Improvement in blood pressure after short-term inorganic nitrate supplementation is attenuated in cigarette smokers compared to non-smoking controls

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ABSTRACT

Dietary supplementation with inorganic nitrate (NO_3^-) has been reported to improve cardiovascular health indices in healthy adults. Cigarette smoking increases circulating thiocyanate (SCN^-), which has been suggested to competitively inhibit salivary nitrate (NO_3^-) uptake, a rate-limiting step in dietary NO_3^- metabolism. Therefore, this study tested the hypothesis that dietary NO_3^- supplementation would be less effective at increasing the circulating plasma nitrite concnetration ($[NO_2^-]$) and lowering blood pressure in smokers (S) compared to non-smokers (NS). Nine healthy smokers and eight healthy non-smoking controls reported to the laboratory at baseline (CON) and following six day supplementation periods with 140 ml·day^{-1} NO_3^--rich (8.4 mmol NO_3^-·day^{-1}; NIT) and NO_3^--depleted (0.08 mmol NO_3^-·day^{-1}; PLA) beetroot juice in a cross-over experiment. Plasma and salivary $[SCN^-]$ were elevated in smokers compared to non-smokers in all experimental conditions (P<0.05). Plasma and salivary $[NO_3^-]$ and nitrite ($[NO_2^-]$) were elevated in the NIT condition compared to CON and PLA conditions in smokers and non-smokers (P<0.05). However, the change in salivary $[NO_3^-]$ (S: 3.5 ± 2.1 vs. NS: 7.5 ± 4.4 mM), plasma $[NO_3^-]$ (S: 484 ± 198 vs. NS: 802 ± 199 μM) and plasma $[NO_2^-]$ (S: 218 ± 128 vs. NS: 559 ± 419 nM) between the CON and NIT conditions was lower in the smokers compared to the non-smokers (P<0.05). Salivary $[NO_2^-]$ increased above CON to a similar extent with NIT in smokers and non-smokers (P>0.05). Systolic blood pressure was lowered compared to PLA with NIT in non-smokers (P<0.05), but not smokers (P>0.05). These findings suggest that dietary NO_3^- metabolism is compromised in smokers leading to an attenuated blood pressure reduction compared to non-smokers after NO_3^- supplementation. These observations may provide novel insights into the cardiovascular risks associated with cigarette smoking and suggest that this population may be less likely to benefit from improved cardiovascular health if they increase dietary NO_3^- intake.

Key Words: nitric oxide; thiocyanate; cardiovascular health; tobacco; fatigue

1. INTRODUCTION

Cardiovascular disease is the leading cause of mortality in developed countries and costs the global economy approximately US$3.7 trillion per annum [1]. As such, interventions that can lower cardiovascular disease morbidity will be of epidemiological and economic importance. It has long been appreciated that a diet rich in fruit and vegetables lowers the risk of developing cardiovascular diseases and the incidence of adverse cardiovascular events such as stroke, heart failure and coronary heart disease [2,3]. Consequently, there are numerous government-driven initiatives to increase fruit and vegetable consumption including the Dietary Approaches to Stop Hypertension (DASH) diet in the United States of America [4], the 5-A-Day diet in the United Kingdom [5] and variations of this latter diet in countries within the European Union [6].

It has been suggested that the cardio-protective effects of diets rich in fruit and vegetables might be linked to their high inorganic nitrate (NO_3^-) content [7-9]. Vegetable consumption accounts for 60-80% of dietary NO_3^- intake [10] with leafy-green vegetables (e.g., spinach and lettuce varieties) and beetroot being particularly rich in NO_3^- [7]. It has been reported that consuming 5 portions of NO_3^--rich vegetables for 7 days, which provided a daily NO_3^- intake of ~ 317 mg (5.1 mmol), lowered systolic blood pressure, whereas a control diet where participants avoided NO_3^--rich vegetables, resulting in a daily NO_3^- intake of ~ 8 mg (0.1 mmol), did not [9]. Increased dietary NO_3^- intake in the form of NO_3^--rich beetroot [11-13] or spinach [14,15] supplementation alone has also been shown to lower resting blood pressure. These findings are consistent with the emerging body of evidence to support improved vascular health following dietary NO_3^- supplementation (4-16 $mmol \cdot day^{-1}$) in younger [11-13] and older [16,17] normotensive adults, and in individuals with hypertension [18], peripheral artery disease [19] and heart failure [20]. Therefore, enriching the diet with NO_3^-, at a dose that can be readily achieved by a diet high in vegetables [4,7,9], might represent a practical and cost-effective intervention to lower cardiovascular disease morbidity and mortality.

After oral ingestion, approximately 25% of NO_3^- passes into the entero-salivary circulation [21]. Subsequently, NO_3^- is concentrated and delivered within saliva to the oral cavity where facultative microflora reduce NO_3^- to nitrite (NO_2^-) [21-25]. NO_2^--rich saliva is then ingested

and NO_2^- is further reduced to nitric oxide (NO) and other reactive nitrogen intermediates in the acidic environment of the stomach [26-27]. It is also clear that a portion (in the nM range) of the ingested NO_2^- passes into the systemic circulation [24] where it can impact vascular function directly [28,29] or through its subsequent reduction to NO via a number of NO_2^- reductases [30]. Although mammalian tissues have the capacity to directly metabolise NO_3^- [34], the entero-salivary delivery of NO_3^- to the oral cavity and its subsequent reduction to NO_2^- by lingual anaerobes, are key rate limiting steps of NO_3^- metabolism in mammals [35]. There is evidence to suggest that the uptake of NO_3^- into the salivary circulation occurs in competition with perchlorate, thiocyanate (SCN^-) and iodide [36]. Therefore, increased exposure to perchlorate, SCN^- or iodide may interfere with dietary NO_3^- metabolism and might subsequently blunt the improvements in vascular health that have typically been observed after increased dietary NO_3^- intake.

Cigarette smoking is a major risk factor for cardiovascular disease morbidity and mortality [37-39], and a leading cause of preventable death worldwide [38,40]. In spite of global government initiatives to facilitate smoking cessation, there are still an estimated 1 billion smokers worldwide [41]. Cigarette smoke contains over 7000 noxious chemicals, including cyanide [38]. Following consumption, cyanide is rapidly detoxified to thiocyanate (SCN^-) via transsulfuration reactions catalysed by the enzymes, thiosulfate sulfotransferase (rhodanase) and 3-mercaptopyruvate sulfurtransferase [42]. Consequently, cigarette smokers have elevated plasma and salivary [SCN^-] compared to non-smoking controls [e.g., 43]. Importantly, and consistent with a competitive inhibition of salivary NO_3^- uptake by SCN^- [36], it has been reported that salivary [NO_3^-] is lower in cigarette smokers after NO_3^- ingestion, compared to non-smoking controls [44,45]. However, in spite of a lower salivary [NO_3^-] after NO_3^- ingestion in smokers, the increase in salivary [NO_2^-] was not different between the smokers and non-smokers [44]. It is therefore unclear whether cigarette smoking interferes with the increases in plasma [NO_2^-] and the associated reduction of blood pressure that has been observed following dietary NO_3^- ingestion in non-smokers [12,13,31,33]. Further research is required to elucidate the effects of cigarette smoking on dietary NO_3^- metabolism and its implications for vascular health. If dietary NO_3^- metabolism is indeed perturbed by cigarette smoking, this may provide new insights into the mechanisms by which cigarette smoking increases cardiovascular disease morbidity.

The purpose of this study was to assess the effects of six days dietary NO_3^- supplementation on plasma and salivary [NO_3^-], [NO_2^-] and [SCN^-] and resting blood pressure in smokers and non-smoking controls. It was hypothesized that the increases in salivary [NO_3^-], plasma [NO_3^-] and plasma [NO_2^-], but not salivary [NO_2^-], after dietary NO_3^- supplementation would be attenuated in cigarette smokers compared to non-smoking controls. It was also hypothesised that dietary NO_3^- supplementation would lower blood pressure in non-smokers, but not in smokers.

2. MATERIALS AND METHODS

2.1 Subjects

We recruited nine cigarette smokers (5 males, mean ± SD, age 24 ± 7 yr, body mass index 23 ± 2 kg·m^2; smoking history 7 ± 6 pack years) and eight age- and BMI-matched non-smoking controls (4 males, mean ± SD, age 24 ± 5 yr, body mass index 23 ± 4 kg·m^2) from the University staff and student communities to participate in this study. Both the smokers [forced vital capacity (FVC) 4.68 ± 1.02 L; forced expiratory volume in 1-s (FEV_1) 4.26 ± 0.99 L; FEV_1/ FVC 91 ± 6 %] and non-smokers (FVC, 4.17 ± 0.76 L; FEV_1, 3.64 ± 0.57 L; FEV_1/ FVC 88 ± 5 %] exhibited normal resting pulmonary function and had a similar level of habitual physical activity, as assessed by the Baecke et al. [46] questionnaire (smokers, 7.2 ± 1.7; non-smokers 7.2 ± 1.7), upon recruitment to the study. All procedures employed in this study were approved by the Institutional Research Ethics Committee and subjects gave their written informed consent to participate prior to the commencement of the study, after the experimental procedures, associated risks, and potential benefits of participation had been explained. Subjects were instructed to arrive at each laboratory testing session in a rested and fully hydrated state, at least 3 h postprandial. Since the reduction of NO_3^- to NO_2^- in the oral cavity is compromised by antibacterial mouthwash [47], subjects were required to refrain from mouthwash use for the duration of the study. Each subject was also asked to avoid consumption of nitrate-rich foods for the duration of the study, and from caffeine and alcohol ingestion 6 and 24 h before each test, respectively. All subjects were instructed to maintain their habitual physical activity pattern for the duration of the study, and to avoid strenuous exercise in the 24 h preceding the testing sessions. Smokers were asked to maintain their habitual smoking patterns for the duration of the study, but were required to abstain from

smoking for 3 h before each testing session. All tests were performed at the same time of day (± 2 hours).

2.2 Supplementation Procedures

All subjects were required to report to the laboratory on three occasions over a 3-4 week period. Subjects did not undergo dietary supplementation prior to their first visit to the laboratory (the control condition; CON). Subjects were asked to record their food and beverage consumption on the day of the CON test and for the 5 days preceding this test and to replicate this prior to the subsequent trials. After completing the CON trial, subjects were randomly assigned to receive six days of supplementation with either NO_3^--rich (NIT) or NO_3^--depleted beetroot juice as a placebo (PLA) as part of a double-blind, cross-over experimental design. In the NIT and PLA conditions, subjects ingested 70 ml of concentrated beetroot juice containing 4.2 and 0.04 mmol NO_3^-, respectively, in the morning and evening over the first five days of supplementation. Subjects ingested 140 ml of beetroot juice two hours before reporting to the laboratory on day six of NIT and PLA supplementation period. This was selected to coincide with the peak plasma [NO_2^-] attained following ingestion of 8.4 mmol NO_3^- [13]. A 7-10 day washout separated the supplementation periods.

2.3 Measurements

2.3.1 Expired carbon monoxide and resting respiratory function

Upon arrival at the laboratory the carbon monoxide (CO) content of subjects' expired air was assessed in a standing position using a hand-held CO analyzer (Micro$^+$ Smokerlyzer, Bedfont Scientific Ltd, Kent, UK). For this assessment subjects were instructed to inhale to total lung capacity and then exhale slowly into the CO analyzer until they attained residual volume. Three manoeuvres were completed with the highest CO measurement used for analysis. Subjects then completed three FVC manoeuvres using a hand-held micro spirometer (Micro Plus, Micro Medical Ltd, Kent, UK) for the assessment of resting respiratory function (FVC, FEV_1 and FEV_1/ FVC). For this assessment subjects were instructed to inhale to the total lung capacity and then exhale as quickly and forcefully as possible into the micro spirometer until they attained residual volume. The data from the manoeuvre that produced the highest FVC were used for analysis. Subjects were fitted with a nose clip during all these measurements to prevent the expiration of air through the nasal passage.

2.3.2 Blood Pressure

Subjects were required to rest supine for 10 min in an isolated room. Thereafter, blood pressure of the brachial artery was measured whilst the subject was supine using an automated sphygmomanometer (Dinamap Pro, GE Medical Systems, Tampa, USA). Five measurements were taken and the mean of the measurements 2-5 was used for analysis.

2.3.3 Blood and saliva collection

Venous blood samples were drawn into 6 mL lithium-heparin tubes (Sarstedt, Leicester, UK). Samples were centrifuged at 4,000 rpm and 4°C for 10 min, within 1 min of collection. Plasma was subsequently extracted and immediately frozen at -80°C for later analysis of $[NO_2^-]$, $[NO_3^-]$ and $[SCN^-]$. Unstimulated saliva samples (~ 3 mL) were collected into 30 mL universal containers and 1 mL aliquots were frozen at -80°C for later analysis of $[NO_2^-]$, $[NO_3^-]$ and $[SCN^-]$.

2.4 Data analysis procedures

2.4.1 $[NO_3^-]$ and $[NO_2^-]$ determination

All glassware, utensils, and surfaces were rinsed with deionized water to remove residual NO prior to $[NO_2^-]$ and $[NO_3^-]$ analysis. Plasma samples were deproteinized using zinc sulfate/sodium hydroxide precipitation prior to determination of $[NO_3^-]$. Firstly, 500 μL of 0.18 N NaOH was added to 100 μL of sample followed by 5 min incubation at room temperature. Subsequently, samples were treated with 300 μL aqueous $ZnSO_4$ (5% w/v) and vortexed for 30 seconds before undergoing an additional 10 min incubation period at room temperature. Samples were then centrifuged at 4,000 rpm for 5 min, and the supernatant was removed for subsequent analysis. The $[NO_3^-]$ of the deproteinized plasma sample was determined by its reduction to NO in the presence of 0.8 % (w/v) VCl_3 in 1 M HCl within an air-tight purging vessel. Plasma samples were introduced to the vessel via 100 μL injections into the septum at the top of the vessel. The spectral emission of electronically excited nitrogen dioxide, derived from the reaction of NO with ozone, was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence nitric oxide analyzer (Sievers NOA 280i. Analytix Ltd, Durham, UK). The $[NO_3^-]$ was determined by plotting signal (mV) area against a calibration plot of sodium nitrate standards. The $[NO_2^-]$ of the undiluted (non-deproteinized) plasma was determined by its reduction to NO in the presence of glacial acetic acid and aqueous NaI (4% w/v), and calibrated using sodium nitrite standards. After thawing at room temperature, saliva samples

were centrifuged for 10 min at 14000 rpm and the supernatant was removed for subsequent analysis. The supernatant was diluted 100 fold with deionized water and [NO_3^-] and [NO_2^-] were determined from 50 μL injections using the same reagents describe above for the plasma analyses.

2.4.2 [SCN^-] determination

Plasma and salivary [SCN^-] were measured in duplicate using the procedures described by Tsuge et al. [43]. Briefly, 300 μL of plasma was treated with 200 μL of trichloroacetic acid (25% w/v) and centrifuged for 10 min at 14000 rpm. The supernatant was then removed for subsequent analysis. 50 μL of sample, 15 μL x 1 M of ice-cold potassium hydrogen phosphate solution (pH 5.5), 5 μL of 5M NaOH solution, and 10 μL of 6.25mg/ml chloramine T solution were added to a 96-well microtiter plate (Sterilin Ltd., Caerphilly, UK) and incubated on ice for 2 min. Subsequently, 120 μL of cyanoline blue solution (0.27 % w/v) was added to the microtiter plate and the plate was incubated for 20 min at room temperature. Following incubation, sample absorbance was measured at 620 nm using a microplate reader (EnSpire 2300, Perkin Elmer, Hamburg, Germany). Thawed saliva samples were centrifuged for 10 min at 14000 rpm and the supernatant was removed for subsequent analysis. Samples were diluted 25 fold, and 40 μL of the diluted sample was added to a 96-well microtiter plate in addition to 20 μL of 1M potassium phosphate buffer solution and 20 μL of 6.25mg/ml choramine T solution, and incubated on ice for 2 min. 140 μL of cyanoline blue solution (0.27 % w/v) in pyridine-water (1:5, v/v) was added to the microtiter plate followed by 20 min incubation at room temperature. Following incubation, sample absorbance was measured at 620 nm using a microplate reader.

2.5 Statistics

A two-way (treatment by group) ANOVA with repeated measures for treatment (CON, PLA and NIT) was employed to determine the effects of the different dietary interventions on plasma and salivary [NO_3^-] and [NO_2^-] in the smokers and non-smokers. Where the analysis revealed a significant main or interaction effect, simple follow-up contrasts were employed to determine the origin of such effects. Paired samples *t*-tests were employed to compare the effects of PLA and NIT on blood pressure variables. Pearson's product moment correlation coefficient was used to assess the relationship between changes in variables across conditions in the smokers and non-smokers. All data are presented as mean ± SD unless otherwise indicated. Statistical significance was accepted when $P<0.05$.

3. RESULTS

The PLA and BR supplements administered in this study were well tolerated by all subjects with no negative side effects reported. Subjects self-reported that they consumed all doses of the supplement for each experimental condition and that their diet and physical activity patterns were consistent across all the dietary interventions. Smokers confirmed that their smoking habits remained consistent across the experimental testing period and that they avoided smoking a cigarette for at least 3 hours before each experimental testing session as instructed. There was a main effect for group on expired [CO] (P<0.01) with smokers exhibiting a higher expired [CO] than non-smokers in CON (6 ± 3 ppm vs. 2 ± 1 ppm), PLA (6 ± 2 ppm vs. 2 ± 1 ppm) and NIT (6 ± 3 ppm vs. 2 ± 0 ppm; P<0.01 for all comparisons), with no differences in expired [CO] between treatments in either the smokers or non-smokers (P>0.05 for all comparisons).

3.1 Salivary and plasma [SCN^-]

Salivary [SCN^-] was higher in smokers than non-smokers in CON, PLA and NIT (P<0.05 for all comparisons; Figure 1). Salivary [SCN^-] was lower in NIT than CON and PLA in both smokers and non-smokers (P<0.05 for all comparisons). Plasma [SCN^-] was higher in smokers than non-smokers in CON, PLA and NIT (P<0.05 for all comparisons). There were no between-treatment differences in either the smoking or non-smoking groups (P>0.05 for all comparisons).

3.2 Salivary and plasma [NO_3^-] and [NO_2^-]

There was a group × treatment interaction effect for salivary [NO_3^-] (P<0.05). Further analyses revealed that salivary [NO_3^-] was higher than CON and PLA in NIT in both smokers and non-smokers (P<0.01 for all comparisons; Figure 2), with a lower salivary [NO_3^-] observed in smokers compared to non-smokers in NIT (P<0.05). A negative correlation was observed between the salivary [SCN^-] in the CON condition and the change in the salivary [NO_3^-] between the CON and NIT conditions in the smokers (r = -0.78, P<0.01; Figure 3) There was a main effect for treatment on salivary [NO_2^-] (P<0.01) with salivary [NO_2^-] being higher than CON and PLA in NIT in both smokers and non-smokers (P<0.01 for all comparisons, Figure 2). However, there were no differences between smokers and non-smokers in any of the experimental conditions (P>0.05).

There was a group × treatment interaction effect for both plasma [NO_3^-] and [NO_2^-] (P<0.01). Plasma [NO_3^-] and [NO_2^-] were not different between smokers and non-smokers in CON and PLA (P>0.05). While plasma [NO_3^-] and [NO_2^-] were higher than CON and PLA with NIT in both smokers and non-smokers (P<0.01), plasma [NO_3^-] and [NO_2^-] were lower in smokers than non-smokers in NIT (P<0.01, Figure 2).

3.3 Blood pressure

Systolic, diastolic and mean arterial blood pressures were not different between the smokers and non-smokers in the NIT and PLA conditions (P>0.05, Table 1). Systolic blood pressure was lowered in the NIT condition compared to PLA in non-smokers (P<0.05), but not smokers (P>0.05; Figure 4). There were no differences in diastolic blood pressure or mean arterial pressure between the smoking and non-smoking groups in the PLA and NIT conditions (P>0.05). There was a negative correlation between the change in in plasma [NO_2^-] and the change in systolic blood pressure (r = -0.71, P<0.05), but not diastolic (r = -0.17) or mean arterial blood pressure (r = -0.36) in the non-smokers between the PLA and NIT trials (P>0.05). There were no correlations between the change in plasma [NO_2^-] and the changes in systolic (r = 0.25) diastolic (r = 0.08) and mean arterial (r = 0.24) blood pressure in the smokers between the PLA and NIT trials (P>0.05).

4. DISCUSSION

The important novel findings from this study are that, despite consuming the same absolute NO_3^- dose, the increases in salivary [NO_3^-], plasma [NO_3^-] and plasma [NO_2^-] were essentially halved in smokers compared to non-smokers, and blood pressure was only improved in non-smokers. These findings are important because they suggest that cigarette smokers may not derive the same vascular benefits, compared to age-, BMI- and activity-matched non-smoking controls, if they increase dietary NO_3^- intake. Therefore, our findings might provide novel insights into the potential risk factors that predispose cigarette smokers to increased cardiovascular disease morbidity.

In the CON condition, where participants did not receive any dietary supplementation, plasma and salivary [SCN^-] were 122% and 89% higher in the smokers compared to the non-smokers. Plasma and salivary [SCN^-] were also higher in the smokers compared to the non-

smokers after PLA and NIT supplementation. Several previous studies have reported increased plasma and salivary [SCN^-] in smokers compared to non-smokers [43,48]. It is known that cigarette smoke contains cyanide [38] and that the increase in systemic [SCN^-] in cigarette smokers is consequent to cyanide detoxification facilitated by the enzymes, thiosulfate sulfotransferase (rhodanase) and 3-mercaptopyruvate sulfurtransferase [42]. Consequently, salivary/plasma [SCN^-] has been recommended as an objective marker of smoking status [43,49]. However, it has been suggested that expired [CO] might be a more sensitive bio-marker of smoking status than salivary/plasma [SCN^-] [50-52]. Expired [CO] was 200% higher in the smokers compared to the non-smokers in all experimental conditions in this study. Taken together, these findings confirm that the participants in our smoking group were indeed active smokers, and that they consistently adhered to the requirement to abstain from smoking for 3 h before each testing session.

In line with previous studies, salivary and plasma [NO_3^-] and [NO_2^-] were increased following dietary NO_3^- supplementation in this study [47,53]. However, despite oral consumption of the same absolute NO_3^- dose (8.4 mmol), the increase in salivary [NO_3^-] above CON in the smokers (3510 μM) was only 48% of that observed in the non-smokers (7289 μM). These findings are consistent with previous observations of a lower salivary NO_3^- uptake after oral NO_3^- consumption in smokers compared to non-smoking controls [44,45]. This antagonism of salivary NO_3^- uptake after NO_3^- supplementation is likely mediated by greater plasma and salivary [SCN^-] in the smokers since it has been suggested that NO_3^- and SCN^- share a common transporter for uptake into the salivary circulation and that SCN^- has a higher affinity for salivary uptake than NO_3^- [36]. In support of a competitive inhibition of salivary NO_3^- uptake by SCN^-, the change in the salivary [NO_3^-] between the CON and NIT conditions was negatively correlated with the salivary [SCN^-] in CON in the smokers (figure 3). Further support for competition between NO_3^- and SCN^- for passage into the salivary circulation is evidenced by the lower salivary [SCN^-] in NIT compared to CON in both the smokers (-33%) and non-smokers (-38%) in this study. Collectively, these findings lend support to the notion that NO_3^- and SCN^- share a common salivary transporter(s), possibly sialin [54], and that SCN^- can competitively inhibit salivary NO_3^- uptake.

Although salivary [NO_3^-] was lower after NIT in the smokers compared to the non-smokers, salivary [NO_2^-] was not different between smokers and non-smokers after NIT. These

observations are consistent with previous findings of a similar salivary [NO_2^-] in smokers and non-smokers after NO_3^- ingestion in spite of a lower salivary [NO_3^-] [44]. It has been reported that the K_m of the oral bacteria NO_3^- reductases for NO_3^- is ~ 1000 μM [55,56]. Salivary [NO_3^-] increased to a mean concentration 7450 μM after NIT in the non-smokers and a mean value of 3708 μM in the smokers in this study. Therefore, it is possible that the similar salivary [NO_2^-] after NIT in the smokers and non-smokers might be related to both groups obtaining a saturating salivary [NO_3^-] for the oral NO_3^- reductases after NIT. However, more recent studies have reported that increasing the salivary [NO_3^-] to a greater extent than achieved in the current study (~12-16 mM) can lead to further increases in salivary [NO_2^-] (~2.5 mM vs. ~1.5 mM in the current study), at least in non-smokers [47, 53]. These results challenge the previously reported K_m of the oral bacteria NO_3^- reductases for NO_3^- [55,56]. Therefore, further research is required to resolve the mechanisms than underlie the similar increase in salivary [NO_2^-] in spite of a lower increase in salivary [NO_3^-] after NO_3^- supplementation in smokers, and the K_m of the oral bacteria NO_3^- reductases for NO_3^-.

Circulating plasma [NO_3^-] and [NO_2^-] were both increased compared to CON and PLA after NIT in non-smokers, as reported elsewhere [9,11-13,24,31,32,33,57]. However, despite plasma [NO_3^-] and [NO_2^-] also increasing above values observed in the CON condition in the smokers, plasma [NO_3^-] and [NO_2^-] were only increased to 60% and 39% of the values observed in the non-smokers after NIT. It has been reported that smoking a single cigarette transiently lowers plasma [NO_3^-] and [NO_2^-] for 60 minutes [58]. However, since our participants were asked to abstain from smoking for 3 hours prior to reporting to the laboratory, and since the expired [CO] was not different in the smokers across the experimental conditions, it is unlikely that the blunted increases in plasma [NO_3^-] and [NO_2^-] after NIT in the smokers can be ascribed to the acute effects of smoking. Given that salivary NO_3^- uptake was lower in the smokers, the lower plasma [NO_3^-] after NIT in this group might have been a function of increased NO_3^- excretion in urine to offset excessive plasma NO_3^- accumulation. The lower plasma [NO_2^-] after NIT in the smokers compared to the non-smokers, in spite of a similar salivary [NO_2^-], might also be linked to increased NO_2^- excretion. Alternatively, it is possible that the SCN^--catalysed reduction of NO_2^- to NO in the stomach [59] was increased in the smokers, which might have attenuated NO_2^- uptake into the systemic circulation. In addition, smokers have an elevated myloperoxidase activity in neutrophils [60], which has been reported to catalyse NO_2^- oxidation [61] and might have contributed to the lower plasma [NO_2^-] after NIT in the smokers. Finally, it has been

suggested that cigarette smoking impairs NO generation through eNOS, by lowering eNOS expression [62] and/or promoting eNOS uncoupling [63], and that the chemical reduction of NO_2^- can compensate for the perturbed cardiovascular function associated with NOS dysfunction [64-66]. Therefore, the lower plasma [NO_2^-] after NIT in the smokers in this study might be reflective of an increased NO_2^- reduction to NO through xanthine oxidase [67] or other NO_2^- reductases [30] as a compensatory mechanism for a potential shortfall in eNOS-derived NO.

Compared to PLA, systolic blood pressure was significantly lowered by 3 mmHg after NIT in the normotensive non-smokers who participated in this study. These findings are consistent with several [9,11-15], but not all [e.g., 68], previous reports of lower blood pressure after NIT in normotensive non-smoking participants. In addition, we observed a significant negative correlation between the change in plasma [NO_2^-] and the change in systolic blood pressure between the PLA and NIT conditions in the non-smokers, consistent with the effects of NO_2^- [28,29] or NO [30, 57] on smooth muscle relaxation, and with previous reports of negative correlations between the changes in plasma [NO_2^-] and blood pressure [57]. However, while NIT increased plasma [NO_2^-], and therefore the potential for O_2-independent NO generation [30], it is unclear whether the lowering of blood pressure is mediated through classical NO-cyclic guanosine monophosphate (cGMP) signalling. NO_3^- supplementation has been reported to increase plasma [NO_2^-] and lower blood pressure in association with increased plasma [cGMP] in some studies [57], but recent evidence also suggests that the lowering of blood pressure following NO_3^- supplementation might be mediated by modulating renal physiology [69]. Therefore, the mechanisms that underlie the lowering of blood pressure after short-term NO_3^- supplementation requires further investigation.

In the smokers, blood pressure was not different between the NIT and PLA trials. It is important to note that the mean increase in plasma [NO_2^-] after ingesting 8.4 mmol of NO_3^--rich beetroot juice was 218 nM in the smokers, which was only 39% of the mean plasma [NO_2^-] observed in the non-smokers after NIT, and is comparable with the mean 220 nM increase in plasma [NO_2^-] after ingesting only 4.2 mmol of NO_3^--rich beetroot juice in our previous dose-response study [13]. While the lack of a significant blood pressure reduction might be ascribed, at least in part, to a lower plasma [NO_2^-] in smokers after NIT, systolic blood pressure but not diastolic or mean arterial blood pressures, was significantly lower after the ingestion of 4.2 mmol of NO_3^--rich beetroot juice in our previous dose-response study

[13]. Smokers have been reported to exhibit increased plasma nitrotyrosine levels [70], potentially indicative of increased scavenging of NO by superoxide [71]. This suggests that the bioavailability of NO_2^--derived NO, and the accompanying lowering of blood pressure, might be compromised in smokers at a given NO_3^- dose. Moreover, cigarette smoking is associated with heightened inflammatory, oxidative and nitrative stress, which contributes to vascular remodelling and biochemical dysfunction [72-74]. These negative effects on the vasculature might lower the responsiveness of the blood vessels to dilate at a given plasma [NO_2^-] which could account for our findings of improved blood pressure in non-smokers, but not smokers, after short-term dietary NO_3^- supplementation.

It is well documented that hypertension [77] is an independent predictor of cardiovascular disease morbidity and mortality. The magnitude of systolic blood pressure reductions in this study would be expected to lower the incidence of stroke and ischemic heart disease [79]. These observations suggest that the blood pressure reductions after short-term NIT in non-smokers might be expected to confer a lower risk for cardiovascular disease morbidity and adverse cardiovascular events. Conversely, and despite ingesting the same absolute NO_3^- dose as the non-smokers, blood pressure was not lowered after NIT in the smokers. By suggesting that cigarette smokers might not improve blood pressure if they increase dietary NO_3^- intake, our data provide potential novel insights into the risk factors for cardiovascular disease morbidity in cigarette smokers. Since the attenuated effects of NIT on blood pressure appear to be linked, at least in part, to a SCN^--mediated perturbation to dietary NO_3^- metabolism, and since circulating SCN^- is increased dose-dependently with smoking status [43], it is possible that the antagonistic effects of SCN^- on dietary NO_3^- metabolism and its associated physiological responses are even greater in individuals with a higher number of pack years than the light smokers (7 ± 6 pack years) who participated in this study. Similarly, since we instructed smokers to abstain from cigarette smoking for 3 hours prior to reporting to the laboratory, the potential for NO_3^- supplementation to offset the transient increase in blood pressure [80] and lowering of plasma [NO_2^-] [58] was not explored in this study and could represent a positive effect of dietary NO_3^- supplementation in cigarette smokers. It is also important to stress that, although the findings of this study suggest that cigarette smokers might not improve blood pressure if they increase dietary NO_3^- intake, increased NO_3^--rich vegetable consumption will provide other nutrients that can benefit human health [2,3]. Moreover, it is possible that increased NO_3^- intake might have improved aspects of health in smokers that were not investigated in this study or that cigarette smokers

might need to consume a greater NO_3^- dose to lower blood pressure compared to their non-smoking counterparts. Therefore, further research is required to assess the health outcomes and underlying mechanisms of compromised dietary NO_3^- metabolism in cigarette smokers.

5. CONCLUSION

In conclusion, salivary [NO_3^-], plasma [NO_3^-] and plasma [NO_2^-] were lower by ~ 50% in cigarette smokers compared to non-smoking controls after ingesting the same absolute NO_3^- dose. This perturbation to dietary NO_3^- metabolism in cigarette smokers abolished the improvement in blood pressure that was observed in the non-smokers after NO_3^- supplementation. These findings suggest that cigarette smokers are less likely to improve blood pressure if they were to increase dietary NO_3^- intake. These observations might provide important novel insights into the mechanisms by which cigarette smoking predisposes to increased cardiovascular disease morbidity and mortality.

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Figure Legends

Figure 1: Mean salivary (*upper panel*) and plasma (*lower panel*) thiocyanate concentrations ($[SCN^-]$) following no dietary supplementation (CON), supplementation with nitrate-depleted beetroot juice (PLA) and supplementation with nitrate-rich beetroot juice (NIT) in smokers and non-smokers. The *filled bars* represent the group mean ± SEM responses in the CON, PLA and NIT conditions in non-smokers, while the *open bars* represent the group mean ± SEM responses in the CON, PLA and NIT conditions in smokers. # indicates significantly different from the smokers in the same experimental * indicates significantly different from responses in CON and PLA. Note condition. the higher mean salivary and plasma $[SCN^-]$ in smokers in all experimental conditions and the lower salivary $[SCN^-]$ in NIT compared to CON and PLA in both smokers and no-smokers.

Figure 2: Mean salivary and plasma nitrate ($[NO_3^-]$) and nitrite ($[NO_2^-]$) concentrations following no dietary supplementation (CON), supplementation with nitrate-depleted beetroot juice (PLA) and supplementation with nitrate-rich beetroot juice (NIT) in smokers and non-smokers. Salivary $[NO_3^-]$ responses are shown in the *upper left panel*, salivary $[NO_2^-]$ responses are shown in the *upper right panel*, plasma $[NO_3^-]$ responses are shown in the *lower left panel* and salivary $[NO_2^-]$ responses are shown in the *lower right panel*. The *filled bars* represent the group mean ± SEM responses in the CON, PLA and NIT conditions in non-smokers, while the *open bars* represent the group mean ± SEM responses in the CON, PLA and NIT conditions in smokers. # indicates significantly different from the smokers in the same experimental condition. * indicates significantly different from responses in CON and PLA. Note the significant increases in mean salivary and plasma $[NO_3^-]$ and $[NO_2^-]$ with NIT supplementation in both smokers and non-smokers and that the increases in the salivary $[NO_3^-]$ and plasma $[NO_3^-]$ and $[NO_2^-]$ were lower following oral consumption of the same NIT dose in smokers compared to non-smokers.

Figure 3: The relationship between salivary thiocyanate concentration ($[SCN^-]$) with no supplementation (CON) and the change (Δ) in salivary nitrate concentration ($[NO_3^-]$) between the CON and NIT conditions in smokers. Note that the salivary $[SCN^-]$ in CON was negatively correlated with Δ CON-NIT salivary $[NO_3^-]$.

Figure 4: The individual changes in systolic, diastolic and mean arterial blood pressures following supplementation with nitrate-depleted beetroot juice (PLA) and supplementation with nitrate-rich beetroot juice (NIT) in smokers and non-smokers. * indicates significantly different from responses in PLA.

Table 1. Resting supine blood pressure measures following nitrate-depleted beetroot juice supplementation (PLA) and nitrate-rich beetroot juice supplementation (NIT) in smokers and non-smokers.

	Smokers	
	PLA	NIT
Systolic blood pressure (mmHg)	109 ± 8	110 ± 7
Diastolic blood pressure (mmHg)	59 ± 6	59 ± 5
Mean arterial pressure (mmHg)	79 ± 5	79 ± 4
	Non-smokers	
	PLA	NIT
Systolic blood pressure (mmHg)	103 ± 8	100 ± 10*
Diastolic blood pressure (mmHg)	59 ± 4	58 ± 8
Mean arterial pressure (mmHg)	76 ± 6	74 ± 9

Values are presented as the mean ± SD. * = significantly different from PLA (P<0.05).

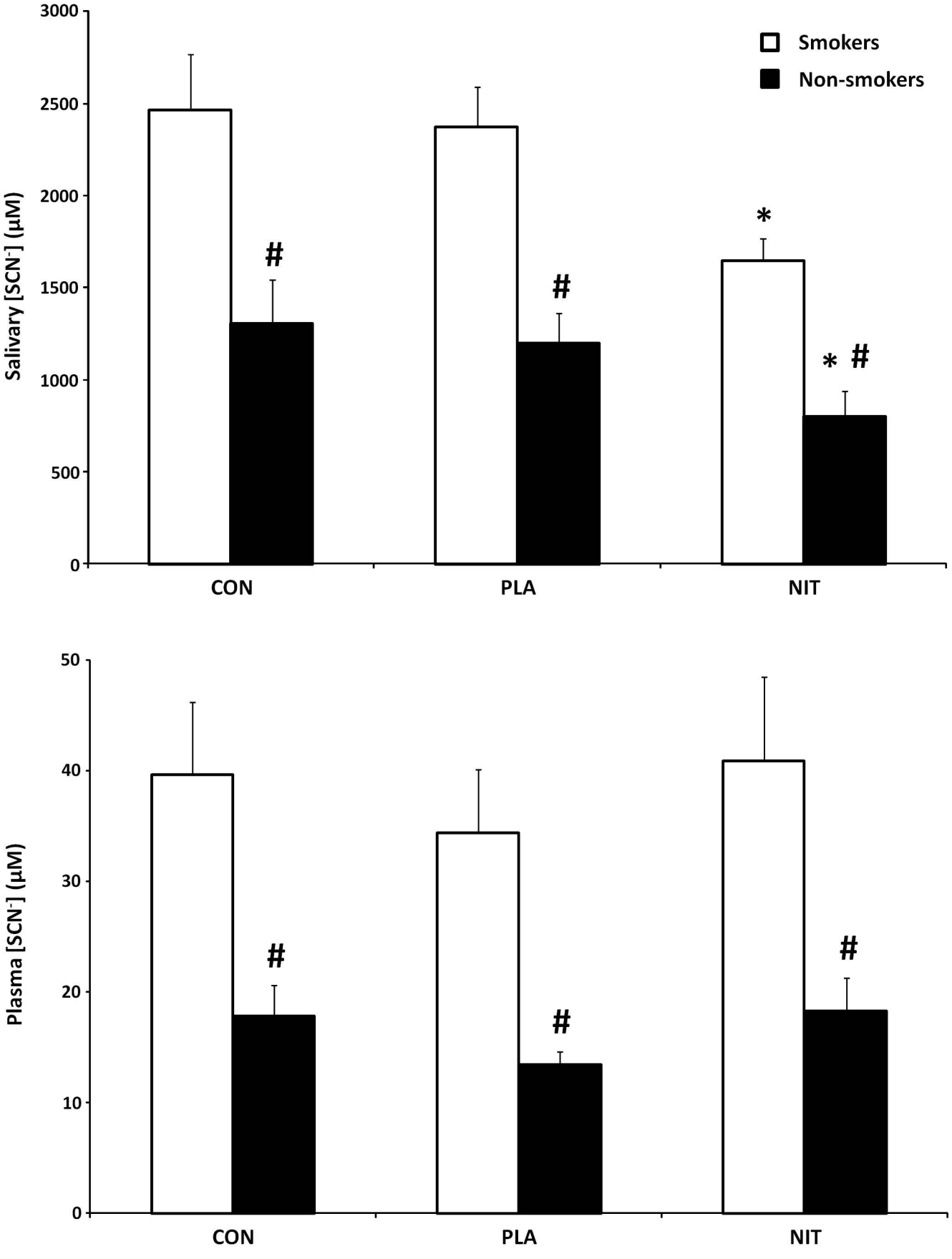
Smokers
Non-smokers
Salivary [SCN-] (µM)
3000
2500
2000
1500
1000
500
0
#
#
*
* #
CON
PLA
NIT
Plasma [SCN-] (µM)
50
40
30
20
10
0
#
#
#
CON
PLA
NIT

Figure 1

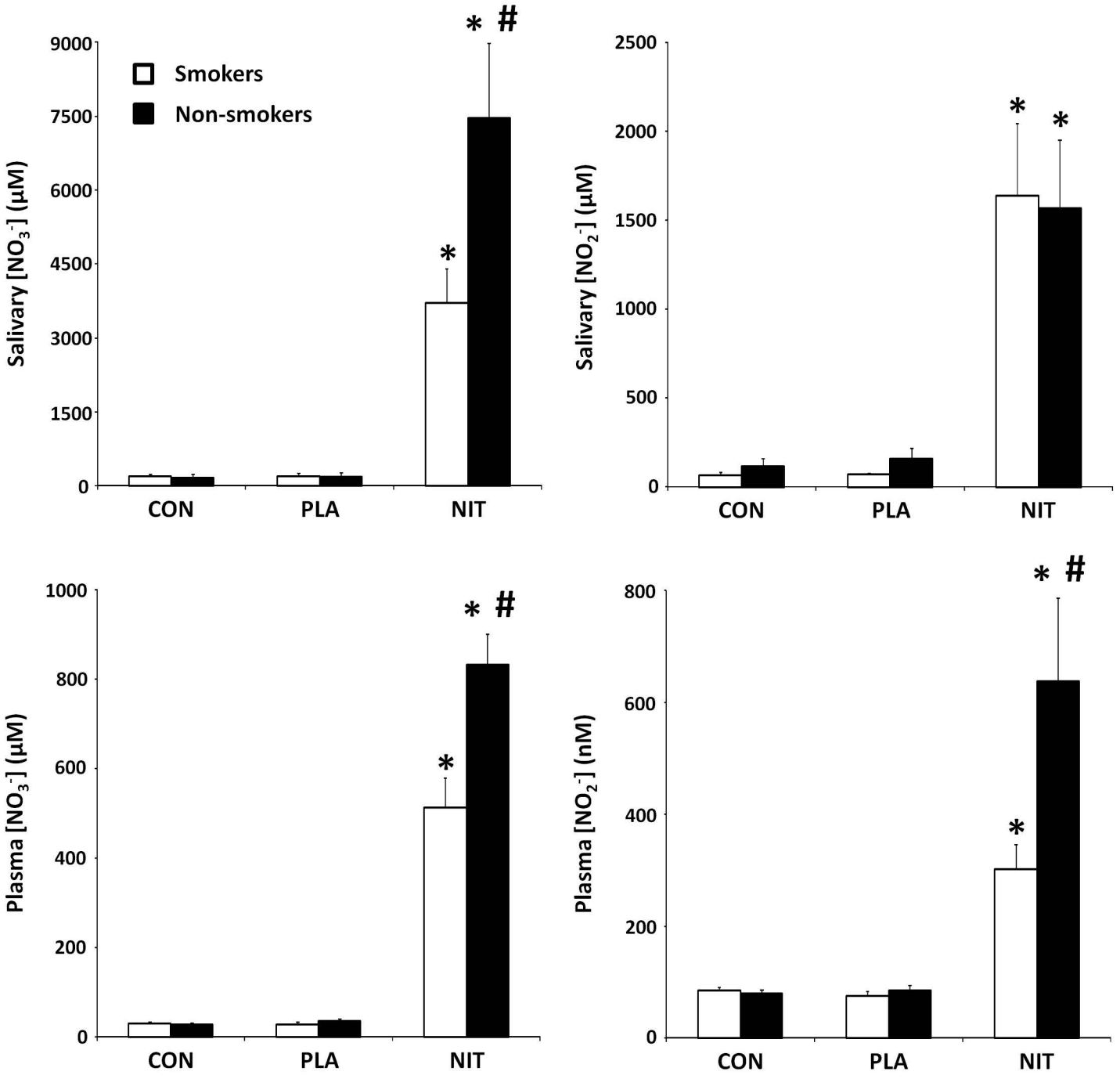
Smokers
Non-smokers
Salivary [NO3-] (µM)
9000
7500
6000
4500
3000
1500
0
CON
PLA
NIT
* #
*
Salivary [NO2-] (µM)
2500
2000
1500
1000
500
0
CON
PLA
NIT
* #
*
Plasma [NO3-] (µM)
1000
800
600
400
200
0
CON
PLA
NIT
* #
*
Plasma [NO2-] (nM)
800
600
400
200
0
CON
PLA
NIT
* #
*

Figure 2

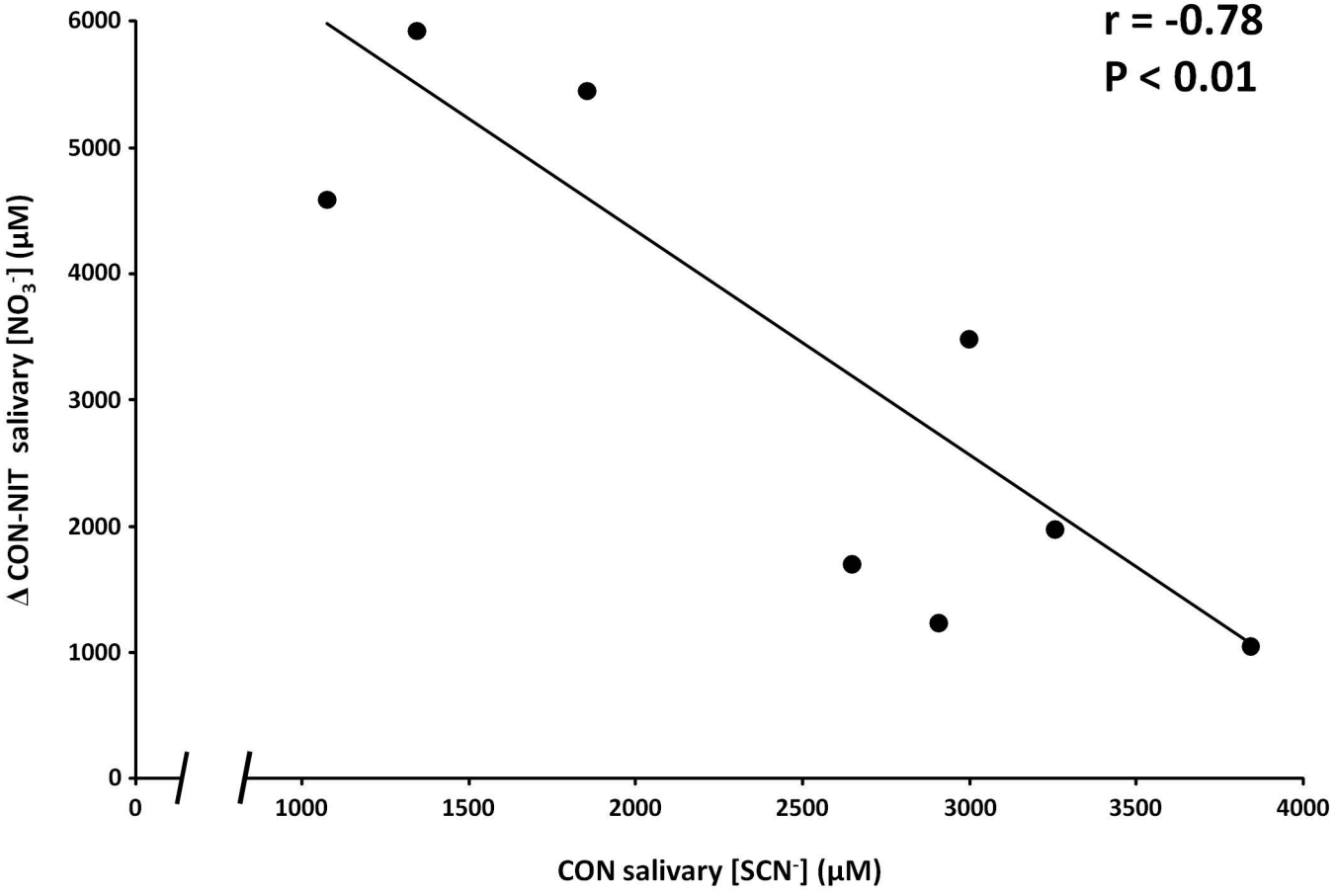
r = -0.78
P < 0.01
Δ CON-NIT salivary [NO3-] (μM)
CON salivary [SCN-] (μM)
6000
5000
4000
3000
2000
1000
0
0
1000
1500
2000
2500
3000
3500
4000

Figure 3

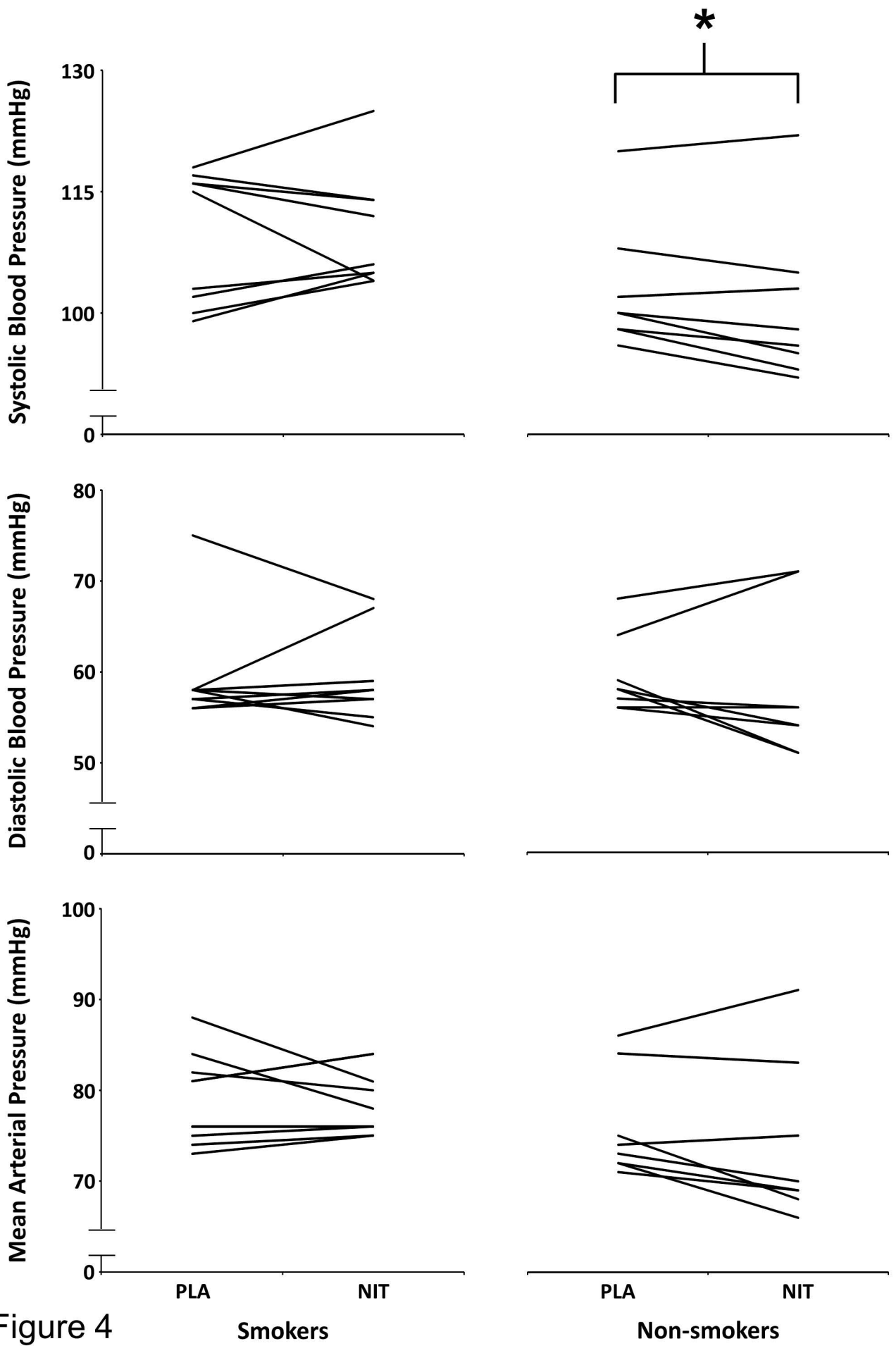
*
Systolic Blood Pressure (mmHg)
130
115
100
0
Diastolic Blood Pressure (mmHg)
80
70
60
50
0
Mean Arterial Pressure (mmHg)
100
90
80
70
0
PLA
NIT
PLA
NIT
Smokers
Non-smokers

Figure 4