated over the extended period with dietary nitrate restriction. Interestingly, as mentioned above, nitrate excretion normalized by body weight was not significantly different between sexes at Baseline. However, following additional 5 weeks of nitrate depletion, women had significantly lower nitrate excretion measured as (mg/24h). Considering that our muscles represent a large storage pool of nitrate one could speculate that a rather long period of nitrate depletion is necessary before any renal adaptation kicks in to maintain circulatory nitrate/nitrite homeostasis. Moreover, men have in general a larger relative muscle mass compared to women, and this may contribute to the observed sex differences regarding renal nitrate handling following prolonged nitrate depletion.

One limitation of the study is the lack of the precise amount of nitrate intake from each individual. However, all participants were clearly instructed not to eat any nitrate-rich vegetables and we have no reason to believe that the men violated the study protocol and the instructions more than the women. Another limitation in this study is that all subjects were prehypertensive or hypertensive and within a specific age range, which makes the generalization of our findings somewhat less applicable.

The underlying mechanisms for this previously non-described sex difference in the renal handling of nitrate are yet unclear. Obviously, the lower fractional excretion of nitrate in the women suggests higher tubular reabsorption or less tubular secretion of nitrate, but how this is actually achieved along the nephron is still unclear. Another possible explanation for the observed sex difference in renal nitrate handling is that men have relatively greater muscle mass than women, with increased capacity to store nitrate, which is continuously released and subsequently handled by the kidneys to maintain circulatory homeostasis of nitrate. If true, this would mean that more nitrate is being filtered in
the kidneys of men, but that absolute reabsorption is similar between sexes. However, Forte and colleagues estimated endogenous NO synthesis in men and women by the intake of $^{15}\text{N}$ L-arginine and measuring $^{15}\text{N}$-nitrate excretion in urine. In their study, urinary nitrate excretion was significantly higher in women compared to men.\textsuperscript{32} Even though their subjects were much younger than in our study it speaks against body composition underlying our opposite results. Importantly, during high dietary nitrate supplementation the difference between men and women in renal nitrate handling was actually reversed such that women excreted more nitrate than men adjusted for body weight. This can possibly be explained by the fact that women received a greater amount of nitrate per kg and day than men (both sexes got 300 mg per day). This assumption is supported by the finding of significantly higher plasma nitrate concentration in women following nitrate supplementation. Fractional excretion of nitrate increased in both women and men, indicative of saturable mechanism(s) in the kidneys that now masked the intrinsic differences between the sexes. From a nutrition physiology-perspective, it should be noted that the effects of nitrate supplementation, using a pill, was similar to those observed following 5 weeks intake of leafy green vegetables containing the same amount of nitrate (data not shown).

In our study, we cannot rule out a sex difference in other routes of nitrate elimination such as the bacterial reduction in the gut\textsuperscript{3,33} or that pools of nitrate in various organs may differ between sexes.\textsuperscript{34} One caveat in using urinary nitrate excretion as a measure of body NO synthesis is that it may, at least partly, reflect local NO generation within the kidney,\textsuperscript{35} but there is little evidence in the literature for that assumption. Moreover, in the case of asymptomatic bacteriuria the bacteria could reduce nitrate to nitrite and ammonia in the bladder or ex vivo in the sampling vial, which could lead to the underestimation of renal nitrate excretion.

Ambulatory blood pressure was lower in the women but whether this has any coupling to the differences in the renal handling of nitrate and nitrite cannot be fully evaluated in the present study. Multiple studies have shown antihypertensive effects of dietary nitrate\textsuperscript{36} and conversely hypertensive effects when blocking the nitrate-nitrite-NO pathway.\textsuperscript{37,38} Some studies show a correlation between plasma nitrite and cardiovascular regulation including endothelial function and blood pressure.\textsuperscript{18,37} However, this is not a consistent finding and variable results regarding circulating nitrite and nitrate levels may possibly be due to varying degrees of iNOS activation.\textsuperscript{39} Further studies are clearly needed to reveal any sex differences regarding nitrate and nitrite handling and blood pressure and to explore the specific tubular handling of these anions.

To conclude, filtered nitrate is highly spared from renal excretion via mechanism(s) that are saturable at high plasma levels of nitrate. There are significant sex differences in renal nitrate handling in individuals with elevated blood pressure, as evident from reduced fractional excretion and clearance of nitrate in women. Additional prospectively designed studies are needed to mechanistically explore the specific tubular handling of these anions along the nephron.

4 \ MATERIALS AND METHODS

4.1 \ Study population

The study was approved by the local research ethics committee in Stockholm and performed, according to the declaration of Helsinki, at Karolinska University Hospital, Department of Cardiology Clinical Research Unit, between September 2014 and December 2018. This work is conforming with good publishing practice in physiology.\textsuperscript{40} Subjects gave their written informed consent before inclusion in the study. The subjects consist of 50 to 70-years-old prehypertensive and hypertensive men and women, according to guidelines from the European Society of Cardiology,\textsuperscript{41} with systolic blood pressure (SBP) of 130-159 mmHg, recruited in Stockholm County, Sweden. Detailed information on the study design, recruitment process and characteristics of the study subjects is described in a recent publication.\textsuperscript{22}

Here we have included all subjects (n = 231) that completed a run-in period of two weeks with a controlled low nitrate diet after which blood, saliva and 24 hours urine samples were collected for analysis of nitrate and nitrite (ie, Baseline). Matched plasma and urine samples were used to analyse nitrate and nitrite excretion as well as to calculate renal nitrate clearance and excretion. Ambulatory blood pressure for 24h was measured at the end of the two-week period. Subjects were given low nitrate vegetables (125 g/d) during the study period and were instructed to avoid all other vegetables. Following baseline characterization, one group (n = 78) received placebo pills (300 mg potassium chloride/day) and one group (n = 77) received nitrate pills (300 mg potassium nitrate pill/day) for another 5 weeks (ie, Intervention). Thereafter, blood pressure was monitored and plasma, saliva and urine samples were collected in the same manner as during the Baseline period. Throughout the study, all individuals maintained a nitrate-restricted diet.

4.2 \ Tissue sampling

Blood samples were collected into tubes containing EDTA (Sigma-Aldrich, #E9884), final concentration 2 mmol/L and was immediately centrifuged at 4700×g for 5 minutes.
Plasma samples, as well as urine and saliva samples, were thereafter frozen and stored at −80°C until analysed.

4.3 | Nitrate and nitrite analysis

The nitrate and nitrite concentrations in the plasma, saliva and urine samples were measured using a high-performance liquid chromatography (HPLC) system (ENO-20; EiCom) which has previously been described.²⁰ A cut-off level for suspected measurement error was set to nitrate >100 µM in the fasting (nitrate depleted) state. Urine samples were diluted 1:50. Levels of nitrate in the low-nitrate-containing vegetables (tomatoes, sweet corn, capsicum and carrots) were measured using a chemiluminescence method described in detail previously.⁴² Daily nitrate intake from these vegetables did not exceed 10 mg.

4.4 | cGMP analysis

The levels of the NO downstream signalling marker cGMP were measured in plasma samples before and after intervention. To prevent the degradation of cGMP, the plasma was transferred to tubes containing the PDE inhibitor IBMX (3-Isobutyl-1methylxanthine; Sigma-Aldrich #I5879) to give a final concentration (10 µM). Samples were thereafter frozen and stored at −80°C before analysing cGMP with an ELISA kit (Cayman Chemical #581021), according to the manufacturers' instructions. All absorbance reading was performed in SpectraMax iD3 from Molecular Devices.

4.5 | Blood pressure recordings

24 hours ambulatory BP monitoring was performed using WatchBP® O3 (Microlife Corporation, Switzerland), validated by the European Society of Hypertension (ESH). The monitor was programmed for reading every 30 minutes.

4.6 | Renal parameters

The study subjects were instructed on how to collect urine during 24 hours and samples were obtained from all individuals. Less than 500 mL/24 h was an exclusion criterion. In total, only two subjects were excluded. GFR calculation was based on creatinine clearance using the 24h urine collection, ie, GFR (mL/min) = \( U_{\text{creatinine}} \times U_{\text{flow}} / P_{\text{creatinine}} \). Data on GFR were adjusted for body surface area and presented as mL/min/m². Renal excretion of nitrate was calculated as \( E_{\text{nitrate}} (\text{mg/24 h}) = 24 \text{ h urine volume} \times \text{nitrate}_{\text{urine}} \). Renal nitrate clearance was calculated as \( C_{\text{nitrate}} (\text{mL/min}) = \text{nitrate}_{\text{urine}} \times \text{urine volume/nitrate}_{\text{plasma}} \). Renal fractional excretion (FE) of nitrate in % was calculated as \( \text{FE}_{\text{nitrate}} (%) = [C_{\text{nitrate}} (\text{mL/min/kg}) / \text{GFR (mL/min/kg})] = 100 \times (\text{nitrate}_{\text{urine}} \times \text{creatinine}_{\text{plasma}}) / (\text{nitrate}_{\text{plasma}} \times \text{creatinine}_{\text{urine}}) \).

5 | STATISTICAL ANALYSIS

D’Agostino & Pearson omnibus normality test was used to determine the normal distribution of data. For comparisons of data between men and women unpaired t tests were used for normally distributed data sets and Mann Whitney test for non-normally distributed data. In the analysis of differences between matched Baseline and Intervention periods, Students paired t-test was used for normally distributed data and Wilcoxon matched-paired signed-rank test for non-normally distributed data. Spearman's correlation was used to test any relationship between plasma nitrate levels and GFR and between or between \( \text{FE}_{\text{nitrate}} \) and GFR. To determine any differences in medication between women and men we used Fisher's exact test. Statistical analyses were performed in PRISM 5 software (Graph Pad). Data are presented as mean ± SD. For multiple t tests, Bonferroni correction was utilized and a \( P \) value less than .0125 was considered to be statistically significant.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

JOL and EW are co-inventors on patent applications related to the therapeutic use of inorganic nitrate and nitrite. The other authors have no conflicts of interest.

DATA CITATION

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Mattias Carlström https://orcid.org/0000-0001-9923-8729
REFERENCES


**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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