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The Effect of Sodium on Sympathetic Nervous System Activity and Endothelial Function in People with Type 2 Diabetes.

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Abstract

Lowering dietary sodium intake is recommended by public health bodies to lower blood pressure with the ultimate aim of reducing cardiovascular related disease. However, lower sodium intake has been demonstrated in observational studies to be associated with adverse health outcomes such as higher cardiovascular related morbidity and mortality in people with type 2 diabetes. The may be due to pleiotropic effects of sodium lowering, such as activation of the renin-angiotensin- aldosterone system (RAAS) and increases in circulating adrenaline and noradrenaline. Dedicated intervention trials are lacking but greatly needed to investigate the complex role of sodium on cardiovascular outcomes further.

In an effort to underpin the mechanistic links behind the associations between low sodium intake and poorer cardiovascular outcomes in people with type 2 diabetes, the studies presented in this thesis aimed to examine the effect of sodium on cardiovascular health by measuring the primary endpoints of sympathetic nervous system activity, as assessed by microneurography, and endothelial function, as assessed by endoPAT and endothelial microparticle measurements, and the secondary endpoints of cardiac baroreflex, serum aldosterone, as markers of RAAS activity, and simultaneous measures of 24h blood pressure and heart rate variability.

We demonstrated, that compared to other groups along the glucose continuum, individuals with treated type 2 diabetes who had low sodium intake presented with worse endothelial and baroreflex function. This occurred even though these individuals were appropriately managed for their cardio-metabolic risk factors and importantly, adhered to recommended low sodium intake guidelines. This study provided the rationale to conduct an interventional study where, for the first time, we assessed the effect of salt supplementation on cardiovascular endpoints of sympathetic activity and endothelial function as assessed by endoPAT, in people with type 2 diabetes. Salt supplementation was associated with a small increase in sympathetic activity. However, this did not reach levels associated with pathological disease states. Importantly, there were no detrimental effects on endothelial function or alterations in blood pressure. Interestingly improvements in baroreflex function and RAAS activity were seen. When participants were categorized based on salt-sensitivity, salt-resistant individuals demonstrated a trend towards improved endothelial function after salt supplementation.

To explore the endothelial response further, we examined the association between habitual sodium intake, RAAS blockade and endothelial function as assessed by circulating microparticles. Despite expecting higher endothelial microparticles being associated with lower sodium intake, we did not demonstrate any significant association

between sodium intake and endothelial microparticles. However, we observed a trend towards higher erythrocyte-derived microparticle levels being associated with lower sodium excretion and higher platelet-derived microparticles being associated with the lowest tertile of 24hUNa excretion. Additionally, RAAS blockade was not associated with lower microparticles counts in the setting of a low sodium intake, questioning whether the therapeutic benefits of RAAS blockade may be attenuated during lower sodium intake.

Despite public health bodies advocating for lowering dietary sodium, we demonstrated that these guidelines are simply not being met and sodium intake is unlikely to change over time. Furthermore, we compared the two most common methodologies used to estimate sodium intake in cardiovascular outcome trials which have largely formulated the basis of dietary sodium guidelines. We demonstrated poor agreement between 24h dietary recall and 24h urine sodium excretions, likely due to underestimation and underreporting of sodium intake via 24h dietary recall.

Taken together, these findings begin to provide mechanistic insight that sodium lowering in people with type 2 diabetes may not provide the benefit on the sympathetic and endothelial system as intended. Therefore, the necessity for stringent sodium lowering is questioned in this population. Our studies additionally demonstrate the need for future studies to use sound methodological approaches to determine the ideal yet feasible dietary sodium intake targets in people with type 2 diabetes.

Declaration

This is to certify that :

I. This thesis comprises only my original work towards the PhD except where indicated in the Preface.
II. Due acknowledgement has been made in the text to all other material used.
III. The thesis is less than 100,000 words in length, exclusive of tables, bibliographies and appendices.
IV. The completion seminar was held on the 29th of May 2020 during the Endocrine Department Meeting, Austin Health.

Sara Baqar
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Preface

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Author	Contribution(%)	Description of contribution
Baqar S	70%	Study execution, recruitment, assistance for clinical investigations, preparation of database, data analysis, preparation and review of manuscript.
Straznicky NE	5%	Execution of clinical investigations, preparation of database, review of manuscript.
Kong YW	5%	Assistance with recruitment, review of manuscript.
Lambert GW, Dixon J and Jerums J	5% (combined)	Review and editing of manuscript.
Ekinci EI	7.5%	Study conception, design, review and approval of final manuscript.
Lambert E	7.5%	Study conception, design, execution of clinical investigations, review and approval of final manuscript.

Chapter 4:
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Baqar S	70	Study execution, recruitment, assistance for clinical investigations, , data analysis, preparation and review of manuscript.

Kong YW	5	Assistance with recruitment and clinical investigations, review of manuscript.
Bouterakos	5	Appointed Trial Dietitian conducting dietary recalls.
Chen AX, O'Callaghan C, MacIsaac R, Lambert GW and Jerums J	5 (combined)	Review and editing of manuscript.
Lambert E	7.5	Study conception, design, execution of clinical investigations, review and approval of final manuscript.
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Baqar S	70	Study conception, design and execution, recruitment, preparation of microparticles, data analysis, preparation and review of manuscript.
Liu D	7.5	Recruitment, preparation of microparticles, data interpretation, review of manuscript.
Lincz LF	7.5	Flow cytometry and laboratory analysis, data interpretation, review of manuscript.
Kong YW	5	Recruitment, preparation of microparticles, review of manuscript.
Jerums J	2.5	Review and editing of manuscript.
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Baqar S	70	Study conception, design and execution, recruitment, data analysis, preparation and review of manuscript.
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Wickramasinghe S	5	Data analysis and interpretation.
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Baqar S, Straznicky NE, Lambert GW, Kong YW, Dixon J, Jerums J, Ekinci EI and Lambert E. *Comparison of endothelial function and sympathetic nervous system activity along the glucose continuum in individuals with differing metabolic risk profiles and low dietary sodium intake*.
Poster presentation at the Joint Annual Scientific Meeting of the AAS, HBPRCA and AVBS, Adelaide (2018).

Baqar S, Kong YW, Chen AX, O'Callaghan C, MacIsaac R, Bouterakos M, Lambert GW, Jerums J, Lambert E and Ekinci EI. *Can salt supplementation alter sympathetic nervous activity and endothelial function in people with type 2 diabetes?*
Australian Diabetes Annual Scientific Meeting, Perth, Australia (2017).
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Michalopoulos A, **Baqar S**, Jerums G, Libianto R, MacIsaac RJ, Chen AX, Stebbing M and Ekinci EI. *The Effect of Salt Versus Placebo Supplementation on Haematocrit Levels in Type Two Diabetes Mellitus*.
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Abbreviations

ABPM	Ambulatory Blood Pressure Monitor
AI	Augmentation Index
ACE	Angiotensin Converting Enzyme
cfPWV	Carotid-Femoral Pulse Wave Velocity
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
FMD	Flow-Mediated Dilatation
ICAM-1	Intracellular Adhesion Molecule 1
IOM	Institute of Medicine
INTERSALT	International Cooperative Study on Salt, Other Factors, and Blood Pressure
HRV	Heart Rate Variability
HDL	High-Density Lipoprotein
LDL	Low-Density Lipoprotein
MSNA	Muscle Sympathetic Nerve Activity
MDCT	Multidetector computed tomography
NHANES1	first National Health and Nutrition Examination Survey
ONTARGET	Ongoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial
PAT	Peripheral Arterial Tone
PBS	Phosphate Buffered Saline
PS	Phosphatidylserine
PECAM-1	Platelet/Endothelial Cell Adhesion Molecule 1
PURE	Prospective Urban Rural Epidemiology
PWA	Pulse Wave Amplitude
RAAS	Renin-Angiotensin- Aldosterone System
RHI	Reactive Hyperemia Index
ROCK I	Rho-associated protein kinase
RNA	Ribonucleic acid
SNS	Sympathetic Nervous System
TRANSCEND	Telmisartan Randomized Assessment Study in ACE Intolerant Subjects with Cardiovascular Disease

VCAM	Vascular Cell Adhesion Molecule
WHO	World Health Organization

Chapter One: Introduction

Abstract

Objective: This comprehensive narrative review aims to provide an account of how the current dietary sodium guidelines were developed, an explanation of the debate that surrounds the role of sodium on cardiovascular health and how biologically plausible mechanisms, such as an impaired sympathetic nervous system and endothelial dysfunction, may provide clues to the paradoxical association between low sodium and adverse cardiovascular outcomes in individuals with type 2 diabetes. This review will conclude by outlining how this thesis will contribute to understanding the mechanisms behind this complex issue.

Study Selection: Manuscripts were reviewed which examined the effects of sodium on blood pressure, cardiovascular disease, sympathetic nervous system and endothelial function. In particular, where possible, manuscripts, which focused on individuals with diabetes, were prioritised.

Data Extraction: A literature search in MEDLINE (1946 – May 2020) was performed using a combination of the following search terms: salt, salt intake, dietary salt intake, dietary sodium intake, dietary sodium, dietary sodium chloride, sodium excretion, urine sodium, 24 hour urinary sodium, hypertension, heart rate, cardiovascular, cardiovascular disease, cardiovascular mortality, mortality, diabetes, type 2 diabetes, sympathetic nervous system, microneurography, endothelial function, endoPAT, microparticles. Combinations of limitations including English language, core clinical journals and journal article were placed on the search terms. Relevant citations were also retrieved.

Conclusions: In individuals with type 2 diabetes, lower sodium intake has been associated with poorer cardiovascular health outcomes. Studies investigating the mechanistic links behind this paradoxical relationship are lacking yet greatly needed given the diabetes population has a higher prevalence of cardiovascular related morbidity and mortality as compared to the general population. The studies presented in this thesis therefore aim to examine the effects of sodium on the sympathetic nervous system and endothelial function, considered surrogates for cardiovascular health, in individuals with type 2 diabetes.

General Overview

This thesis explores the association between sodium intake and cardiovascular health by examining the sympathetic nervous system activity and endothelial function as surrogates for cardiovascular outcomes. There is an ongoing debate regarding the complex interplay between sodium intake and cardiovascular outcomes, as studies, mainly observational in design, suggest a paradoxical association between low sodium intake and higher cardiovascular morbidity and mortality, especially in individuals with diabetes. The mechanisms underpinning these observations are unclear and require further understanding to improve public health policy recommendations and health outcomes for the general and high-risk population, such as those with diabetes. This literature review will summarise the present understanding of the effect of sodium on cardiovascular health and outline the potential roles of the sympathetic nervous system and endothelial systems in providing further mechanistic insights.

The Effect of Sodium Intake on Cardiovascular Health

How Salt Seasoned History

Historically, in brief, salt made of sodium and chloride (NaCl), was once considered of high value, important for the development of life in communities given its use in the flavouring and preservation of food [1]. Additionally, it served as an excellent antiseptic and the Roman word for salt, Salus, is given to the goddess of health. To further highlight its valued status in history, it is claimed that Roman soldiers were paid their wages in salt and the word "salary" arises from the Latin word for salt, salarium. Furthermore, the tax and trade of salt is well recognised in countries such as France.

However, the adverse effects of high dietary sodium on health began to emerge and thus too the fall of salt from its place as a highly valued commodity. Indeed, as far back as 2700 BC in China, The Yellow Emperor's Classic of Internal Medicine described *"if too much salt is taken in food, the pulse hardens"* [2] and the concept of high dietary sodium intake elevating blood pressure, which was apparent for more than 4000 years [3], started to garner interest.

In the 18th century Stephen Hales proposed that fluid volume determined arterial pressure [4]. Hales further reasoned this scientific opinion stating that blood volume was largely determined by its sodium and water content [4]. At the beginning of the 20th Century, two scientists Ambard and Beaujard [5], went on to compare a high sodium diet (5800mg/24h or 252 mmol/24h) versus a low sodium diet (1200mg/24h or 52 mmol/24h) in six hypertensive patients, demonstrating the high sodium intake contributed to high blood pressure[3]. They have thus been credited for formulating the 'Salt-Blood Pressure Hypothesis' and effectively, igniting the 'Salt War'[6].

Later, Goldblatt [7] was able to show in the 1930s that excessive sodium intake in rats led to hypertension. The Kempner diet, consisting of rice and fruit with low sodium content, was used to treat many people with hypertension in the early 1940s. In 1948, Kempner et al [8] published the first case report utilising sodium restriction in 500 patients as a method of managing severe hypertension. However, during this time, Chapman and Gibbons[9] were critical of Kempner's recommendations[8] after having reviewed the available literature from 1904 to 1949 concluding that out of the 28 studies available, the majority were uncontrolled case reports[9]. Of these, 21 studies were in favour for a low sodium diet as a treatment for blood pressure reduction, however seven studies reported "unfavourable" health outcomes with significant difficulties adhering to such restrictions being noted [9]. Therefore in the first half of the 1900's clinicians were at odds as to whether to recommend low sodium intake as strategy to treat hypertension[6].

Follow up epidemiological and observational studies of communities with habitual low sodium intake [10, 11] demonstrated that blood pressure had a tendency of being lower in this population that did not rise with age. These findings led to the hypothesis that at a population level, blood pressure may in fact be correlated with sodium intake [10, 11]. Indeed the first randomized cross-over study examining the effect of reduced sodium intake on blood pressure by Parijs et al in 1973 [12] demonstrated that in 14 patients with mild hypertension, reducing sodium excretion lead to a decrease in blood pressure. However, the study was criticized for the wide range of reduction in sodium excretion and blood pressure changes seen. Nevertheless, this renewed interest in trying to link sodium intake with hypertension with further studies, epidemiological and

experimental in design, going on to confirm the association between high sodium intake and high blood pressure as described below.

Sodium terminology

At this point, before further embarking upon this thesis, it is important to establish the sodium terminology that will be presented in this thesis. The commonly known salt we use is made of sodium and chloride (NaCl). However, salt is not synonymous with sodium. Sodium refers specifically to dietary sodium. Furthermore, estimation of dietary salt that is measured by urinary excretion is measured in terms of sodium content by weight, represented in the units millimole (mmol). To calculate this, the following principles are important. Firstly, a mole is the gram molecular weight of a substance. Thus, given the molecular weight of sodium is approximately 23, sodium is equivalent to 23 grams. Secondly, converting one gram of sodium to the unit mmol requires the following equation: 1/23 gram = 0.0435 moles = 43.5 mmol. Alternatively, 1mmol of sodium = 23mg. This thesis will report sodium in both mmol/24h and mg/24h.

Epidemiological studies assessing sodium intake and blood pressure outcomes

The International Cooperative Study on Salt, Other Factors, and Blood Pressure or more commonly known as the INTERSALT study, arguably the most influential sodium and blood pressure outcome study, was an observational study designed to assess the relationship between electrolyte excretion and blood pressure outcomes[13]. Utilising 24h urinary electrolyte excretion, considered a major strength of the study, 10079 men and women aged between 20 to 59 years, from 52 centres from 32 countries around the world, were studied in a cross-sectional manner, taking into account body mass index and alcohol consumption as potential variables [13]. Overall, the study reported a positive association between dietary sodium intake and blood pressure[13] given populations with low sodium intake had lower mean blood pressure and hypertension, with no increase in blood pressure with increasing age[13] and subsequent adverse cardiovascular outcomes[13].

However, these findings were met with fierce criticism from the Salt Institute, the salt producer's trade organisation. The INTERSALT investigators were asked to hand over the raw data for reanalysis almost a decade later. The findings of the repeat analysis were published in 1996[14] and demonstrated similar results with a 100mmol decrease in sodium intake being associated with a 3mmHg decrease in systolic blood pressure[14]. Sodium consumption was reported to be between 100-200mmol/24h or 2300-4600mg/24h in 48 of the 52 centres. It is worth noting that four of these 52 communities were considered outliers as their sodium intake was far less, at 50mmol/24h or less[14]. Thus, when excluding these four communities in the repeat analysis, the remaining 48 centres demonstrated that the relationship between sodium and blood pressure became negative, or inverse, which may somewhat contradict the original interpretation[15].

Furthermore, the INTERSALT study has been criticized as the initial recommendations[13] were made without understanding the genetic predispositions of the communities studied. Of the four communities which demonstrated the lowest sodium intake, the Yanomamo tribe, who live in the Amazon rainforests, the Xingu tribes, who reside near the Xingu river in Brazil, and another tribe in Papua New Guinea, were all found to have a low frequency of the angiotensin converting enzyme (ACE) D/D genotype[16], which leads to high ACE levels and in turn a higher risk of myocardial infarction[17] and sudden cardiac arrest[18]. Of these tribes, the Yanomamo tribe had the lowest sodium intake [19] yet they have a life expectancy of only 47 years[16]. Therefore, the original observations from INTERSALT study of a low sodium intake lowering age-related increases in blood pressure[13], is met with concern.

Despite the above criticisms, in 2004, the Norfolk Cohort of the European Prospective Investigation into Cancer (EPIC-Norfolk) study assessed the relationship between dietary sodium intake and blood pressure in 23104 community-living adults aged 45-79. Urinary sodium was estimated from spot urine samples rather than 24hour urinary excretion. The findings demonstrated an increase in urine sodium was associated with an increase in mean blood pressure, independent of age, body mass index, urinary potassium, smoking sex and prior history of hypertension [20]. Furthermore, positive relationships between urinary sodium excretion and both systolic and diastolic blood pressure have been demonstrated from studies in mainland China [21].

Intervention studies reviewing sodium intake and blood pressure outcomes

The TONE study[22] aimed to determine, in about 900 elderly participants with and without obesity on a single antihypertensive agent, whether sodium reduction (80mmol/24h or 1840mg/24h as measured by 24h urinary sodium excretion) and weight loss of 4.5 kilograms was effective in being able to withdraw the single antihypertensive medication [22]. The obese participants (n=585) were randomized to receive reduced sodium intake, weight loss, both, or randomized to usual care. The nonobese participants (n=390) were randomized to reduced sodium intake or usual care [22]. The primary endpoint was evidence of high blood pressure following withdrawal of the antihypertensive agent. The study found a 30% reduction in the need for antihypertensive agents when sodium intake was reduced to 40mmol/24h or 920mg/24h or body weight was reduced by 3.5 kilograms[22]. Overall, the TONE study concluded that reduction in weight and sodium, either alone or in combination, are a feasible nonpharmacologic approach in managing hypertension in the elderly [22].

The Dietary Approaches to Stop Hypertension (DASH)[23] and DASH-Sodium diets[24] were designed to assess the effects of various nutrients and specifically sodium on blood pressure respectively. Participants, who were either pre-hypertensive or in the early stages of hypertension, consumed two experimental diets against each other and a third control diet typical of the average American diet at the time, low in key nutrients such as potassium. The first experimental diet was essentially higher in fruits and vegetables but otherwise comparable to the control diet[23]. The second experimental diet, the DASH diet, consisting of fruits, vegetables and low overall fat and dairy fat products, was rich in potassium, magnesium and calcium, and was based on the recommendations by the National Institute of Health [23]. The DASH trial (n=459) demonstrated that compared to the control diet, the DASH diet was able to reduce systolic blood pressure by 6mmHg and diastolic blood pressure by 3mmHg in the pre-hypertensive group. A further reduction occurred in the hypertensive group of 11mmHg and 6mmHg for systolic and diastolic blood pressure respectively within two weeks of consuming the diet[23].

The DASH-sodium diet was aimed to determine whether the results from the DASH diet could be improved further by lowering sodium content. Similar to the DASH study,

in the DASH-sodium study, 412 participants with either pre-hypertension or hypertension were randomly assigned to consume either a control diet or the DASH diet in a crossover fashion. Post randomisation, participants were given diets with three varying sodium contents in random order for 30 consecutive days; high (3000mg/day or 130mmol/24h), intermediate (2400mg/ day, or 104mmol/24h) or low (1500mg/day or 65 mmol/24h) levels of sodium. The main findings were that low sodium intake lowered blood pressure in either the DASH or the control diet. However, the largest reductions in blood pressure were observed when the combination of the DASH diet along with the lowest sodium intake of 1500mg/day, translating to an average systolic and diastolic blood pressure reduction of approximately 9mmHg and 5 mmHg respectively.

Following the results of the abovementioned trials, He and MacGregor[25] conducted a meta-analysis of randomised salt reduction trials from 1966 to 2001. They included only those trials where salt reduction was of at least four weeks duration given concerns arising from two[26] [27]previous meta-analyses that included trials with a median duration of acute salt reduction for eight [27] and 14 days[26]. Reviewing 17 randomised trials of dietary sodium restriction in individuals with hypertension (n=734) and 11 trials in individuals with normotension (n=2220) [25] they found in hypertensive individuals, a median reduction in 24h urinary sodium excretion of 78 mmol/24h (4600mg/24h) was associated with a mean reduction in systolic blood pressure of 5.0 mmHg[25]. Furthermore, in normotensive individuals, a median reduction in 24h urinary sodium excretion of 74 mmol/24h (4400mg/24h of salt), was associated with a mean reduction in systolic blood pressure of 2.0 mmHg. Based on this meta-analysis and the previous findings of hypertension being associated with an increase in cardiovascular disease [28], assumptions arose that high dietary sodium intake was also associated with increased cardiovascular morbidity and mortality [28, 29] which later formed recommendations for population wide sodium restriction[25].

The basis of dietary sodium intake recommendations

Based on extensive evidence from varied study designs demonstrating that a reduction in sodium intake not only lowers blood pressure [30, 31] but also lowers cardiovascular risk [32, 33], which was further backed by a meta-analysis likewise demonstrating that increased

sodium intake was associated with an increased incidence of cardiovascular disease[34]. As cardiovascular disease is a major global cause of disability and premature death, and contributes substantially to the escalating costs of health care[35, 36], it is not surprising sodium public health bodies thus advocate for low sodium intake [30].

Early studies suggested that each one gram reduction in dietary sodium intake roughly leads to a one mmHg reduction in blood pressure[37]. More recently, He and MacGregor demonstrated in their meta-analysis through a weighted linear regression, a dose response relationship exists behind their observations of reduced urinary sodium being associated with reduced blood pressure [25]. For every 100mmol/24h or 2300mg/24h reduction in sodium, systolic blood pressure reduced by 7.11mmHg in hypertensive individuals and 3.57 mmHg in normotensive individuals[25]. Global public health bodies, such as The World Health Organization (WHO), recommended that adults should reduce sodium intake to roughly 2000mg/24h (87 mmol/24h) in order to have beneficial effects on blood pressure reduction [38, 39]. However, most guidelines interestingly fail to mention what a lower limit or what a dangerous level of sodium intake is, leading to an assumption that there is no lower limit of sodium intake[40].

The demonstration of a positive relationship between sodium intake and cardiovascular outcomes.

The support for dietary sodium restriction is fuelled by studies suggesting a higher sodium intake was associated with a higher risk of cardiovascular disease[41, 42]. It is largely assumed that a linear relationship exists between reduction in sodium intake and blood pressure and between blood pressure and cardiovascular disease outcomes [43]. The prospective cohort study, the first National Health and Nutrition Examination Survey (NHANES 1) Epidemiologic Follow-up Study[41], was conducted on participants (n=9485) aged 25 to 74 years who were entered in the study during 1971 to 1975 and followed up between 1982 to 1992 to identify those who had cardiovascular related incidents or mortality from medical record data. The range of sodium intake for participants was between 1047 mg/day (45.5mmol/24h) to 2983 mg/day (129.7mmol/24h). The study demonstrated higher sodium intake was significantly associated with higher stroke incidence and mortality from stroke, as well as higher mortality from coronary heart disease, cardiovascular disease all-causes[41]. However,

this observation was only seen in the participants who were overweight and not in those with normal weight and as such, cannot be generalised to the general population. Despite the strengths of the large number of participants with a long follow-up, of concern is that the sodium intake was estimated from a single collection of 24h dietary recall, which is considered a main limitation. In particular, at the time of the recall, discretionary sodium use, such as the salt that may be added in cooking or at the table, which is known to account for up to 30 % of sodium intake[44] was not accounted for and may therefore give misleading results.

To overcome the issues of dietary recall, in a Finnish study[42] with similar participant characteristics, 24h urinary sodium excretion and cardiovascular risk factor data was prospectively followed. Despite the higher mean 24h urine sodium excretion values of 4968mg/day (216mmol/24h) for men and 3726mg/day (162mmol/24h) for women as compared to the above study, similar associations were observed A 100mmol/24h increased in 24h urinary sodium excretion was associated with a hazards ratio of 1.51 (95% CI 1.14-2.00) for coronary heart disease, 1.45 (95% CI 1.14-1.84) for cardiovascular disease and 1.26 (95% CI 1.06 -1.50) for all-cause mortality with the study concluding that a high sodium intake was an independent predictor of cardiovascular risk, separate even to blood pressure[42].

Furthermore, the Trials of Hypertension Prevention (TOHP) Phases I and II [45] demonstrated a positive association between urinary sodium excretion and mortality. However, the mean blood pressure reduction in the TOHP trials were minimal with systolic blood pressure reduction of 1.7mmHg in Phase I and 1.2 mmHg in Phase II[46].

However, the studies[41, 42 45, 47-49] demonstrating a positive linear relationship between sodium intake and cardiovascular outcomes, were mostly prospective in nature with differing methodologies used to estimate sodium intake ranging from dietary recall[41], food frequency questionnaires[47, 48] to multiple 24h urine excretions[45] or single 24h urine excretions in a meta-analysis[49] of randomised trials. There are, however, no interventional, randomised clinical trial studies to date that have examined these observations with hard cardiovascular endpoints .

The start of the great salt debate: Evidence for a negative relationship between sodium intake and cardiovascular health

Despite the beneficial effects of sodium lowering on blood pressure, on the contrary, Alderman et al initially showed that in treated hypertensive individuals, the 24h urinary sodium excretion was inversely associated with myocardial infarction [50]. Alderman et al went on to further demonstrate with a cox multiple regression analysis that sodium restriction was associated with a higher incidence of all-cause and cardiovascular mortality by examining the NHANES 1 dataset[51]. This was followed up by Cohen et al [52, 53] who repeatedly described lower sodium intake may be associated with higher cardiovascular and all-cause mortality. However, the use of 24h dietary recall remains a limitation for these studies[51-53]. Through the use of 24h urinary excretion measurements, in a prospective longitudinal study in 3681 participants with no prior cardiovascular disease who were followed for a median of 7.9 years, Stolarz-Skrzypek et al[54] showed cardiovascular related deaths were lower as the tertiles of 24h urinary sodium were higher. Analysis of a subgroup of 1499 participants who had measures of both blood pressure and 24h urinary sodium excretion at baseline and the final follow-up, revealed a 100mmol increase in 24h urine sodium was associated with a significant increase in systolic blood pressure of 1.71mmHg, but not diastolic pressure. Despite this change in systolic blood pressure, lower sodium excretion was associated with higher cardiovascular related mortality[54].

Evidence for non-linear relationships between sodium intake and cardiovascular outcomes

Two multi-centred prospective cohort studies suggested a non-linear, J-shaped, association between the estimated 24h urine, using the Kawasaki formula, and cardiovascular outcomes [40, 55]. In an observational analyses of two cohorts from the ONTARGET (Ongoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial) [56]and TRANSCEND (Telmisartan Randomized Assessment Study in ACE Intolerant Subjects With Cardiovascular Disease)[57] trial databases, 28,880 participants who provided baseline urinary samples were analysed. When comparing 24h urine sodium of 4,000 to 5,990mg/day (174-260mmol/24h), the levels of sodium excretion less than 3,000mg/day (130mmol/24h) were associated with higher risk of cardiovascular mortality. Additionally, sodium excretion levels greater than 7,000mg/

day (304mmol/24h) were associated with a higher risk of cardiovascular mortality and hospitalisation for congestive heart failure[55].

Following on from this, the Prospective Urban Rural Epidemiology (PURE) study [40]analysed the 24h urinary sodium measurements, estimated using the Kawasaki formula from a fasting midstream urine sample, from 101,945 enrolled participants from 17 countries. Likewise, this study demonstrated that the lowest risk of death and cardiovascular events was seen in those participants with a sodium excretion between 3000 to 6000mg/ day (130-260 mmol/24h). A J-shaped association curve was demonstrated as above with higher (7,000mg/ day (304mmol/24h)) and lower (3,000mg/day (130mmol/24h)) levels of sodium excretion being associated with higher overall risk. After adjusting for blood pressure or previous history of hypertension, the associations remained significant for lower, but not higher, urinary sodium excretion and cardiovascular risk, suggesting mechanisms other than blood pressure may play a role at lower levels of sodium excretion[40].

In a study pooling data from four international prospective studies[58], 133,118 individuals with and without hypertension were assessed from 49 countries. Using the Kawasaki-estimation of 24h urine sodium, levels below 3,000mg/day (130mmol/24h) were associated with a higher risk of cardiovascular events and mortality as compared to 24h urine excretion levels between 4,000mg/day to 5,000mg/day (174mmol/24h to 217mmol/24h)[58]. However, when 24h urine sodium levels were greater than 7000mg/day (304mmol/34h), only those individuals with hypertension had an association with higher cardiovascular events and mortality. However, the association of low sodium intake with higher risk of cardiovascular events and mortality was seen in individuals with and without hypertension[58]. Reverse causality has been thought to contribute to these findings. These studies are limited, being observational in design, and hypothesis generating at present. Additionally, despite the international representation of the cohort, it must be appreciated that the Kawasaki formula was originally developed in Asians without cardiovascular disease [59]. Nevertheless, the concerns raised require validation with large-scaled randomised controlled trials to further understand the complex interplay between sodium and cardiovascular outcomes.

The relationship between sodium intake and cardiovascular outcomes in diabetes

Limited studies exploring these observations are available in those with diabetes. Our group [60] and others [61] have previously demonstrated that a low sodium intake was associated with an increased overall mortality in patients with diabetes. Our group has shown in a prospective cohort study of 638 individuals with type 2 diabetes followed up over a median of 9.9 years, the lowest tertile of sodium excretion was paradoxically associated with higher cumulative hazard for all-cause mortality[60]. Specifically, for every 100mmol rise in 24h urine sodium, the all-cause mortality was 28 % lower (p=.02) [60]. Similarly, an observational cohort study from Japan found that lower 24h urine sodium excretion was not associated with lower cardiovascular related events such as myocardial infarction, angina pectoris, stroke and peripheral vascular disease[62]. Thomas et al demonstrated in 2807 individuals with type 1 diabetes, a U-shaped relationship between single 24h urine excretion measurements and all-cause mortality[61]. Whilst acknowledging the concerns about reverse causality from the original observations[60], in a follow up study based on the same cohort of participants, we were able to again demonstrate using serial 24h urinary sodium measures, after adjusting for covariates associated with 24h urine sodium and estimated glomerular filtration rate (eGFR), that lower 24h urinary sodium excretion over time was associated with higher mortality in people with type 2 diabetes[63].

Conversely, a Japanese study demonstrated in 1588 participants with type 2 diabetes, a positive relationship between sodium intake and cardiovascular disease was observed[64]. Interestingly, no significant associations between sodium intake and all-cause mortality was seen[64]. However, this study is limited by the use of food frequency questionnaires to estimate sodium intake. Additionally, results are not generalisable to the population at large given participants were predominantly of Asian ethnicity[64]. In particular, Asian individuals are noted to have lower body mass index (BMI) [65], lower overall cardiovascular disease related risk [66] despite their habitual higher consumption of sodium [67] as compared to those individuals of non-Asian ethnicity. Therefore, participant ethnicity must be accounted for when interpreting these results and may explain, in part, the reasons for differences in observations seen with the other studies[60, 63].

Revisiting the sodium dietary guidelines

Given the inconsistencies in the available literature, likely due to differences in methodology used to estimate dietary sodium intake, population characteristics and cardiovascular outcome measures, the debate into optimal sodium intake prompted the Institute of Medicine (IOM) to assess public health outcomes with a sodium intake with an emphasis placed on the high risk subgroups [68, 69]. The IOM found no benefit and possible harm in restricting sodium intake to 1500 to 2300mg/day (65-100mmol/24h) in the high-risk population, such as those with diabetes, chronic kidney disease or underlying cardiovascular disease. The IOM concluded that given the insufficient evidence to suggest either harm or benefit for further sodium restriction to less than 2300mg/day (100mmol/24h) in these high-risk subgroups, they should be treated no differently to the general population guidelines. Furthermore, IOM did not support recommendations to lower sodium intake to less than 1500mg/day (65 mmol/24h) given no evidence on improved health outcomes. Additionally, this report promoted the American Diabetes Association to update their guidelines and recommendations for individuals with diabetes stating sodium intake recommendations should be in line with those advocated for the general public of 2300mg/day (100mmol/24h) [70]. Overall, the IOM committee recommended more randomized controlled trials were needed focusing on the higher-risk subgroups such as those with diabetes[69].

Sodium restriction guidelines are not being adhered to

Despite these guidelines, the general population consumes more than the recommended sodium intake, with figures from United States showing ninety percent of adults consume more than 2300mg/24h and ninety-eight percent of adults in high-risk subgroups consume more than 1500mg/day[71]. Recently the PURE study, which reviewed sodium intake from 17 countries, showed similar findings of 90% of participants having urinary sodium excretion more than 3000mg/day[72]. Alarmingly only 10% of the population had sodium excretion less than 3000mg/day with only four percent meeting the current guideline targets of less than 2300mg/day[72]. Specific to the diabetes population, a meta-analysis of the Australian population found that the mean sodium intake in people with diabetes was well above recommended targets at 9660mg/24h (420 mmol/24h) [73]. Our group has likewise previously shown that individuals with type 2 diabetes did not meet national sodium intake guidelines with

only 3% of males and 14% of females being able to meet dietary sodium guidelines[74]. Given that recent studies have shown that only a small minority of the general worldwide population is consuming a low sodium diet[72, 75] and that in these people the relationship between sodium intake and blood pressure was non-linear[72], a very plausible question arises; is there any meaningful purpose in advocating for sodium restriction as a current approach for population based blood pressure reduction? Additionally, without data from definitive randomised controlled trials specifically evaluating the association between low sodium intake and cardiovascular endpoints, current dietary guideline recommendations at present may need to be viewed with caution, especially in high-risk groups, such as those with diabetes.

Methods for estimating sodium consumption

Dietary sodium intake is reflective of a combination of food availability and personal preferences [76]. Estimating dietary sodium intake is difficult, as currently no method is able to accurately account for the discretionary salt added either during or after the cooking process. This is important as the majority of dietary sodium intake, up to 30% of sodium intake[44], is through the addition of salt to food during processing. Not-withstanding these limitations, the current accepted 'gold standard' for assessing dietary sodium intake is the measurement of 24-hour urinary excretion of sodium, given approximately 90% of ingested sodium is excreted in the urine [77, 78]. However, whilst the long-term sodium balance studies in Russian cosmonauts during a space travel simulation program recently demonstrated that 95 % of sodium ingested is excreted in urine, this was surprisingly not on a daily basis[79]. Constant sodium intake was found to be excreted on a weeklyand monthly rhythm. [79] Therefore, to get a more accurate understanding of habitual sodium intake, it is advisable to have multiple, timed urine collections to more accurately interpret an individual's intake [80] which is also the recommended approach for assessment of population intake [81].

However, many countries prefer to rely on dietary recall, given the increased burden placed on both researchers and individuals to collect 24h urine, with both under and over collections being reported [82] with overall low overall response rates in population surveys[83]. Should recall methods be used, a multiple-pass method with food composition databases are preferred[84]. The US Department of Agriculture Multiple-

Pass method relies on trained professionals using a 5-step interview process to conduct the recall[85]. In brief, the steps involved are to firstly, collect a quick list of all foods and beverages consumed the previous day. This is followed up by probe questions to ask about possible forgotten foods from the quick list. The time and eating occasion as well as detailed descriptions of the amounts and additions used during the day are detailed. Therefore, this method is advantageous as it includes asking about discretionary salt use. Final probe questions identifying any other foods consumed then complete the recall[85]. However, if recall methods are used, it must be acknowledged that food composition databases may be inconsistent depending on food manufacturers, the methods used to measure nutrient content, natural variations in food composition and the frequency of updates to the food databases [86].

A recent systematic review, however, concluded that methods such as 24h dietary recall are deemed to be less accurate in estimating an individual's sodium intake[84], as they often underestimate sodium intake, as compared to 24h urine excretions[84]. For example, in a study performed over 3 days, in adults who were knowledgeable regarding nutrition, neither food record estimates nor analyses of food portions provided valid measures of individual urine sodium excretion [87]. Individuals can underestimate their sodium intake by 30% to 50% [88], due to factors such as recall bias[89], especially if they are overweight or obese[85]. Recently, when evaluating for the measurement error in sodium intake assessed using 24h urine excretion and 24h dietary recall during the NHANES, it was concluded that 24h dietary recall underestimates mean sodium intake[90]. Importantly, the study raised concern that 24h dietary recall attenuates any true associations between sodium intake and health outcomes and any associations would be biased towards the null[90]. Therefore, estimation of sodium intake by 24h dietary recall in cardiovascular outcome studies have been criticized [91-93].

The use of a random spot urine sample with application of various formulas [59, 94, 95] to estimate levels of 24h urine electrolyte excretion, the most commonly used being the Kawasaki method (second morning spot urine)[59], the Tanaka method (single random spot urine)[94] and the INTERSALT method (single random spot urine)[95] are based on the assumption that 24h urine sodium measurements, after correcting for creatinine excretion, are proportionate to spot urine sodium excretion. Given the ease of

collection, the reduced costs[96], as well as random spot urines having been previously shown to have minimal impact on the circadian patterns of 24h urine excretion [94], spot urine samples have become a popular method for estimating sodium intake. However, whilst these methods are advantageous for estimating the mean population levels of sodium intake[59], they are not reliable at an individual level[94] due to the strong influence of age and gender on urinary creatinine excretion [97]. Furthermore, a recent population validation study in 365 elderly individuals, considered to be at high-risk of stroke, from a province in China, compared 24h urine sodium excretion measurements with estimated 24h urine sodium values calculated from spot urines using the above Kawasaki[59], Tanaka[94] and the INTERSALT[95] methods[98]. The study concluded that all the formulas applied to the spot urine samples showed little agreement between with 24h urine sodium excretion measurements at both an individual and population level[98]. Therefore, there has been concern for cardiovascular outcome studies that have used spot urine methods of assessing sodium intake.

To add complexity to the understanding of sodium balance, in the aforementioned Mars flight simulation study[79], sodium was demonstrated to be stored in the body, independent of blood pressure and water content. The same group had previously demonstrated in animal studies that a high-salt diet in rats resulted in sodium accumulation in the skin with hyperplasia of the lymph-capillary network and subsequent hypertension, driven by signaling mechanisms involving the vascular endothelial growth factor-C [99]. Using the techniques of ^{23}Na magnetic resonance imaging, people with cardiovascular[100] and chronic kidney disease[101], as well as the elderly[102] and those with diabetes [103] have likewise been shown to store large amounts of sodium in their bodies, predominantly in skin and skeletal muscles[104]. However, more recently, these findings have been questioned as tissue sodium accumulation was found to be systemic and isotonic and was deemed unlikely to induce hypertensive vascular dysfunction in animal studies[105]. Similarly, analysis of skin punch biopsies in humans did not demonstrate hypertonic sodium accumaltion[105]. At this stage, further research is needed to explore the concept of tissue sodium storage as well as determining the potential role of 23Na magnetic resonance imaging as a tool for assessing the relationship between sodium homeostasis and cardiovascular morbidity.

Therefore, taken together, these findings suggest that at present, the most accurate method of estimating dietary sodium intake appears to be 24h urine sodium excretion measurements and hence was the method of choice for the studies presented in this thesis.

The physiological basis of sodium restriction leading to poorer cardiovascular outcomes

The physiological basis and reasoning for the paradoxical observations of low sodium intake being associated with adverse health outcomes is not fully understood. However in a meta-analysis[106], potential cardiovascular benefits seen with sodium restriction through blood pressure reduction were offset by the adverse effects of sodium restriction on plasma renin activity, with activation of the renin-angiotensin-aldosterone system activation (RAAS) [106], occurring when sodium intake falls below 3000mg/ day (130mmol/24h)[107] which is higher than the currently recommended target of 2300mg/day (100mmol/24h) [69, 70]. Coupled with RAAS activation, increases in circulating adrenaline and noradrenaline, as well as elevated levels of cholesterol and triglycerides [106], concern arose for subsequent progression to cardiovascular related diseases due to the disturbed endothelial and sympathetic nervous system pathways[106]. Therefore, whether low dietary sodium intake has the potential for pleotropic effects and activation of neurohormonal pathways, which in turn could contribute to poorer cardiovascular outcomes, calls for further investigation.

The role of the sympathetic nervous system in cardiovascular disease

Overview of the sympathetic nervous system

The autonomic nervous system, responsible for the unconscious and involuntary functions of the human body, is composed of two distinct anatomical and functional divisions: the sympathetic nervous system (SNS) and the parasympathetic nervous system [108] with transmission of messages traversing a bidirectional flow, in the afferent or efferent direction.

The sympathetic nervous system and the parasympathetic system have opposing effects, resulting in rapid, precise control of tissue function. The parasympathetic system in brief, is essential for "rest and digestion"[109]. In general, the sympathetic nervous system is important for mediating neuronal and hormonal stress responses, the so-called "flight or fight" response. This thesis will focus on the sympathetic nervous system.

The sympathetic nerves arise in the spinal cord, in the intermediolateral nucleus of the lateral grey column in the thoracolumbar region. The two neurons that are involved in the efferent transmission of any signal along the sympathetic nervous system are the pre-ganglionic and post-ganglionic neurons. The pre-ganglionic neurons, which are short in length, usually travel to the nearby paravertebral ganglia, located alongside the spinal cord. These neurons can also synapse with many ganglia simultaneously or travel rostrally or caudally to synapse with post-ganglionic neurons in ganglia at other levels. These neurons then synapse with the post-ganglionic neuron, which are usually longer and able to traverse most of the body terminating at the target organs. At the synapse, the pre-ganglionic neurons release a neurotransmitter, acetylcholine which acts on the post-ganglionic neuron. In response, the post-ganglionic neuron releases norepinephrine which in turn activates adrenergic receptors on target tissues[109].

There are some noteworthy exceptions to the above generalization. Firstly, at the kidney, dopamine is released instead of norepinephrine. Dopamine is the immediate precursor to norepinephrine. Additionally, within the adrenal gland, the adrenal medulla develops in parallel with the sympathetic nervous system, containing chromaffin cells which have the same embryonic origin as neural tissue. Thus, the adrenal gland can be considered a modified post-ganglionic neuron. It is capable of releasing norepinephrine, however importantly, releases in greater quantity the neurotransmitter epinephrine[109]. Other ganglia outside the spinal cord include the celiac and mesenteric ganglia, which send sympathetic fibres to the gastrointestinal tract.

The sympathetic system is important for cardiovascular homeostasis with four main influences: the cardiac sympathetic nerves, the vascular sympathetic nerves, adrenal

medullary input from circulating norepinephrine and epinephrine and the renal juxtaglomerular cells activating the renin-angiotensin-aldosterone system[110]. Sympathetic nerves act on the heart via the sinoatrial node, allowing increase in heart rate and on the myocardium to increase contractility and in turn stroke volume. On the vascular system, sympathetic activity induces peripheral vasoconstriction via the activation of alpha-1 adrenergic receptors by norepinephrine[110]. Also known as the sympatho-adrenal response, the preganglionic fibres that enter the adrenal medulla, activate the release of norepinephrine and to a greater extent, epinephrine as stated above. Therefore, this response is mediated indirectly by the catecholamines released from the adrenal gland and directly by impulses via the sympathetic nervous system to act on the cardiovascular system.

Measurement of Sympathetic Nerve Activity

Microneurography enables the direct measurement of efferent postganglionic sympathetic nerve firing to skeletal muscle vasculature, which yields a measure of muscle sympathetic nerve activity (MSNA) [111]. Microneurography is considered the 'gold standard' method of assessing the SNS. An advantage of microneurography is that it is highly reproducible in an individual in the short-term and long-term [111]. Microneurography will be further detailed in chapter two.

The influence of physiological states on sympathetic nervous system activity.

In humans, MSNA reflects the vasoconstrictor signal to skeletal muscle vasculature, closely regulated by the arterial and cardiopulmonary baroreflexes. Thus, the primary role of MSNA is to work with the baroreflex, described below, and restrict further any acute reductions in blood pressure, aiming to maintain constant perfusion pressure. An inverse relationship between blood pressure and MSNA was apparent from even the early experiments. As blood pressure decreased, MSNA increased[112]. Despite the suggestion of arterial baroreceptors modulating sympathetic activity, the recording from a cardiac patient with an arrhythmia rationalized the relationship between heartbeat and sympathetic discharge and provided momentum for analyses of arterial baroreflex effects[113]. Other physiological states can affect MSNA. Respiration is known to modulate MSNA with inspiration decreasing MSNA and expiration increasing

MSNA[114]. The duration of each MSNA burst is governed by the cardiac cycle. Ageing is significant as increases in MSNA are common after the age of sixty[115] by approximately one burst per minute per year [116] and thus could explain the risk of hypertension with advancing age. After the age of forty, there is a strong association between MSNA and resting blood pressure, with this not being observed in younger people[116], suggesting that the buffers that maintain a balance between MSNA and blood pressure may diminish with advancing age[113]. Gender plays a role in MSNA levels. Young women have lower MSNA than men[117]. However, post-menopausal women, especially over the age of sixty, having either the same or even higher MSNA levels than their male counterparts[116]. Female sex hormones are deemed responsible, the mechanisms however by which they do so remain unclear. Pre- ovulation surges in estrogen are associated with higher MSNA [118] thus in young women, MSNA measurements are made in the early follicular phase when estrogen and progesterone are low, or in a placebo phase if on oral contraceptive treatment to account for these effects[110]. Elevations in body mass index (BMI), especially above thirty, increases MSNA levels which are able to be decreased following weight loss efforts with exercise and diet [119].

The consequences of overactivation of the sympathetic nervous system

Chronic sympathetic activation is associated with end organ damage[120] and an adverse clinical prognosis [121] (Figure 1). An elevation in MSNA is known to contribute to the hypertension[122, 123] and cardiovascular disease such as ischemic heart disease[124] and chronic heart failure[125], especially amongst obese individuals[123]. Coronary artery disease states, such as unstable angina and myocardial infarction, are associated with an increase in MSNA[126] by up to 20 to 30 bursts per minute immediately after an acute cardiac event[127]. These levels can return to baseline after a period of six months for unstable angina and up to nine months following an episode of acute myocardial infarction[127]. The longer time in sympatho-excitation post cardiac events may be the reason why cardiovascular related morbidity and mortality rates are increased following acute coronary events. Additionally, MSNA is considered an independent predictor of mortality in people with heart failure, where MSNA levels above 49 bursts per minute have been associated with lower rates of survival in this population[128]. Thus

MSNA may be a useful tool in cardiovascular risk stratification with more work needed to identify its role as a prognostic tool[110]. Whilst not the focus of this review, it is worth mentioning in brief that chronic SNS activity is seen in other medical conditions such as chronic kidney disease[129] where SNS overactivity has been demonstrated to be an independent predictor of overall mortality and cardiovascular related morbidity and mortality and is thus associated with a poorer overall prognosis in people with end stage renal disease [130].

The role of increased sympathetic nervous system activity in diabetes

Overactivation of the SNS has been associated with the metabolic syndrome (Figure 1), in particular, insulin resistance, hyperinsulinemia and other metabolic abnormalities such as dyslipidaemia, are increased with SNS activation[131]. Given the prevalence of the metabolic syndrome is high, ranging between 70%[132] to 92 % [133]in individuals with type 2 diabetes[132], it is not surprising that type 2 diabetes has likewise been associated with chronic SNS activation[134], even in the absence of hypertension. Patients with type 2 diabetes have increased total muscle sympathetic nerve activity (MSNA)[135] and arterial noradrenaline concentration as well as cardiac enlargement and diastolic dysfunction, which relate to SNS alterations [136]. In experimental diabetes, sympathetic activity, as assessed by norepinephrine measurements, was increased in the ventricles of rats with diabetes compared to control rats[137] suggesting increases in cardiac sympathetic activity may provide a plausible explanation for myocardial injury and risk in the diabetes population. Given the increased risk of morbidity and mortality from cardiovascular related disease in individuals with type 2 diabetes [138], changes in SNS activity may be more important in people with diabetes.

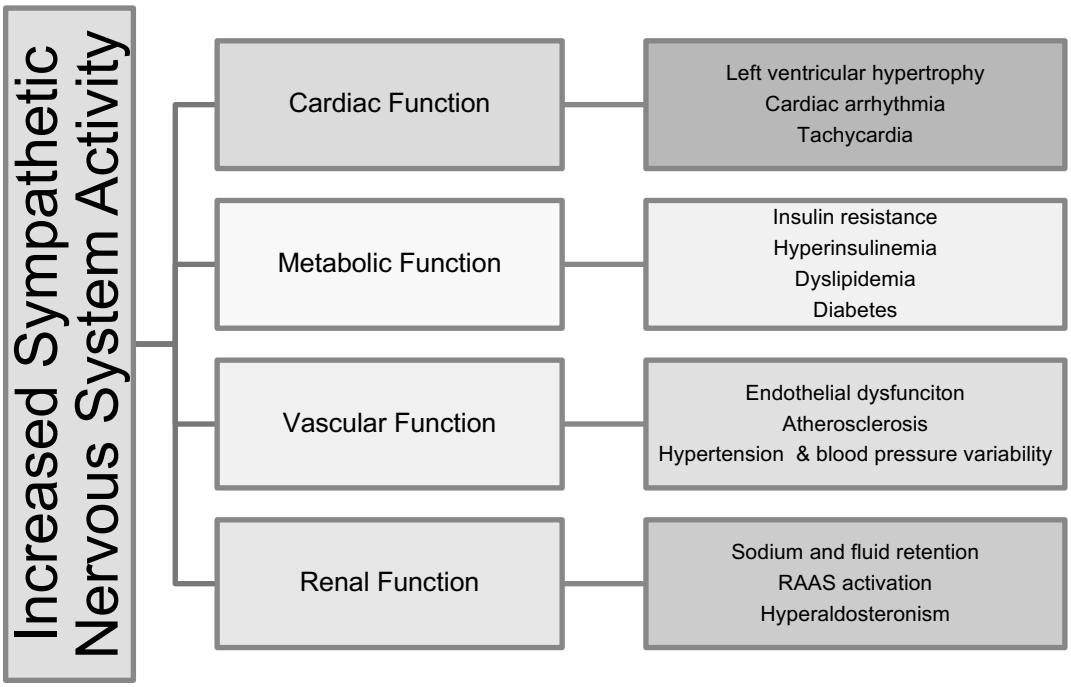

Figure 1: Summary of the consequences of sympathetic nervous system activity overactivation

The effect of sodium intake on sympathetic nervous system activity

Experimental studies are conflicting with demonstration of either an increase in sympathetic activity after high sodium intake[139] or an opposite effect, where sympathetic activity has been enhanced by a low dietary sodium intake and suppressed by a high dietary sodium intake in rats [140, 141]. In human studies, in those without diabetes, low sodium intake has been associated with higher SNS activity[142 143 144]. Additionally, after six days on a low sodium diet (10mmol sodium/ 24h) and a high sodium diet (400mmol sodium /24h) in people with normotension and borderline hypertension, a high sodium diet was associated with reduced MSNA and plasma norepinephrine[145]. Plasma norepinephrine levels were higher on the low sodium diet and lower on the high sodium diet in the borderline hypertensive group as compared to the normotensive group[145]. Furthermore, in a separate study with participants with mild to moderate hypertension, increased MSNA was observed with low dietary sodium intake [146]. Whilst sodium restriction has been shown to lead to hyperinsulinemia [147] and insulin resistance[148] which as above may plausibly lead to SNS activation[131], no studies have directly examined the effects of sodium intake on SNS activity in individuals with type 2 diabetes. In the aforementioned study in individuals that demonstrated higher

MSNA levels in those with diabetes, study participants were reported to have a baseline sodium intake of 400 mmol/24h (9200mg/24h). However, the method of dietary sodium intake estimation was not provided[135] and no further comments were made about sodium intake in relation to MSNA levels. Therefore, there is currently a gap in the literature regarding the effects of sodium intake in individuals with diabetes.

The baroreflex: an important autonomic reflex for acute regulation of sympathetic activity and arterial blood pressure.

As described above, the main role of the sympathetic nervous system is to maintain blood pressure and regulate blood flow tightly via the arterial baroreflex. The baroreceptors can be considered as the sensory receptors of the major systemic arteries, primarily designed to monitor blood pressure via a negative feedback loop, and, are located in the carotid sinus and the aortic arch. The carotid sinus baroreceptor axons travel in the glossopharyngeal nerve and the aortic arch baroreceptor axons travel in the vagus nerve. The baroreceptor activity will travel along these nerves and enter the nucleus of the solitary tract in the brainstem, considered the vasomotor centre of the brain, where the information then reaches the area where the parasympathetic and sympathetic neurons are located in the brainstem. Hence, a reduction in blood pressure will be sensed quickly by the baroreceptors, leading to a reduction in the transmission of signals to the brainstem. This in turn, leads to increased sympathetic activity and decreased parasympathetic activity of the heart and blood vessels with a resultant net increase in vascular resistance and heart rate, restoring blood pressure to normal values. Likewise, when blood pressure increases, the baroreceptors are distended and activated causing a decrease in sympathetic activation, leading to lower peripheral resistance, and an increase in parasympathetic activity, leading to reflex bradycardia and reduced contractility, with a net decrease in blood pressure. Importantly, after activation of the baroreceptors, it is usually the parasympathetic area that is activated within milliseconds, followed by a delay of a couple of seconds which is when the sympathetic area is activated. Thus the baroreflex control of heart rate is usually exerted through vagal, rather than sympathetic control[149]. Even under resting conditions, there is a strong inverse relationship between blood pressure and sympathetic activity[110]. The sensitivity of the baroreflex is characterized as the amount of change in sympathetic or

parasympathetic activity that occurs following a given change in blood pressure. This will thus vary from person to person and over different time periods however a well-preserved baroreflex function is indicated by a baroreflex slope of >6ms/mmHg[149].

A less sensitive baroreflex is not ideal for health, as for any given change in blood pressure, the sympathetic responses may be less pronounced and thus it may take longer to restore blood pressures to baseline[110]. Conditions such as hypertension, coronary artery disease, acute myocardial infarction and heart failure have been shown to be associated with a reduced baroreflex function[149]. A blunted response is in turn predictive of increased cardiovascular risk, especially after an acute coronary event or in heart failure[149]. Experimental studies in a canine model revealed that post an acute myocardial infarction, 70% of animals had reduction in baroreflex control, a higher occurrence of lethal arrhythmias occurred in those with a lower baroreflex function and ventricular fibrillation was inversely related to the baroreflex function. [150, 151]. These animal studies led to human studies, most notable being the ATRAMI (Autonomic Tone and Reflexes After Myocardial Infarction) study[152]. This study demonstrated that in approximately 1300 participants aged less than 80 years, a depressed baroreflex slope (<3ms/mmHg) was a significant predictor of total cardiovascular mortality with a relative risk of 2.8, independent of left ventricular function and ectopic beats [152]. A combination of depressed left ventricular function in addition to depressed baroreflex slope increased the predictive power than either factor alone[152]. Furthermore, in a cohort of 228 individuals with mild to moderate but stable heart failure, a depressed baroreflex slope (<3ms/mmHg) was significantly associated with a higher risk of cardiac death[153]

However, a wide variation in baroreflex slope was seen in ATRAMI suggestive that factors other than the cardiac event, such as environmental or genetic factors, may contribute to baroreflex function[154]. Subsequent studies in twins have confirmed the heritable nature of the baroreflex function [155] and the aldosterone-synthase gene was found to influence the baroreflex function[156]. This then begs the question that the ease of use of baroreflex slope to assess baroreflex function may be considered in the future as an important clinical tool in predicting cardiovascular events in either healthy or high-risk individuals.

Significance of sodium intake on the baroreflex

Experiments in animals such as dogs[157] and rats[158] have shown that sodium restriction attenuates baroreflex function. Studies in individuals without diabetes have likewise demonstrated that a low sodium diet was associated with an impaired baroreflex function with only minimal reduction in blood pressure[142]. This impairment in baroreflex function was improved after restoration of the baseline habitual sodium intake[142]. Similarly, in experimental studies with streptozocin-induced diabetes rats who underwent salt-loading leading to higher sodium intake, no effect on baroreflex function was observed despite an increase in arterial pressure[159]. Yet again, studies targeting the type 2 diabetes population are lacking and greatly needed to understand the baroreflex function and baroreflex control of MSNA.

The role of the endothelial system in cardiovascular disease

Overview of the endothelium system

The endothelium provides the cellular lining to all blood vessels in the circulation. Once considered an inert structure, simply maintaining blood vessel wall permeability, the endothelium is now no longer thought of as an inactive lining of the blood vessels[160]. Rather, the endothelium has been demonstrated to be a key component in determining vascular health outcomes. Weighing approximately one kilogram, comprised of almost ten trillion endothelial cells[160], the endothelial system is now recognised as a dynamic organ with capabilities of exerting endocrine, autocrine and, paracrine functions on vascular smooth muscle cells, platelets and leucocytes[160]

One of the main roles of the endothelium is to regulate arterial tone and blood flow. Endothelial cells regulate arterial tone by balancing the production of vasodilator molecules, such as nitric oxide and prostacyclin, with the production of vasoconstrictor molecules such as endothelin-1 and angiotensin II, which occurs via the conversion of angiotensin I at the endothelial surface[161]. Early experimental studies first demonstrated that relaxation of blood vessels required endothelial cells, as stimulation of endothelial cells by acetylcholine resulted in the release of a ‘substance’ that was able to relax

vascular smooth muscle cells[162]. This 'substance' would later go on to be identified as nitric oxide. Generated from L-arginine by the action of endothelial derived nitric oxide synthase, nitric oxide is the main vasodilator [163]. In situations of shear stress, nitric oxide synthase will be activated to allow appropriate vasodilatory responses. The endothelium is additionally able to regulate blood flow through its effects on haemostasis and coagulation. For example, the tissue factor pathway inhibitor, synthesized primarily by endothelial cells, inhibits, as its name suggests, tissue factor, the receptor that is responsible for the pro-coagulant factor VII [160]. Endothelial cells are additionally capable of producing ectonucleotidases, the enzymes that are responsible for inhibiting platelet aggregation via the dephosphorylation of adenosine diphosphate ultimately to produce adenosine[160]

The endothelium is also essential for maintaining an anti-inflammatory state. Via the release of nitric oxide, endothelial cells are able to prevent leucocyte adhesion by stabilising the inhibitor subunit of nuclear factor kappa B[160, 161]. Nuclear factor kappa B is in an inactive state in the cytoplasm, however, without the inhibitor subunit, it can lead to transcription of genes encoding cytokines, adhesion molecules and growth factors[160]. Endothelial cells also express adhesion molecules, E-selectin, P-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM). These molecules have an important role in regulating leucocyte movement to sites of injury or infection[160]. Furthermore, after being stimulated with mediators such as bacteria and hypoxia, endothelial cells are capable of producing cytokines and growth factors, such as vascular endothelial growth factor, in order to maintain immune responses to disease states such as infection and inflammation[160]

Significance of endothelial dysfunction

Endothelial dysfunction occurs when the endothelium is exhausted by repeated pathological exposures and is no longer able to perform its protective functions. Thus, when the endothelium cannot regulate arterial tone and blood flow to maintain appropriate vascular homeostasis[161], unopposed vasoconstrictor effects will lead to an increase in arterial tone [164]. In experimental models of hypertension, overexpression of endothelin-1 has been demonstrated[165]. In mice, disruption of the gene encoding for nitric oxide synthase type-II, resulted in hypertension [166]. Additionally, in the presence

of vascular risk factors, the endothelium is attenuated due to the increased expression of leucocyte adhesion molecules, chemotactic factors and pro-inflammatory cytokines, which in turn lead to arterial wall inflammation[167]. Endothelial dysfunction therefore predisposes to atherosclerosis and subsequent cardiovascular disease via the resultant vasoconstriction, inflammation and thrombosis formation[164].

Experimental studies have demonstrated that in the presence of cardiovascular risk factors, endothelial dysfunction arises well before any plaque accumulation [168] occurring early in the atherogenesis pathway[169], well before any clinical manifestation of cardiovascular disease is apparent[168]. For example, endothelial dysfunction, due to a reduction of nitric oxide activity, is an early marker of atherogenesis, was seen in high-risk individuals such as those with diabetes[170] and hypertension [171], even when no coronary angiographic evidence of atherosclerosis was apparent. Identifying endothelial dysfunction early is therefore important as it has the potential for improving cardiovascular outcomes in those individuals with known vascular risk factors.

Significance of diabetes on the endothelial system

Diabetes has a variety of negative effects on cell signalling pathways which can ultimately impair the ability to produce nitric oxide, even in response to physiological stimuli[164]. In a study evaluating coronary endothelial function responses to acetylcholine in patients with type 1 and type 2 diabetes and controls, with angiographically normal coronary arteries at baseline, the patients with diabetes had paradoxical vasoconstriction instead of the expected vasodilation after a stepwise acetylcholine infusion[170], suggesting endothelial cells exposed to hyperglycaemia undergo an apoptotic process[172].

Other studies have suggested hyperglycaemia can lead to angiogenesis and a hypertensive state. In human umbilical vein endothelial cells that were exposed to a hyperglycaemic milieu, 25 mmol/l of glucose for a 24-hour period, up-regulation of seven major genes occurred, two of which were genes that encode for ICAM-1, and interleukin-8, a chemokine with chemotactic properties as well as potent angiogenic properties[173]. In a similar fashion, human aortic endothelial cells that were cultured in 25 mmol/L of glucose for seven days, had increased interleukin-8 secretion compared

to control cells that were cultured in 5.5 mmol/L of glucose[174]. Furthermore, in a single-blinded randomised crossover trial, normotensive men with type 2 diabetes had higher plasma levels of endothelin-1 during a two-hour euglycemic hyperinsulinemic clamp as compared to placebo, with a return to baseline of endothelin-1 levels after cessation of the insulin infusion[175]. These findings suggest a stimulatory effect of hyperinsulinemia on endothelin-1 which becomes important in insulin-resistant individuals. In Sprague Dawley rats receiving a high fructose diet, hyperinsulinemia as well as higher levels of endothelin-1 were associated with higher blood pressure as compared to controls[176].

Markers of endothelial damage and repair

The extent of damage to endothelial cells must be balanced with an ability to endogenously repair itself in order to maintain integrity of the endothelium. The primary mechanism of endothelial cell repair had long been considered due to nearby endothelial cells replicating themselves to replenish the loss of damaged cells. However, an alternative model of repair was provided when endothelial progenitor cells were first recognised in 1997[177]. Originating from the bone marrow, endothelial progenitor cells enter the circulation to sites of injury and mature into cells with phenotypic properties similar to endothelial cells in a somewhat nitric oxide dependent fashion[178]. Concern arises for individuals with cardiovascular risk factors such as diabetes, as described above, as they may not mount this nitric oxide dependent response appropriately and therefore this repair process may be impaired. This can then lead to ongoing, unopposed endothelial cell damage with ultimate detachment of endothelial cells into the circulation. These markers of damage are termed endothelial microparticles.

Microparticles

Overview of microparticles

What were initially reported as insignificant platelet membranes or 'platelet dust' in 1967[179], were later found to be microparticles, small extracellular vesicles that are released into the circulation after the exocytic budding of cellular membranes in the setting of activation, injury or apoptosis [180-183]. Microparticles are different in size and composition than exosomes and apoptotic bodies [180-183]. Microparticles are usually defined as 0.1–1 μm membrane fragments exposing the anionic phospholipid phosphatidylserine (PS) and membrane antigens representative of their cellular origin [184]. Exosomes on the other hand, are usually smaller (approximately 40–100 nm) than microparticles and form by the fusion of multivesicular bodies with plasma membrane. Exosomes usually do not expose externalised PS on their membranes unlike in microparticles where exposure of PS is thought to be a key mediator of their formation [180]. Micropartices are smaller, with less nuclear material, than apoptotic bodies (1–5 μm)[180].

In the cellular activation formation of microparticles (Figure 2), exposure to pathogens such as pro-inflammatory cytokines [185] or bacterial lipopolysaccharides[186], triggers the increase in cytosolic calcium[187] which leads to the breakdown of membrane skeleton proteins such as talin[187]. The subsequent disruption in the membrane skeleton allows the release of the microparticle. In a similar fashion, apoptosis induced microparticle formation is dependent on the activation of a kinase, belonging to the family of serine-threonine kinases, the Rho-associated protein kinase (ROCK I)[188]. Activated ROCK I then leads to actin-myosin mediated cellular contraction and disruption of the membrane skeleton[188]. Once released, microparticles retain cell-surface proteins from the cell of origin, along with cytosolic contents including enzymes, ribonucleic acid (RNA), microRNA and, possibly, deoxyribonucleic acid (DNA)[180].

The composition of microparticles therefore depends on the cellular origin. Most microparticles originate from platelets, however other cell types such as monocytes and erythrocytes, as well as endothelial cells, as described above, are all able to release microparticles. Despite endothelial microparticles being fewer in number as compared

to other cell types [189], comprising approximately 15% of all microparticle subtypes[190], they are of most interest to this thesis given they represent endothelial dysfunction and hence will be the focus of this literature review.

As microparticles are capable of retaining the proteins from their parent cell, enumeration of microparticles is possible through methods of identification such as flow cytometry [180], further detailed in chapter two. Thus, the role of microparticles representing a relatively non-invasive biomarker of endothelial disease processes has garnered interest (Figure 2). Biomarkers are useful as they are measurable marker of physiological, pathological and pharmacological responses. Biomarkers are beneficial in identifying risk of pathological disease states earlier leading to earlier therapeutic intervention[180].

Endothelial microparticles in cardiovascular related disease

Whilst being present in the circulation at low levels [191] in healthy individuals, endothelial microparticles have been shown to be increased in cardiovascular related diseases such as hypertension [192-194], acute coronary syndrome [195, 196] and cardiovascular disease[197]. The first demonstration of endothelial microparticles being capable of disrupting vascular function came from studies evaluating the exposure of rat aortic ring endothelium to circulating microparticles derived from human plasma from patients after either nonischemic events or after an acute myocardial infarction[195]. Endothelium-dependent relaxation of the rat aorta was impaired by the microparticles derived from the myocardial infarction patients but not from the nonischemic patients[195]. In vivo evidence suggests apoptotic microparticles are present in atherosclerotic plaques[198]. Furthermore, in a prospective, case-controlled study on people with and without coronary artery disease, who had had a cardiac event such as acute coronary syndrome, myocardial infarct, unstable angina and stable angina, the levels of endothelial microparticles were higher in those with pre-existing coronary artery disease as compared to those without[196]. Within the coronary artery disease cohort, participants who had had an acute coronary syndrome or myocardial infarct had even higher levels of endothelial microparticles compared to those who had stable or unstable angina[196].

Endothelial microparticles in diabetes

Microparticles are known to be elevated in individuals with type 2 diabetes [189, 199, 200]. A mix of *in vitro* and *in vivo* experiments of endothelial microparticles belonging to the cadherin family of receptors, vascular endothelial cadherin, or VE-cadherin, thought to play a role in maintaining vascular permeability and maintenance of the endothelial barrier, were conducted in a cross-sectional study in Japan in individuals with type 2 diabetes[201]. These levels of VE-cadherin were significantly higher in people with type 2 diabetes compared to those without diabetes. Moreover, VE-cadherin was positively associated with the degree of damage, induced by in vitro hydrogen peroxide, of endothelial cells from human coronary arteries [201]. Additionally, the degree of acetylcholine-induced vasodilation was lower as the microparticle counts were higher[201]. Perhaps the most important finding was that VE-cadherin was demonstrated to be the most significant predictive risk factor for coronary artery disease, even more significant than traditional cardiovascular risk factors such as hypertension, dyslipidaemia and duration of diabetes[201]. Despite being limited by the population representative of Asian ethnicity only, thus not being generalisable to a more general population, this study still paved the way for further interest in this area. For example A cross-sectional study from France was conducted in 56 individuals with type 2 diabetes who had pre-existing coronary noncalcified plaques as detected by multidetector computed tomography (MDCT) with differing cardiovascular risk levels[202]. Endothelial microparticles were higher in those with acute coronary syndromes[202]. Additionally, the endothelial microparticle levels were positively associated with coronary noncalcified plaques[202] which is of significance given these plaques themselves are associated with an increased cardiovascular risk [203].

Recognising that most studies contributing to the understanding of microparticles in diabetes have been *in vitro* studies, which face challenges as results cannot fully replicate physiological conditions, Feng et al recently conducted an *in vivo* study in streptozocin-induced diabetes rats to look at the role of circulating microparticles[204]. They demonstrated a greater than 130-fold increase in microparticles in plasma from rats with diabetes compared to those without diabetes with the majority originating from platelet, leucocyte and, endothelial cells[204]. They went on to show that when the plasma from the rats with diabetes was perfused into the microvessels of normal rats,

the microparticles almost immediately mediated leucocyte adhesion and increased vascular permeability causing similar vascular changes as seen in the rats with diabetes[204]. Perhaps the most critical observation is that endothelial microparticles may not only be a by-product of cellular activation or apoptosis but may also contribute to the widespread vascular dysfunction seen in diabetes[204].

Others have shown that the type of diabetes may further be important in determining mechanisms of vascular dysfunction. For example, in a study comparing individuals with type 1 and type 2 diabetes, with age-matched controls, only the individuals with type 1 diabetes, not those with type 2 diabetes, showed that the activity of procoagulant microparticles were higher and the degree of glycaemic control correlated with the levels of these procoagulant microparticles[205]. Nevertheless, the support from existing literature that endothelial microparticles are elevated in individuals with type 2 diabetes[189] and hence reflective endothelial dysfunction support their use as a non-invasive surrogate markers of cardiovascular risk (Figure 2).

Significance of sodium intake on endothelial microparticles

Despite the clear associations between endothelial microparticles with hypertension [192-194] and cardiovascular disease [195-197], together with the call from the IOM[69] to conduct mechanistic studies, exploring the role of sodium on endothelial microparticles as surrogates for cardiovascular health outcomes seems biologically plausible. Aldosterone-salt-induced hypertension in rats has been demonstrated to increase the levels of platelet, endothelial and erythrocyte microparticles associated with endothelial dysfunction [206]. It is however difficult to extrapolate conclusions from this animal study given the intervention of aldosterone rather than sodium was used to measure changes in endothelial function and cardiovascular risk. There are surprisingly, at present, no human studies in any population examining the direct effects of sodium intake on any subtype of microparticle, presenting a considerable gap in knowledge in this area.

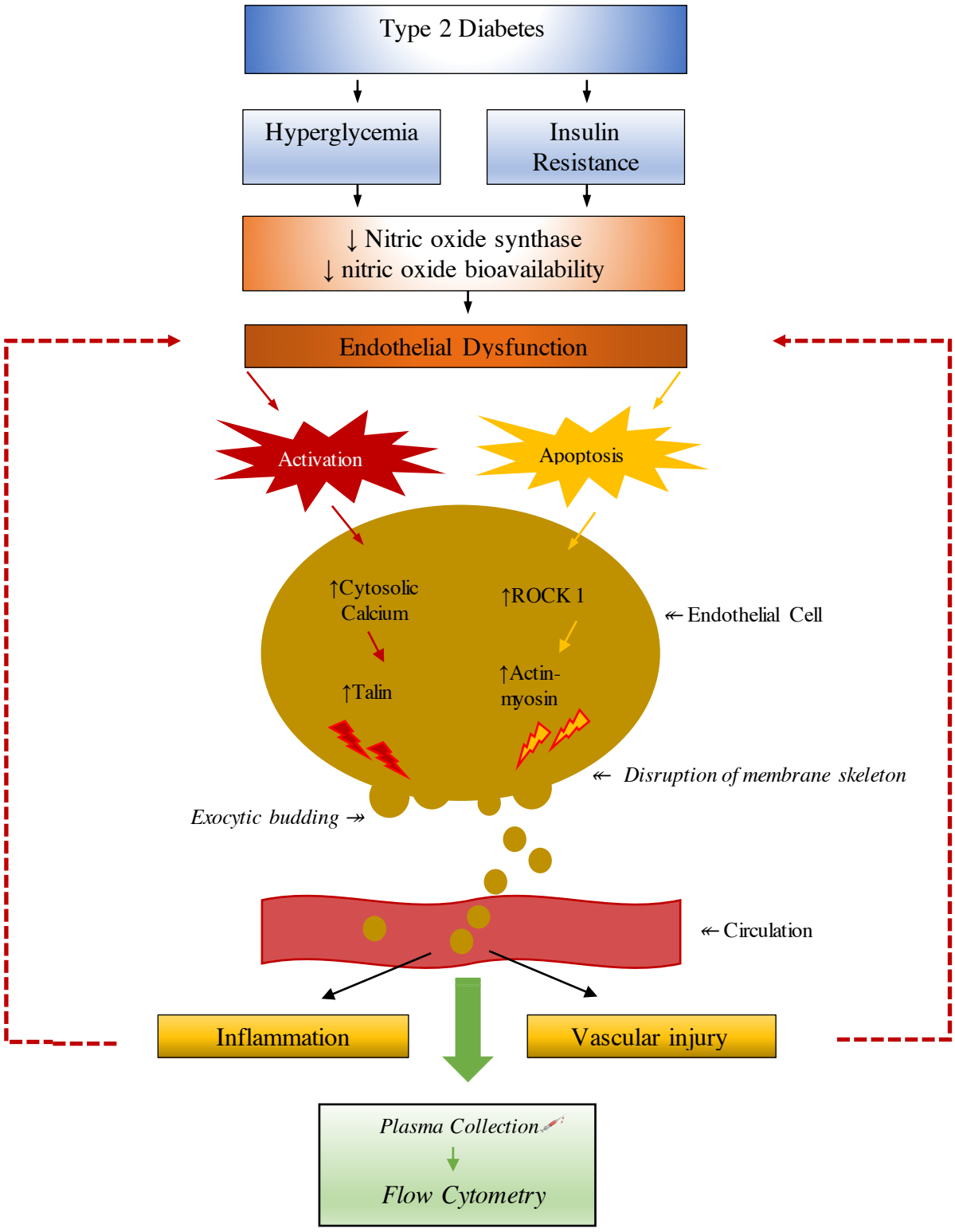

Figure 2: Overview of endothelial microparticle formation and their potential use as biomarkers of cardiovascular disease in high-risk conditions such as type 2 diabetes

The role of the renin-angiotensin-aldosterone system in cardiovascular disease

Overview of the renin-angiotensin-aldosterone system (RAAS)

The renin-angiotensin-aldosterone system (RAAS), comprised of three main hormones, as its name suggests, renin, angiotensin and aldosterone, has an important role to regulate blood volume and vascular resistance. As opposed to the baroreflex, described above, which is involved in the more short-term management of blood pressure, the RAAS is important for both acute and chronic regulation of haemodynamic function.

Activation of RAAS occurs firstly by release of renin from the renal juxtaglomerular apparatus in response to reduced sodium delivery to the macula densa, reduced renal perfusion pressures and sympathetic activation[207]. Renin then goes on to cleave hepatic angiotensinogen to angiotensin I which is then converted to angiotensin II by pulmonary angiotensin-converting enzymes. Angiotensin II then goes on to stimulate the release of aldosterone which leads to enhanced renal tubular sodium reabsorption , vasopressin release, activation of the sympathetic nervous system, vasoconstriction of vascular smooth muscle cells and renal sodium and water retention[208]. These ultimately leads to increased circulating plasma volume, blood pressure and cardiac output (Figure 3). Whilst an important system for vascular haemostasis, the inappropriate activation of RAAS will trigger a cascade of events leading to lead to hypertension and an increase in cardiovascular disease risk[209]. Moreover, independent of blood pressure status, angiotensin II itself is associated with progression of atherosclerosis[210], cardiac remodelling leading to ventricular hypertrophy and fibrosis[211] and myocardial infarction[212].

The role of increased RAAS activity in diabetes

Diabetes itself is capable of RAAS activation. For example, experimental studies have demonstrated that hyperglycaemia is associated with higher levels of angiotensin II in cardiac myocytes which in turn increases the susceptibility of apoptosis of these myocytes [213]. Deficiency in ACE 2, the enzyme responsible for angiotensin II metabolism, has been shown to increase angiotensin II[214]. This enzyme appears to be

down regulated in diabetes[215]. In streptozocin-induced diabetes mice, increased angiotensin II production was seen in mice with ACE 2 deficiency[215]. The ACE 2 is increasingly being recognised as an important component in balancing the RAAS[216]. Furthermore, other components of the RAAS pathway, such as aldosterone stimulation, is also increased in hyperglycaemic states[217] which further increases the progression of diabetes related cardiovascular risk[218].

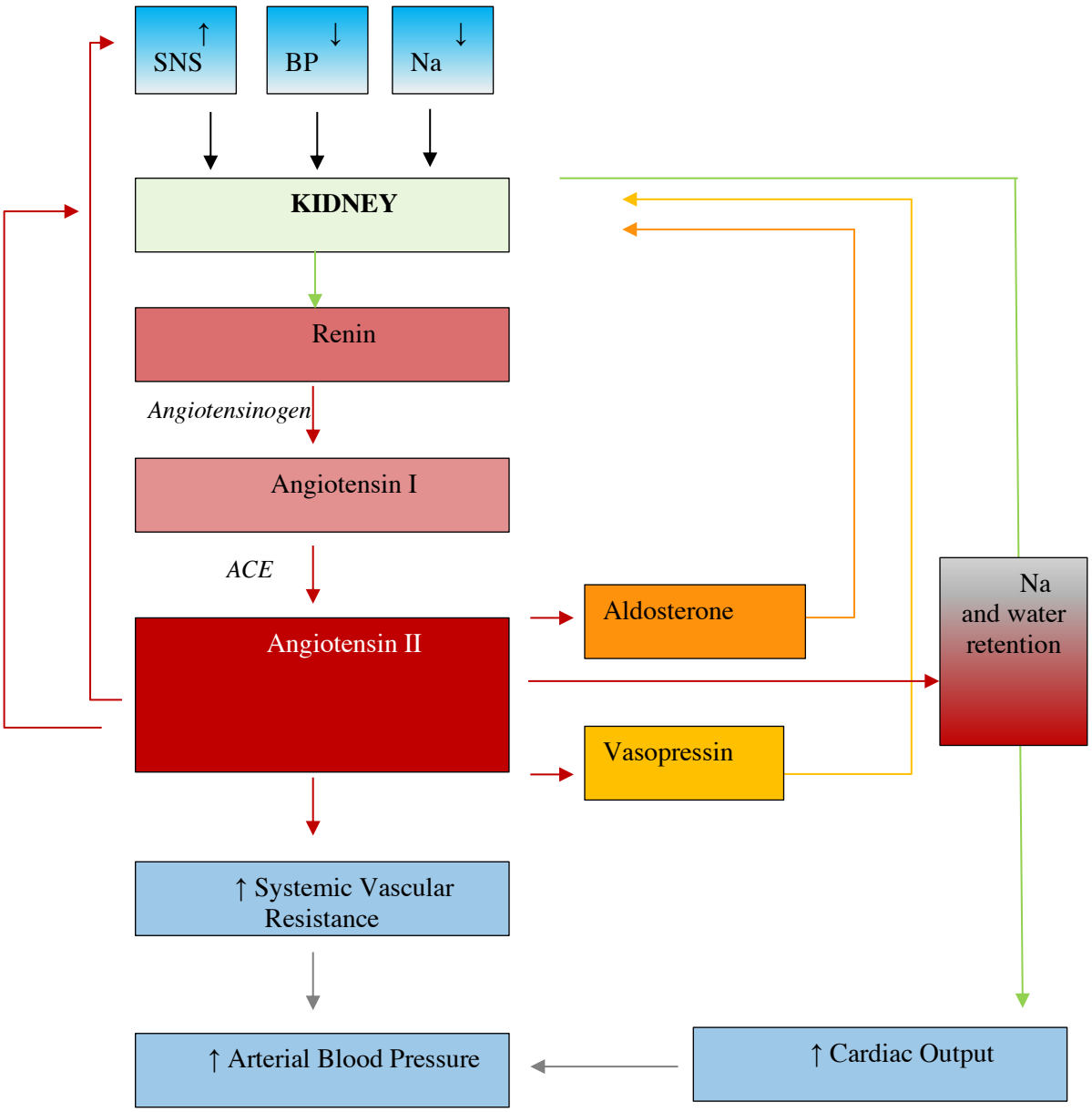

Figure 3: Schematic representation of the cascade of events that occur in renin-angiotensin-aldosterone system activation.
Abbreviations: ACE, angiotensin converting enzyme; BP, blood pressure; Na, sodium; SNS, sympathetic nervous system.

Effect of sodium intake on the renin-angiotensin-aldosterone system

Atherosclerosis-prone apolipoprotein E knockout (ApoE KO) mice given a low sodium diet (0.03% sodium) for six weeks were demonstrated to have a 4-fold increase in aortic plaque accumulation as compared to mice who received a normal sodium diet (0.3% sodium) [219]. A low sodium diet was additionally associated with an increase in the expression of vascular adhesion molecules, such as ICAM-1, and pro-inflammatory cytokines, in both the circulation and at the aortic tissue level. Moreover, the low sodium diet was associated with an activation of the RAAS, as measured by increased aldosterone levels, however, was not increased in mice receiving the high sodium diet. The addition of the ACE inhibitor, perindopril, was able to attenuate these atherogenic changes[219]. This important finding was able to provide the mechanistic understanding behind the previous seminal findings of RAAS being involved in atherosclerosis from the Heart Outcomes Prevention Evaluation Study[220]. Interestingly, the high sodium diet (3% sodium) had similar effects as ACE inhibitors in being able to suppress atherosclerotic plaque formation with only a modest increase in blood pressure (5mmHg) [219]. The data suggest that atherogenesis induced by sodium restriction, via stimulation of endothelial dysfunction and RAAS activation, may be attenuated by lifting the sodium restriction, at least in mice models.

More recently, the effect of a low sodium diet (0.15% sodium chloride) as compared to normal sodium (1.27% sodium chloride) diet was assessed on atherogenesis outcomes in hypertensive, low-density lipoprotein-receptor knockout mice who were treated with or without hypertensive agents (losartan or hydralazine)[221]. Despite a reduction in blood pressure, mice on the low sodium diet had higher arterial lipid infiltration as a result of higher triglyceride and total cholesterol levels. The mice who were on the low sodium diet, who additionally received losartan, showed an attenuation of these changes with prevention of the arterial lipid infiltration[221]. These findings further support the role of angiotensin II induced atherogenesis being induced by low sodium intake[221]. These results have been replicated in streptozotocin-induced diabetic ApoE KO mice who were randomised to either a low sodium diet (0.05%), normal sodium diet (0.3 % sodium) or high sodium diet (3.1% sodium) with or without additional ACE inhibitors (perindopril) [222]. A low sodium diet (0.05%) augmented atherosclerosis whereas a high sodium diet (3.1% sodium) was able to suppress plaque accumulation similar to the

degree of ACE inhibition [222]. Whilst these results are difficult to translate to human outcomes, they create a need for further clarification regarding the clinical impact of such findings.

Reduced sodium intake in human studies has been shown to be associated with higher plasma renin activity and subsequent higher risk of cardiovascular events [107, 223]. Studies focusing on the diabetes population are lacking. We have demonstrated lower 24h urine sodium excretion values were associated with higher serum aldosterone levels in individuals with both type 1 and type 2 diabetes [224]. Similarly, our group further demonstrated in a randomised controlled trial in hypertensive individuals with type 2 diabetes, that lower mean 24h urine sodium excretion was associated with higher plasma renin activity levels[225]. Moreover, short-term salt supplementation (100mmol sodium chloride/day) in this cohort was associated with lower plasma renin activity levels and a trend toward lower aldosterone (p=.05), in the setting of an angiotensin receptor blocker (40mg telmisartan) use [225]. These findings suggest the possibility of RAAS activation occurring despite the presence of RAAS blockade in the setting of low sodium intake. Given that the majority of patients with type 2 diabetes are on angiotensin converting enzyme inhibitors or angiotensin II receptor blockers, a low sodium diet whilst potentiating the beneficial effects on the effectiveness of RAAS blockade on blood pressure, may impair endothelial function via upregulation of local tissue RAAS[226]. Additionally, these findings warrant further assessment as plasma renin activity is also known to be an independent risk factor for adverse cardiovascular outcomes in high-risk individuals such as those with diabetes [227].

Understanding the relationship between combined RAAS activation and endothelial dysfunction on cardiovascular outcomes

As shown earlier[219], RAAS activation and endothelial dysfunction may act together to lead to the progression of atherosclerosis observed in the setting of sodium lowering. Furthermore, RAAS activation may itself lead to endothelial dysfunction given pro-coagulant microparticles have been observed after RAAS activation [228] and RAAS blockade reduces microparticles in circulation [229-231] which subsequently improves endothelial function [230]. Overall, there are limited studies exploring the links between

RAAS activation and endothelial microparticles and only one study has examined the effect of RAAS blockade on microparticle levels in people with type 2 diabetes [231]. There are, however, no studies at present, which have examined both the RAAS and endothelial systems in the presence of varying sodium intakes in any population.

Understanding the relationship between combined sympathetic nervous system activity and endothelial dysfunction on cardiovascular outcomes

Experimental animal studies have demonstrated that chronic sympathetic activation can lead to endothelial dysfunction[232], independent of increases in blood pressure[233] suggesting an interaction exists between the SNS and endothelial system in order to achieve cardiovascular regulation[233]. Trophic actions of the SNS leads to hypertrophy and proliferation of vascular smooth muscle cells [233] with a reduction in these hypertrophic effects observed post sympathetic denervation[234]. Chloralose anaesthesia, used to stimulate sympathetic activation in New Zealand White rabbits, demonstrated a five-fold increase in the number of injured endothelial cells suggesting an association between SNS activation and atherogenesis[232]. Furthermore, peptides released from the vascular endothelium such as endothelin-1, capable of vasoconstriction and vascular smooth muscle proliferation, are stimulated by norepinephrine-infusion in Sprague-Dawley rats[235], which, in turn leads to vascular remodelling and blood pressure variability[236]. As blood pressure elevation and variability have been associated with cardiovascular complications[237], balancing this interplay between the SNS and endothelial function becomes important.

Whilst the relationships between SNS activity and endothelial dysfunction are yet to be fully explored in humans, increased SNS activity has been associated with increased arterial wall thickness, thus highlighting the importance of the SNS in arterial remodelling in humans, especially with advancement of age [238]. Even in healthy normotensive individuals with no pre-existing atherosclerosis, an inverse relationship between SNS activity, as measured by MSNA, and surrogate markers of endothelial function, measured by the reactive hyperaemic index, described in greater detail in chapter two, was observed [239]. Moreover, the antihypertensive agent, moxonidine, a selective imidazoline receptor agonist, capable of central sympathoinhibition, has been

shown to improve endothelial dysfunction in patients with mild hypertension and the metabolic syndrome, further suggesting the possibility of the SNS being capable of regulating endothelial function[240]. This concept becomes important for individuals with type 2 diabetes who, as described above, have higher resting MSNA or SNS activity [135] as well as an increased likelihood of endothelial dysfunction induced arterial stiffness and in turn increased mortality rates[241].

Thesis aims and objectives

Contrary to the established view regarding the beneficial effects of sodium restriction, low sodium intake has been associated with adverse cardiovascular outcomes in individuals with diabetes [60, 61]. These observational findings require further mechanistic exploration. Possible explanations from animal and human studies involving the non-diabetes population have shown that low sodium intake has undesirable effects on hormonal pathways such as activation of the sympathetic nervous system [242] which may facilitate the development of endothelial dysfunction [239] and subsequent cardiovascular disease [161] in turn negating the beneficial effect of blood pressure reduction on the overall cardiovascular outcome. However, there is a paucity of data exploring role of sodium on these systems in people with type 2 diabetes.

The studies represented in this thesis aimed to address the significant gap in literature by underpinning the mechanisms responsible for the association between low sodium intake and poorer cardiovascular health in people with type 2 diabetes by determining the effect of sodium intake on SNS activity and endothelial function. The primary hypothesis was that a low sodium intake would be associated with worse SNS and endothelial function. The specific research aims and hypotheses that formed the basis of this thesis are presented below:

Chapter Three: Comparison of endothelial function and sympathetic nervous system activity along the glucose continuum in individuals with differing metabolic risk profiles and low dietary sodium intake.

Aims: To compare sympathetic nervous system activity and endothelial function in the setting of low sodium intake along the glucose continuum in individuals who had either:

(i) normal glucose tolerance
(ii) impaired glucose tolerance
(iii) treatment naïve, diabetes
(iv) treated type 2 diabetes with established cardio-metabolic risk factors.

Hypothesis: In individuals with higher metabolic risk, low sodium intake is associated with (i) impairment of endothelial function and (ii) higher SNS activity

Chapter Four: Effect of salt supplementation on sympathetic activity and endothelial function in salt sensitive type 2 diabetes

Aims: To determine the effect of salt supplementation (100mmol NaCl/24h) on cardiovascular health by measuring sympathetic nervous activity and endothelial function as surrogate cardiovascular endpoints in people with type 2 diabetes with habitual low sodium intake.

Hypothesis: Compared to placebo, salt supplementation will:

1. lower SNS activity
2. improve endothelial function

Chapter Five: The relationship between habitual dietary sodium intake and RAAS blockade on circulating microparticle levels in type two diabetes

Aims: To determine the associations between habitual dietary sodium intake and RAAS blockade on circulating microparticle levels in individuals with type 2 diabetes.

Hypothesis:

(i) Individuals with lower habitual sodium intake will have higher circulating endothelial microparticle levels, reflective of a damaged endothelium
(ii) The use of RAAS blockade will be associated with lower circulating endothelial microparticle levels.

Chapter Six: Dietary sodium and potassium intake in people with diabetes: are guidelines being met?

Aims: To provide an updated estimate of whether people with diabetes of any type are adhering to current dietary sodium (< 2300mg per day (100mmol/24h))[1] and potassium guidelines (> 4680mg per day (120mmol/24))[2] as assessed by 24h urine excretions from 2009-2015.

Hypothesis:

(i) there will be an overall low adherence to the guidelines

(ii) sodium and potassium intake will not change over time.

Chapter Seven: Comparison of 24hr urine sodium excretion measurements and 24hr dietary sodium recall in determining sodium intake in individuals with type 2 diabetes.

Aims: To compare 24h dietary recall with 24hUNa in estimating dietary sodium intake in people with type 2 diabetes

Hypothesis:

(i) there will be limited agreement between the two methods

(ii) there will be an underreporting of sodium intake from 24h dietary recalls

Chapter two: Appraisal of methodology used to assess the sympathetic nervous system and endothelial function

Abstract

Objective: To explain the concepts and rationale of the methodologies utilized during the studies reported in chapters three to five in assessing the sympathetic and endothelial systems. Specifically, to explain the principles behind microneurography, pulse amplitude tonometry, baroreflex, and endothelial microparticle assessment. Additionally, to outline the development of these techniques and their current clinical applications in cardiovascular disease states.

Study selection and data extraction: A literature search in MEDLINE (1946– May 2020) was performed using a combination of the following search terms: augmentation index, baroreflex, cardiac baroreflex, endothelial function, EndoPAT, epinephrine, norepinephrine, flow cytometry, heart rate variability, microneurography, microparticles, reactive hyperaemic index and sympathetic nervous system. Combinations of limitations including English language, core clinical journals and journal article were placed on the search terms. References from the relevant papers were also sourced.

Conclusions: Microneurography is currently considered the gold standard in the assessment of the sympathetic nervous system. Pulse amplitude tonometry measured by EndoPAT provides an effective, non-invasive approach for endothelial function measurement which can give additional measures of arterial stiffness as determined by the augmentation index. Microparticles, assessed by flow cytometry, provide potential as non-invasive biomarkers in cardiovascular disease states.

Microneurography to assess Sympathetic Activity

Microneurography, briefly introduced in chapter one, assessed the sympathetic activation for studies presented in chapters three and four. Microneurography is an electrophysiological method designed to record sympathetic nerve traffic directly from the peripheral nerves in awake humans to either the muscle, termed muscle sympathetic nerve activity (MSNA), the method used for chapters three and four, or to the skin, termed skin sympathetic nerve activity (SSNA). Microneurography in the skeletal muscle vasculature is representative of 20% of the total body sympathetic activity[243] and has considerably advanced our understanding of the autonomic nervous system in physiological and pathological disease states, such as in those with diabetes[244].

From humble beginnings: development of the microneurography technique

As sympathetic postganglionic neurons are small, before the advent of microneurography, measuring the peripheral nerve impulses in humans was deemed impossible. Available animal studies had required the dissection and splitting of the nerve to record impulses. Whilst seemingly intolerable to humans, a single study in seven healthy male individuals does exist, in which the superficial branch of the radial nerve was dissected to assess cutaneous sensibility[245]. The procedure was laborious, lasting five to six hours in duration, requiring ultra-short acting anaesthetic agent and at times requiring up to seven months for recovery of sensation post procedure[245]. Notwithstanding these concerns, the major criticism was the placement of a needle into the nerve and the potential for permanent nerve damage. Scientist at the time believed it would be impossible to record the nerve impulses via an extracellular needle and cautioned the progression of such techniques due to the plausible damage from trauma, intra-neural infection and bleeding.

In 1965-1966, at the Academic Hospital Neurophysiology department, Uppsala, Sweden, two scientists, Karl-Erik Hagbarth and Ake Vallbo, who performed the initial experiments, on themselves, were able to overcome these medical and ethical concerns[246]. Whilst having the experience of single-unit recordings from muscle afferents in animals, both were not versed in autonomic nervous system activity[246].

Hagbarth took the first steps by percutaneously inserting a needle into his own ulnar nerve[246] to record action potentials from axons. With the needle attached to an amplifier, tapping on the skin produced the initial signal-to- noise ratio, which was quite low. Difficulty was faced in visualizing the signals on the oscilloscope screen, an electronic instrument which allowed the observation of signals such as sound or vibration as converted voltages on display screens[246]. To improve the signal-to-noise ratio, multiple experiments were conducted; utilizing exposed animal nerves and various different metals to coat the needles. It was through these initial experiments where Hagbarth and Vallbo discovered Tungsten had the suitable properties. It was not too hard, not too soft such as steel, nor too brittle such as platinum-iridium. Additionally, the size of the tip diameter, approximately five micrometres (μm), shaft diameter of 100-200 μm and an epoxy-based varnish to coat the needle were deemed successful in optimizing the electrode properties[246]. At a time when computers were not available, all the recording outputs were generated on light sensitive paper[246].

The initial recordings produced from the experiments carried out on Hagbarth and Vallbo were followed up by electromyography and when no nerve damage was apparent, the scientists felt confident to conduct future experiments on healthy volunteers[246]. The term for their technique, microneurography, was suggested by prominent neurophysiologist, Professor Yngve Zotterman, at the Royal Veterinary College in Stockholm[246]. Hagbarth and Vallbo researched three main neuronal areas: cutaneous afference, muscular afference and sympathetic efferent activity[114]. From here onwards, multi-unit sympathetic nerve activity will be described in further detail.

The emergence of a clinical use of microneurography in assessing the sympathetic nervous system

German cardiologist, Wolfram Delius, was recruited to provide the necessary clinical perspective on cardiovascular regulation[246]. The initial multiunit recording of skin sympathetic activity was made in a hypertensive woman[247]. This finding was essential to dispel the common view that the sympathetic nervous system was organized with the same nerve traffic to different organs. It was previously widely considered skin and muscle activity would be similar to each other. However, the data demonstrating different patterns of sympathetic activity in the skin compared to muscle was an

important early achievement in microneurography [247]. To this day, the concept of a highly differentiated sympathetic system, arises from this original body of work[247].

Determining the strength of muscle sympathetic activity with initial approaches of using a planimeter to assess the area under the curve proved to be inconsistent. This led to measuring each burst immediately before and after the burst ended. The mean voltage amplitude of the burst was used to describe the measure of burst strength[246]. Recordings from muscle sympathetic activity at rest demonstrated that inter-individual differences were highly reproducible over a prolonged period of time, with a characteristic burst strength for that individual[248]. These findings had significant clinical implications. Importantly, microneurography became meaningful in comparing resting nerve activity between health and disease states. Additionally, changes with ageing, physical activity and those incurred with therapeutic intervention, seemed possible to demonstrate with microneurography[246, 248]. One of the drawbacks however, was that an upper limit of normal, or a pathological level, was not possible to define, as the inter-individual differences in MSNA were too wide[246]. Nevertheless, being able to use microneurography in awake humans paved the way for pathological disease states to be assessed. Initially, microneurography was a technique primarily used in Sweden. However, with more interest in the autonomic nervous system, visiting scientists initially from Australia, Italy and America during the 1970 to 1980s paved the emergence of microneurography being introduced globally, where it is now practiced in many countries. Sympathetic recordings have now become the largest field in microneurography[246].

Microneurography in the laboratory setting: performing the measurements for evaluation of muscle sympathetic nerve activity

Preparation of study participants in chapters three and four proceeding to microneurography was performed according to pre-specified protocols. As caffeine[249], alcohol [250] and exercise[251] are known to increase the autonomic output, participants are advised to abstain from caffeine for 18 hours and alcohol and exercise for 36 hours to eliminate any potential acute effects on the investigations. Similarly, smoking is advised against prior to microneurography as acute smoking exposure leads to elevations in blood pressure and heart rate[252]. These hemodynamic effects of smoking

are mediated by increases in catecholamine release and sympathetic activity. Usually, the net effects of smoking on sympathetic activity will depend on the balance between the sympatho-excitatory effects of smoking on the central nervous system and the sympatho-inhibitory effects on the baroreflex. The baroreflex is activated by the increase in blood pressure from smoking. In healthy individuals, an intact baroreflex will go on to suppress MSNA. However, in disease states, the underlying baroreflex impairment will lead to an increase in MSNA[252]. These acute effects are similarly observed in chronic, habitual smokers, who usually have an impaired baroreflex. Hering et al [253] confirmed with the use of microneurography that resting sympathetic levels were greater in smokers with pre-existing hypertension compared to those hypertensive participants who were non-smokers[253]. Interestingly, the chronic sympatho-excitatory effects of habitual smoking are reversible, as seen in the study by Middlekauff et al [254]. In this study of 49 women, previous smokers (n=9) had similar sympathetic activity compared to those whom had never smoked (n=19)[254]. Participants are assessed in the fasting state for at least a six hour duration as increase in MSNA occurs after any ingestion of food, in particular carbohydrate based foods, more so than protein or fat based food items[255].

To ensure that background sympathetic activity remains constant [246] thermal conditions must be stable and all forms of potential interference, such as body movement, lighting and background noise minimized. Thus, most laboratories perform experiments in a quiet environment where the light is dimed, and the temperature is set at 22°C. Participants are resting on a bed in the supine position. A tungsten microelectrode is inserted percutaneously, directly into an accessible nerve. This is usually the right peroneal nerve at the fibular head (Figure 4) [256]. To find this nerve, the intraneural stimulation technique is employed[257]. In brief, pulses of electrical stimuli are delivered through the surface probe. An optimal site is identified once the lowest electrical threshold is needed for evoking twitches in the innervated muscle or paraesthesia in the cutaneous area innervated by that nerve[257]. The microneurographer is confident that a nerve fascicle has been entered once twitches or cutaneous parasthesiae are able to be generated at low currents[257]. A separate subcutaneous electrode is then positioned two to three centimetres away from the recording site and acts as a reference.

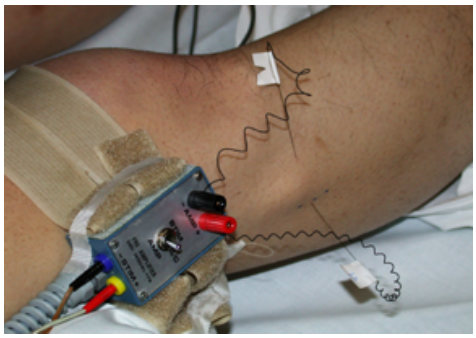

Figure 4: Tungsten electrode inserted into fibular head with separate subcutaneous reference electrode.
Image courtesy of PhD Supervisor, A/Prof Elisabeth Lambert

The nerve signals are small and thus require to be amplified (up to 50, 000 times) via a headstage. This can pre-amplify the signal up to 100 times and is located close to the recording site, ensuring electrical noise from external leads are not picked up[257]. The main amplifier then does the further amplification and filtering via a bandpass filter of 700 to 2000Hz. This device allows only frequencies of the specified ranges to pass and rejects those frequencies outside the range. The filtered neural signal is then rectified and displayed as a mean voltage neurogram as an integrated signal (time constant 100mlliseconds)[257].

Simultaneously, blood pressure measurements are taken from a finger cuff (Finometer; Finapres Medical System, Amsterdam, the Netherlands) as well as heart rate measurements via an electrocardiograph (ECG). The blood pressure, ECG and MSNA measurements are then digitalized with a sampling frequency of 1000Hz (PowerLab recording system, model ML 785/8SP, ADI Instruments) [256]. When a suitable nerve-recording position is attained via identification of spontaneous sympathetic "bursts" both visually and acoustically, resting measurements are recorded over a period of at least 15 minutes [256]. LabChart software (ADInstruments, Sydney, Australia) is then able to extract the beat-to-beat values for MSNA, as well as for R-R interval, systolic and diastolic pressure (Figure 5) .

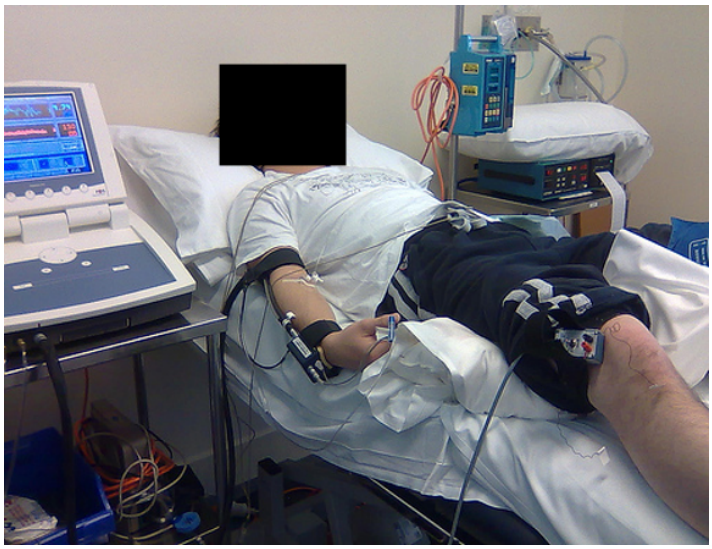

Figure 5: Set up of microneurography equipment and computer software.
Image courtesy of PhD Supervisor, A/Prof Elisabeth Lambert.

Analysis of microneurographic recordings to quantify sympathetic activity

The MSNA is then manually calculated for each participant, ideally by the investigator conducting the MSNA. Results are expressed as burst frequency (number of visible bursts per one minute), burst incidence (number of visible bursts per 100 heartbeats), burst strength (amplitude of the largest burst defined as 100 with all other bursts representing a percentage of the largest one) and median burst amplitude (percentage). Total MSNA activity is calculated by multiplying the mean burst amplitude per min by burst rate, expressed as either units per minute or units per 100 heartbeats[258]**.** Traditionally, articles will cite findings from burst frequency and burst incidence. As burst amplitude is not only determined by the intensity of burst, but also by the proximity of the electrode tip to the group of active nerve fibres, burst activity and or the median amplitude are often not comparable across subjects[257]. The number of bursts per minute at rest is usually between one to sixty. However the median values in healthy individuals is between 20-30.[259]

Strengths and Limitations of microneurography

Microneurography enables the direct measurement of efferent postganglionic sympathetic nerve firing to skeletal muscle vasculature, which yields a measure of MSNA [111] without the use of anaesthetic agents and no evidence of permanent nerve

damage. Microneurography is considered the gold standard for assessing sympathetic nervous system activity [260]. The main advantage of microneurography is that it is highly reproducible in an individual in the short-term and long-term [111, 259]. Hence MSNA obtained on one occasion will be similar to that obtained many months or years later[110]. The exception to this is that given MSNA increases with aging, there should not be too long a delay in measurements, especially if the patient has reached over sixty [115].

However, microneurography is a highly specialized skill and time consuming for both the microneurographer and the participant. It can take a significant period of time to locate the most suitable position for the electrode insertion, relying on visual and auditory cues, and often the necessity of minute adjustments of the electrode by the microneurographer[246]. Additionally, this process can be uncomfortable for the participant due to the invasiveness of the procedure. These limitations prevent its use for routine clinical diagnostic purposes, being confined to a laboratory setting with only highly trained scientists capable of performing the procedure.

Additionally, microneurography provides assessment only of the region innervated, thus the MSNA findings are limited to skeletal muscle and cannot be generalized to other vascular beds. However, not-withstanding this limitation, it is generally accepted that MSNA is the best representative index of the whole-body or the total amount of sympathetic vasoconstrictor activity. As previously described, the rationale for this comes from previous reports where in young men, MSNA strongly correlated with whole-body norepinephrine spillover.[261]

Multi-unit MSNA analysis is further limited in cardiac conditions such as arrhythmias[262]. The low diastolic blood pressure induced by these conditions creates a larger and longer multi-unit burst[263], often followed by prolonged periods of sympathetic inhibition. Thus, in these settings, underestimation of true sympathetic activity may occur. Additionally, heart failure creates extreme sympatho-excitation. For example, in healthy individuals at rest it is common to see 30 to 50 bursts per minute compared to in heart failure individuals where levels can increase up to 90 to 100 bursts per minute [128]. Therefore, in human studies, pre-existing heart failure or arrhythmia are considered exclusion criteria when evaluating sympathetic outflow with this technique.

Additionally, MSNA cannot be used for clinical diagnostic purposes, given there is a large inter-individual variability and there can be between a seven to ten fold variation in healthy people[110]. Furthermore, despite the fact that blood pressure and MSNA are tightly regulated via the baroreflex, MSNA can still vary greatly among individuals and may not be related to blood pressure. For example, for the same measure of blood pressure, two different people can have two very different MSNA levels. Studies aimed at focusing on these inter-individual differences have demonstrated that other physiological mechanisms may come into play. Charkoudian et al [264] highlighted that in young men with high MSNA and high vascular resistance they had a lower cardiac output, thought to act as a balancing mechanism for the higher vascular tone, however, overall more work is deemed needed in this area[110].

Assessment of the sympathetic nervous system through neurotransmitter release

Initially, urine catecholamines were used to measure sympathetic activity which later was superseded by the advent of plasma norepinephrine, a known sympathetic neurotransmitter, enabling a more convenient method of measuring short-term sympathetic changes [265]. In the 1980s, the number of MSNA bursts was found to positively correlate with the concentration of plasma norepinephrine [266]. Later, Esler et al in Melbourne, were able to demonstrate that norepinephrine release is in parallel between the sympathetic output to the muscles, heart [267] and kidney[261]. However, circulating norepinephrine measurements are overall limited as they represent only a small percentage of the neurotransmitter release from nerve terminals, are subject to modulation at the pre-synaptic level, and once released, circulating levels are governed by the tissue clearance, metabolism and uptake from the circulation. For example, an increase in catecholamines may be reflective of either a true increase in release of norepinephrine or may be due to a reduction in reuptake or a decrease in metabolism. Furthermore, a combination of all three variables is possible[110]. Thus, plasma levels released centrally are not accurately reflected in peripheral measurements. Additionally, further studies demonstrated that plasma norepinephrine was not equally as sensitive and reproducible as compared with microneurography[268].

To overcome these issues, Esler et al[269] used the norepinephrine radiolabelled method to measure "spillover" of norepinephrine from specific regional circulations. This method utilized intravenous infusion of titrated norepinephrine, enabling tissue clearance to be subtracted from plasma values, leaving the remainder level as a marker of norepinephrine spillover. In steady-state conditions, this spillover concentration was considered to mirror the secretion of norepinephrine from sympathetic terminals. This technique permitted the assessment of norepinephrine from target organs such as the cardiac and renal areas, avoiding the issues of tissue clearance confounding plasma levels[269]. The benefit with this technique is that it can access those regions not accessible by percutaneous microneurography. However, whilst allowing a direct measurement of regional sympathetic activity, this approach is clearly complex, invasive and is restricted to the laboratory setting and hence the reason this method was not used for chapters three and four.

Assessment of the Baroreflex

Baroreflex sensitivity is the measure used to express the baroreflex function and essentially evaluates the responsiveness or gain in the baroreflex during changes in arterial blood pressure [270]. The sympathetic portion of the baroreflex is assessed through changes in the MSNA and the parasympathetic portion is assessed through changes in the cardiac efferent arm. Both measures are useful to reflect the overall baroreflex control[270]. Within individuals, it was shown that no correlation existed between the cardiac and sympathetic arms of the baroreflex mechanism, thus using only one measure may not necessarily provide enough information about the baroreflex and conclusions should not be made on one measure alone[270].

Computer-based technology has enabled the assessment of the cardiac baroreceptor. This cardiac baroreflex is defined as the changes in the interbeat intervals or the R-R interval measured in milliseconds (ms), per unit change in blood pressure[271] and gives an assessment of the efferent vagal activity of the heart. Using an example to demonstrate this concept, if blood pressure rises by 20mmHg, this will activate the baroreflex to increase the R-R interval. For example, if this was by 100ms, then the sensitivity of the baroreflex would be 100ms/ 20 mmHg = 5ms/mmHg. It is important to highlight that this measure focuses on the sinoatrial node responses rather than the

blood pressure buffering capacity of the baroreflex. Importantly, this measure cannot describe the way in which the R-R interval changes. In the example above, the rise in R-R interval by 100ms is usually due to an increase in parasympathetic firing, but it could also be due to a decrease in sympathetic firing or a combination of both[271]. The benefit of this technique is that it is simple to carry out, non-invasive and associated with low cost[149].

In chapters three and four the baroreflex slope (ms/mmHg) and baroreflex effectiveness index (BEI) were determined using the sequence method of Parati [272] as we had simultaneous blood pressure measurements from the finger cuff (Finometer; Finapres Medical System, Amsterdam, the Netherlands) and heart rate via the ECG recordings for each participant whilst their MSNA was being assessed. This method relies on identifying three or more cardiac cycles for which each successive rise or fall in systolic blood pressure is followed by a successive lengthening or shortening of the R-R interval. The threshold values set for including the systolic blood pressure changes are 1mmHg and for the R-R interval changes it is 6ms[272]. Three or more sequences were required for analysis. The sensitivity of the baroreflex was calculated by computing the slope of the regression line which reflects the changes between systolic blood pressure and the R-R interval. This was calculated for each validated sequence and averaged over 15 minutes for the resting recording. The advantages of the sequence method are that the computation analysis allows a standardized approach which eliminates intra- and inter-individual variability and by assessing changes in blood pressure whether it be an increase or decrease, the flexibility of the baroreceptor is taken into account.

Other methodologies for assessing baroreflex function

At present, the currently accepted 'gold standard' method for assessing baroreflex function is the 'Modified Oxford Method', that involves the infusion of vasoactive drugs to alter blood pressure. Initially described in 1969, the original 'Oxford Method' consisted of injecting intravenous angiotensin, into participants with and without hypertension, to transiently increase arterial pressure[273]. The reflex decrease in heart rate was then measured to determine baroreflex sensitivity[273]. Successive investigators used agents such as phenylephrine or sodium nitroprusside to measure both baroreflex sensitivity and MSNA[274] which was later accepted as the gold standard technique for

evaluating baroreflex sensitivity[275]. This technique is however limited by its invasive nature. Furthermore, nitroprusside may inhibit the central nuclei which in turn could reduce sympathetic outflow[276], as well as attenuate blood vessel responsiveness to MSNA[277]. Importantly, injection of these vasoactive drugs may alter autonomic responses to such a degree in which profound hypotension ensues [278]. As such, studies reported in chapter three and four focused on using the sequence method of Parati given this utilizes a relatively non-invasive approach, as aforementioned, and was considered more suitable for our study population.

Heart Rate Variability

Importance of heart rate variability

Analysis of heart rate variability (HRV) has been used as a non-invasive tool to assess the autonomic control of the heart for over thirty years now [279]. One of the pioneering studies was in 1965, by Hon and Le who discovered that foetal distress was preceded by changes in the intervals between each successive heartbeat, before any changes in the heart rate were detected[280]. It was not until the 1980s when studies demonstrated the clinical significance of HRV as an independent predictor of mortality after a myocardial infarction [281, 282]. Analysing HRV may thus provide understanding about the variation of cardiac vagal and sympathetic nerve activities.

Measuring heart rate variability

Essentially, HRV is based on the physiological principle that the time interval between each heartbeat will vary and is considered a quantifiable marker of autonomic activity[283]. Measurements of HRV are best derived from short term (five minutes) or longer-term (24 to 48 hours) ECG recordings, however, can also be determined from blood pressure monitoring and from a photoplethysmograph. An ECG recording is considered superior given analysing the heartbeats is clearer and those heartbeats not arising from the sinoatrial node can be easily excluded[283]. Therefore, in chapter four, we utilized the card(X)plore (Meditech Ltd, Budapest, Hungary), a combined ECG Holter and ABPM recorder system based on the ABPM-04 (Meditech Ltd) ABPM monitor which has been validated by the Association for the Advancement of Medical

Instrumentation and the British Hypertension Society[284]. This battery-operated device requires very little training to use and is able to provide a fully automated report via the CardioVisions software, making it ideal for both research and clinical use. The use of the card(X)plore device is further able to provide information on the concept of sodium sensitivity [285] which was important for the study presented in chapter four, in addition to measuring heart rate variability. Participants for the study presented in chapter four

On the ECG waveform, the QRS complexes are first detected. From this the R-R interval is then determined by calculating the time between successive R points, R being the peak of the QRS complex. Reporting the analysis of HRV using ECG replaces RR interval with normal-to-normal or NN intervals, signifying that the analysed beats are normal beats, representative of those beats arising from sinoatrial depolarization[283]. At present, the two main methods of quantifying HRV are the time domain (statistical and geometrical) and frequency domain measurements (power spectral density)[283] .

Time domain statistical methods is based on the NN intervals and calculates variables from the analysis of the total ECG recording or from a smaller segment. Commonly reported variables include the standard deviation of NN intervals (SDNN). This is calculated from a 24 hour recording and is representative of total variability, reflecting all the cyclic components for variability [283] in different physiological states such as sleep, wake and activity[286]. A shorter period, usually of five minutes, is depicted by the variable standard deviation of the average of NN intervals (SDANN) and estimates the heart rate changes due to cycles longer than five minutes. The SDNN index is the mean of SDANN and thus measures variability of cycles usually shorter than five minutes[283]. The square root of the mean of the squares of the successive differences between adjacent NN intervals (RMSSD) is associated with rapid changes in heart rate over a short time period and is associated with the vagus- mediated component of HRV[286]. Other variables include the number of interval differences of successive NN intervals greater than 50 milliseconds (NN50) and the proportion derived by dividing NN50 by the total number of NN intervals (pNN50)[283]. The popularity in utilizing HRV in this way lies in its ease in deriving this measure, with ready access by automated devices enabling its use in clinical practice and research studies [287]

Endothelial Function Assessment: Pulse Amplitude Tonometry

Assessment of endothelial function with EndoPAT device

Whilst there are a number of methods available to assess endothelial vasodilator function, for chapters three and four, endothelial function was assessed using the EndoPAT 2000 (Itamar Medical Ltd, Caesarea, Israel) device. This is a rapid, operator-independent and non-invasive, FDA approved device, capable of measuring endothelium–dependent dilatation in response to reactive hyperaemia[288]. Specifically, it measures arterial tone changes in the peripheral arterial vasculature, which is where a significant proportion of sympathetic vasoconstrictor activity occurs [289].

This device relies on technology that comprises of plethysmographic bio-sensors placed at the fingertip to measure the pulse wave amplitude (PWA) [289] providing an assessment of Peripheral Arterial Tone (PAT). These bio-sensors are covered by an outer probe and provide uniform pressure approaching sub-diastolic levels to the fingertips. This application of pressure is advantageous as it prevents any distal venous pooling which in turn prevents a veno-arteriolar vasoconstriction reflex. Furthermore, by placing the PAT bio-sensor on the fingertip, there is reduction in movement artefact [289].

Methodological advantages of using the EndoPAT device

The gold standard method for assessment of endothelial function is to assess coronary arteries[290]. However, as endothelial dysfunction is not restricted solely to coronary arteries, with additional limitations of the invasiveness of this procedure, cardiac catheterization as an assessment of the peripheral vasculature is not suitable. Development of non-invasive techniques to assess endothelial function thus became important.

As arterial blood flow increases, the shear stress of that artery increases. Physiologically, the impact of shear stress is minimized by increasing the diameter of the artery. Thus, flow-mediated dilatation (FMD), characterized by arterial

vasodilatation stimulated after an increase in blood flow and internal-wall shear stress[291], is essential for arteries to remain intact. Moreover, as FMD is in turn dependent on an intact endothelial lining [291], any underlying endothelial dysfunction will prevent FMD from occurring effectively. Thus, in conditions such as diabetes or other vascular disease states which may lead to a compromised endothelial system, FMD is considered an important indicator of underlying endothelial function[161].

Currently, brachial ultrasound measuring changes in the brachial artery diameter in response to reactive hyperaemia, is considered the gold standard non-invasive method to assess flow-mediated dilatation (FMD). In a seminal paper, the use of high-resolution ultrasound was employed as a non-invasive alternative to invasive angiography to determine the underlying endothelial function in asymptomatic individuals at high risk of atherosclerosis[292]. Subsequent studies [293,294] suggested that FMD, as described earlier [292], was an endothelium-dependent and nitric oxide dependent marker of endothelial function. Apart from being non-invasive, FMD is able to predict cardiovascular events in asymptomatic people with either a high risk of cardiovascular disease or those with already established cardiovascular disease, which is comparable to the use of traditional risk factors [295]. However, this methodology requires expensive high-resolution sonography equipment. Further training of sonographers is required before they are specialized enough in being able to use this equipment. Therefore, the high cost and need for highly skilled sonographers to be available limits its practicality for either research based or clinical based use[296].

Unlike FMD as assessed through ultrasound, the EndoPAT device is operator-independent. Therefore, it does not rely on highly skilled technicians being available. Furthermore, the analysis of the recordings is predominantly automated using pre-specified calculations from the manufacturer. Moreover, abnormal PAT results have been shown to correlate with the 'gold standard' coronary microvascular endothelial dysfunction, as assessed by measuring coronary blood flow from cardiac catherization in response to acetylcholine[297] as well as to FMD [298]. Importantly, PAT derived measures of endothelial function have been demonstrated to have similar predictive capabilities as FMD [288]. In a longitudinal study, EndoPAT was able to predict cardiovascular adverse events, consisting of cardiac death, myocardial infarction,

revascularization or hospitalization due to a cardiac cause, over a seven-year follow up period, beyond the Framingham Risk Score[288]. For these reasons, EndoPAT is being more commonly used alternative to study endothelium-dependent dilatation and formed the basis of its use for studies presented in chapters three and four.

EndoPAT protocol

The protocol implemented in chapters three and four is described below. All participants were tested between the hours of midday to 1400hours. Participants were advised to eat a light breakfast and have completed their meal by 0600 hours. This was to ensure at least a six-hour fasting period was provided. Participants were advised to abstain from caffeine, smoking and significant exercise such as running the 24 hours prior so as not to affect the vascular tone[299]. Prior to testing, participants were rested in the supine position with their arms resting on arm supports for 20 minutes in a quiet, temperature-controlled (21–24◦ C) room with reduced lighting to ensure participants were in a cardiovascular steady-state prior to formal testing[299]. Participants were requested not to move or talk during the testing period. The EndoPAT probe was placed on each index finger into the designated areas in the arm supports to record peripheral arterial tone (PAT) (Figure 6) [256]. Care was taken to ensure these probes were free from touching any other object.

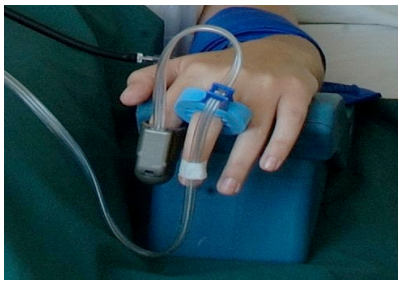

Figure 6: Finger PAT probes and arm supports used during EndoPAT testing.
Image courtesy of PhD Supervisor A/Prof Elisabeth Lambert.

The control arm would not be occluded during the study however blood pressure was measured from this arm. The contra-lateral arm or the test arm had a blood pressure cuff applied which would later be used as below. Once the patient was settled, the EndoPAT 2000 software on the computer was opened and a patient file was created recording the participant study identification number. Measurements were obtained

during three phases of the test arm. Firstly, measurements were recorded for five minutes at baseline. This was then followed by the arterial occlusion phase where measurements were recorded for five minutes after inflating the cuff to supra systolic blood pressures (60mmHg above systolic blood pressure or 200mmHg). Finally, measurements were recorded for a further five to ten minutes in the post-occlusion phase after releasing the cuff to induce the reactive flow-mediated hyperaemic response (Figure 7) [256]. All PAT probes used were then discarded appropriately.

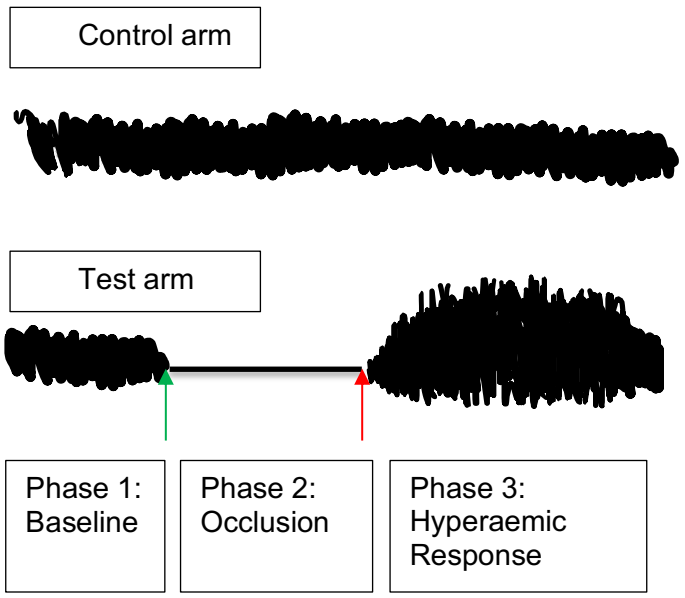

Figure 7: Schematic representation of EndoPAT recordings.
The black shading represents blood flow. The green arrow represents the start of the cuff occlusion and the red arrow represents the release of the cuff.

To analyse the results, this was performed automatically by the EndoPAT software with the occlusion period highlighted in blue and the test results displayed as the reactive hyperaemic index, described in further detail below. The PAT ratio was calculated using the calculation used to assess endothelial function in the Framingham Heart Study [256, 300].

Reactive hyperaemia scores

The reactive hyperaemia index (RHI) is based on measuring nitric-oxide dependent changes in arterial tone[301] and thus reflects the bio-availability of nitric-oxide[301]. This is

calculated automatically by the EndoPAT software firstly by quantifying the hyperaemic response as a ratio, which compares the amount of blood volume that can reach the periphery pre and post occlusion, as follows:

$$\frac{ratio\ of\ occluded\ (test)\ arm\ mean\ PWA\ \boldsymbol{post\ occlusion}\ to\ the\ mean\ PWA\ at\ \boldsymbol{baseline}}{ratio\ of\ non{-}occluded\ (control)\ arm\ mean\ PWA\ \boldsymbol{post\ occulsion}\ to\ the\ mean\ PWA\ at\ \boldsymbol{baseline}}$$

The ratio is then further multiplied by a pre-set baseline correction factor (Itamar Medical, Israel). The above calculation allows for any changes in systemic vascular changes during testing to be accounted for. An inverse association exists between RHI scores and cardiovascular disease risk factors[300]. The RHI values of <1.67 indicate poor endothelial function whereas levels > 2 are indicative of good endothelial function [297].

Augmentation index

The EndoPAT device is able to give the additional measure of augmentation index (AI) from the same dataset which measures arterial stiffness. This provides further information on underlying vascular health given the RHI and AI are considered functionally distinct[302]. The AI is automatically calculated from the analysis of the pulse waveform that arises from the PAT signals recorded during the baseline measurements. In general, the lower the value, the more elastic the artery. Hence higher AI values, which are more reflective of arterial stiffness, are associated with higher cardiovascular related morbidity and mortality[303].

The most commonly accepted 'gold standard' method of evaluating arterial stiffness is obtained, using tonometry, the measures of carotid artery to the femoral artery pulse wave velocity (cfPWV)[304]. Automated devices are available such as the most commonly used Complior System (Colson, Les Lilas, France). The cfPWV method corresponds to the propagative model of the arterial system and has been shown to independently predict cardiovascular events [304]. This method however requires applying pressure to the carotid artery which is of concern if individuals have underlying plaque disease. Additionally, in those individuals with diabetes, obesity and the metabolic syndrome, the femoral pressure waveforms may be difficult to obtain and results may therefore be inaccurate [304]. Therefore, based on these limitations, we determined the augmentation

index from EndoPAT. Additionally, the AI derived measures from EndoPAT have been shown to correlate with other methods of estimating AI[302].

Endothelial Function Assessment: Microparticles

Currently, it is possible to measure microparticles in plasma samples and thus evaluate endothelial, platelet, erythrocyte and leucocyte -derived microparticles. However, it is much more difficult to measure microparticles derived from other samples with non-circulating cells such as vascular smooth muscle cells or from other bodily fluids such as urine, saliva, synovial or ascetic fluid[180]. Therefore, for chapter five, microparticles were assessed using plasma.

Preparation of platelet-free plasma

The first step in being able to assess for microparticles, as was required for the study reported in chapter five, is to take care in preparing whole blood into platelet-free plasma. After fasting blood was drawn from participants into sodium citrate tubes (BD vacutainer), which were placed upright, platelet-free plasma was separated from whole blood using a double centrifugation protocol (Thermo Scientific Heraeus Pico 21 centrifuge) at the Austin Health endocrine department laboratory (Figure 8).

We opted to use a double step protocol as a single centrifugation step is considered to less reliable in its ability to remove platelet from plasma[305]. It is important to remove as much platelet from plasma as possible during this step, as later on during the freeze and subsequent thawing stage, described below, any platelets present will continue to release microparticles and in turn will affect the overall results. We followed a protocol previously described[305] as follows: whole blood was first spun at 1500xg for 20 minutes to obtain platelet-rich plasma. The second spin was at a higher speed of 13000xg for two minutes to obtain the supernatant that was removed without disturbance of the buffy coat. The higher speed during the second spin allows up to a 65 % reduction in platelet counts in plasma[306].

Figure 8: Centrifuge used for double spin protocol.
Image taken by Dr Sara Baqar, Endocrine Department, Austin Health 2014.

Aliquots of platelet-free plasma were then placed in Eppendorf tubes and stored at -80˚c immediately. The time from venepuncture to centrifugation was within fifteen minutes and the time to storage in the freezer was within 120 minutes. This is considered a strength of our protocol as a time delay of more than two hours between venepuncture and centrifugation may lead to abnormally higher levels of microparticles perhaps due to ongoing release of microparticles in the tubes after venepuncture [305]. Additionally, the processing and handling each sample was identical to improve interpretability of results.

Freezing for this study was essential, as batch analysis was required, especially given platelet-free plasma samples were later transported on dry ice by World Courier to our Associate Investigator, Dr Lisa Lincz, at the Hunter Haematology Research Group Laboratory, Newcastle, Australia, for further phenotyping and quantification of microparticles by flow cytometry. All platelet-free samples were stored for similar lengths of time and retrieved from the freezer within 12 months of storage. Usually it is after approximately 20 months of freezer storage that the level of microparticles may be less reliable[305]. Whilst it has previously been shown that freezing of samples does not later affect the microparticle assessment [307], there is no formal consensus on the ideal freeze/thawing method[308]. For this study, platelet-free plasma samples were later thawed at 37 ºC immediately prior to analysis which is an accepted approach[308].

Protocol for the analysis of microparticles

Antibody staining of platelet-free plasma for microparticle analysis

Fluorescent antibodies against common cell surface antigens were used to identify microparticle cellular origins. Identification of platelet microparticles helped to confirm that final endothelial microparticle counts were not contaminated with other subtypes. As previously described [309] and, according to guidelines established by the International Society on Thrombosis and Haemostasis Vascular Biology Scientific and Standardization Subcommittee [310], a 10 microliter aliquot of platelet-free plasma was combined with antibodies (Table 1) at room temperature for 30 minutes. Additionally, Annexin V-APC (eBioscience, San Diego, USA) was used to measure phosphatidylserine exposure which was important to determine whether microparticles were from vesicles derived from activated or apoptotic cells.

All assays were diluted to a final volume of 500 microliters in phosphate buffered saline (PBS) (without Ca^{+2} and Mg^{+2}) or calcium-rich binding buffer (for those stained with Annexin V) with the addition of a known quantity of CountBright (Molecular Probes, Eugene, OR, USA) counting beads[310] and 15 micrometre PPAK (D-Phe-Pro-Arg-CMK-HCl) to inhibit clumping. Samples were then diluted in 500 microliters of either PBS or a calcium-rich buffer (if Annexin V) was used[311]. To be able to quantify microparticle counts, counting beads(TruCount, BD Biosciences), larger than the diameter of microparticles, were additionally added to the samples.

Rationale for choice of endothelial markers

The endothelial markers aforementioned (Table 1) were selected for the study in chapter five as they have previously been shown to represent biomarkers of endothelial dysfunction and cardiovascular risk in previous clinical research as described below.

Cell origin marker	Target Antigen	Clone ID	Supplier
Platelet	CD41-PE	Clone PL2-49	BioCytex, Marseille, France
Platelet	CD42b-FITC	Clone HIP1	BD Pharmingen, San Diego, USA
Erythrocyte	CD235a-APC	Clone GA-R2 (HIR2)	BD Pharmingen, San Diego, USA
Endothelial	CD 54-BB515	Clone HA58	BD Horizon, New Jersey, USA
Endothelial	CD105-PE	Clone 1G2	Beckman Coulter, Brea, USA
Endothelial	CD31-PE	Clone WM59	BD Pharmingen, San Diego, USA
Endothelial	CD62e PE-Cy5	Clone 68-5H11	BD Pharmingen, San Diego, USA
Endothelial	CD36	Clone 11H5 conjugated to DyLight-488	

Table 1: Antibodies used to identify cellular origins of microparticles

The Intracellular Adhesion Molecule 1 (ICAM-1), known as CD54, is a transmembrane protein present in low concentrations in endothelial cells, as well as leucocytes, that are upregulated by cytokines. The main role of CD54 is to primarily facilitate adhesion in the leucocyte transendothelial migration process[312]. Of interest for our study, elevated levels have been shown to be present in atherosclerotic lesions [313]. Experimental studies with mice further demonstrated that in addition to plaque formation, CD54 is involved in the progression of plaque lesions[314] and may thus be considered a mediator in atherosclerotic disease progression.

Endoglin, assigned the number CD105 [315], is a transmembrane accessory glycoprotein of the transforming growth factorβ system, expressed in high levels on activated vascular endothelial cells[316]. Mutations in CD105 are associated with the Osler-Weber-Rendu syndrome leading to haemorrhagic telangiectasias [317] demonstrating its importance in vascular homeostasis. Of relevance to our study, CD105 levels are higher

in individuals with type 2 diabetes[189]. Moreover, higher CD105 levels have been associated with plaque haemorrhage and inflammation and as such are considered makers of ensuing coronary thrombotic complications[318].

Of particular interest is CD31 which is also known as Platelet/Endothelial Cell Adhesion Molecule 1 (PECAM-1), capable of functioning as both an adhesive and signalling protein [319]. It is important to note that CD31 occurs on both platelet microparticles as well as endothelial microparticles. However, as CD42b exclusively occurs on platelets, for our study in chapter five, we defined endothelial CD31 microparticles as CD31+/CD42– and platelet microparticles as CD31+/CD42+. It has been previously demonstrated that individuals with type 2 diabetes have higher levels of CD31+/CD42b-[189]. Additionally CD31 is positively associated in atherosclerosis [320]. Higher CD31+/CD42b- endothelial microparticle levels were associated with higher brachial to ankle pulse wave velocity and lower FMD [321]. Therefore CD31+/CD42b- endothelial microparticle levels were identified as an independent risk factor for vascular dysfunction as assessed by brachial artery pulse wave velocity and FMD[321]. The same group went on to demonstrate, in a cross-sectional study, that CD31+/CD42b- endothelial microparticles were found to be the most significant contributor to severe endothelial dysfunction as determined by the measurement of arterial stiffness from brachial to ankle pulse wave velocity. Severe arterial stiffness was defined when the brachial to ankle pulse wave velocity was above the 75th percentile of the results for the study cohort [322]. Despite both these abovementioned studies enrolling participants of Asian ethnicity who had relatively less diabetes duration and co-morbidities than the study cohort in chapter five, CD105 and CD31 were still deemed to be markers of interest given they have been found to be the predominantly expressed endothelial microparticles in individuals with type 2 diabetes[189].

E-selectin, a glycoprotein specific to the endothelial membrane, also known as CD62E, is expressed on vascular endothelial cells after activation predominantly by tumour necrosis factor α[323]. It acts as an adhesion molecule to slow down leucocyte rolling on the endothelium[323] . Elevated levels of CD62E have been observed in individuals with type 2 diabetes[189] and in cardiovascular disease states and are considered to be

associated with higher cardiovascular risk [324]. For example, higher CD62E levels have been associated with carotid atherosclerosis[325]. Of note, in a prospective longitudinal study, higher levels of soluble E-selectin were found to be an independent predictor of cardiovascular events in Japanese individuals with type 2 diabetes without prior cardiovascular disease [326].

Additionally, CD36, a glycoprotein found on a range of cell types such as endothelial cells, platelets and monocytes, strongly involved in lipid metabolism[327], as well as innate immunity, thrombosis and atherosclerosis[328], was included for analysis given its previous demonstration as a biomarker of type 2 diabetes and atherosclerotic plaque instability [329] . Furthermore, CD36 has previously been shown to be one of the 'core' plasma microparticle proteins in people with a high prevalence of cardiovascular disease and diabetes[330] .

Analysis of microparticles by flow cytometry

Flow cytometry, at present, remains the preferred method for the analysis of microparticles [331] as it allows not only enumeration but also identifies the cellular origins of microparticles by the use of the antibodies as described. Additionally, flow cytometry is now becoming widely available in pathology laboratories attached to research-based institutions making it ideal for future research and clinical use. It is highly reproducible, with an inter-assay variability of 7-12% and an intra-assay variability of 2–6%[180]. For the study in chapter five, a FACS Canto Flow Cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) was used as previously described [309] according to established guidelines [310]. The cytometer was calibrated using fluorescent Megamix beads (Biocytex, Marseille, France) of varying sizes (0.5, 0.9 and 3 micrometres in diameter). Prior to formal data analysis, events were collected for 60 seconds at a low flow rate to be able to classify the fluorescent beads before being subsequently analysed using FACS Diva software (BD Biosciences). This was essential to be able to adequately detect forward scatter resolution as well as being able to determine the lower microparticle detection limits. These were set according to the manufacturer's instructions. We allowed detection of the 0.5 micrometre beads (median level) and an upper limit was set using the 0.9 micrometre beads to detect microparticles ranging from 0.5 – 1.0 micrometres. This method provides intra-instrument (coefficient

of variation < 10%) and inter-instrument (coefficient of variation < 12%)reproducibility [332].

To calculate the number of microparticles for each microliter of platelet-free plasma, the following formula was used:

$$\frac{Microparticles}{microliter} = \left(\frac{microparticle\ count}{bead\ count}\right) x \left(\frac{total\ number\ of\ beads}{test\ volume}\right)$$

Limitations of flow cytometry

Whilst being the most commonly used method for detecting microparticles, flow cytometry is poor at detecting smaller microparticles, less than 0.5 micrometres in diameter, with concern that multiple smaller microparticles may be inaccurately considered as a single microparticle. However, the newer generation flow cytometers have the capability of detecting microparticles as small as 0.1 micrometres due to improved resolution[333]. These new-generation cytometers are unfortunately, at present, not yet widely available for research or clinical use however do provide promising abilities in being able to detected microparticle counts more accurately[334]. In addition, as detection relies on a strong bond of the antibodies as the labelling particles with the respective antigen, failure of this would potentially lead to under-detection[180]. On a practical level, in order for flow cytometry to be useful in a clinical setting, current protocols will most likely need to be standardised to allow minimal difference in results if samples are stored in the freezer for later analysis, as opposed to freshly prepared samples. However, despite these limitations, at this stage, flow cytometry is considered the gold standard for microparticle detection[180].

Chapter 3: Comparison of endothelial function and sympathetic nervous system activity along the glucose continuum in individuals with differing metabolic risk profiles and low dietary sodium intake.

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Abstract

Low sodium intake may trigger sympathetic nervous system (SNS) activation and endothelial dysfunction. Studies have not explored these associations along the glucose continuum. Accordingly, we compared endothelial function and SNS activity in individuals with low sodium intake and differing categories of metabolic risk along the glucose continuum. We hypothesized that low sodium intake is associated with (i) impairment of endothelial function and (ii) higher SNS activity in individuals with higher metabolic risk. In this prospective observational study, participants (n=54) with low sodium intake (single 24-hour urine sodium excretion <150mmol/24h) were categorized based on oral glucose tolerance testing as: normal glucose tolerance (NGT, n=10), impaired glucose tolerance (IGT, n=15), treatment naive type 2 diabetes (T2D-) (n=12) or treated type 2 diabetes (T2D+) (n=17). We assessed endothelial function using pulse amplitude tonometry (PAT) derived reactive hyperemic index and PAT ratio; arterial stiffness via augmentation index; muscle sympathetic nerve activity (MSNA) using microneurography; cardiac baroreflex; heart rate; blood pressure; glycosylated hemoglobin A1c (HbA1c) and lipid profile. Mean (SD) sodium excretion was 110.6 (26) mmol/24hr. Compared to NGT, IGT and T2D-, the T2D+ group had lower MSNA (p=0.005), PAT ratio (p=0.04) and baroreflex sensitivity (p=0.0002) and an augmented heart rate (p=0.02). The T2D+ group had appropriate mean (SD) glycemic (HbA1c 7.2(1.72)%), total cholesterol (4.2(1.0) mmol/L), LDL (2.2(1.0) mmol/L) and blood pressure (systolic136 (13), diastolic 78 (12) (mmHg) control. Individuals with T2D+ have impaired endothelial and baroreflex function, despite low sodium intake, appropriately managed cardio-metabolic risk factors and lower SNS activity, compared to others along the glucose continuum. Whether low sodium intake is associated with modulation of the sympatho-vascular profile in T2D requires further investigation.

Significance of this study

What is already known about this subject?

Reduction in sodium intake is believed to lead to a reduction in blood pressure and has been advocated by public health bodies as a strategy to reduce progression of cardiovascular disease (CVD). However, these recommendations have recently been questioned as emerging observational and experimental evidence has shown the paradoxical association between a low sodium intake and adverse cardiovascular outcomes in individuals with type 2 diabetes, possibly through mechanisms such as increased sympathetic nervous system activity and endothelial dysfunction. However, it is not known whether low sodium intake has effects on cardiovascular health outcomes along the glucose continuum.

What are the new findings?

- The findings demonstrate that compared to other groups along the glucose continuum, individuals with treated type 2 diabetes who had low sodium intake presented with worse endothelial and baroreflex function
- This was despite a better sympathetic profile and being appropriately managed for cardio-metabolic risk factors
- To the best of our knowledge, we are the first to report such observations.

How might these results change the focus of research or clinical practice?

- By determining endothelial function and sympathetic nervous activity in individuals with a varied range of sodium intake (ie low, average and high sodium intake) along the glucose continuum, we may be able to provide an optimal and practical level of sodium intake to optimize cardiovascular health outcomes.
- Future research is required to:
    - To determine if differences in insulin levels or other unmeasured factors such as fitness levels may have played a role in the findings.

- To assess the association between the renin-angiotensin aldosterone system and sodium intake on cardiovascular health outcomes along the glucose continuum.

Introduction

The burden of type 2 diabetes is rising due to its increasing prevalence and the associated higher likelihood of this population acquiring cardiovascular disease [1]. As cardiovascular disease is the leading cause of death worldwide [2], its prevention has become a global health priority[3]. The World Health Organization has urged a targeted strategic approach[3] to reduce this cardiovascular burden. Lessons learnt from randomized controlled trials assessing intensified glycemic control on macrovascular complications [4-6] suggest that in older people with longstanding type 2 diabetes at high risk of cardiovascular disease, intensive glycemic control may not significantly improve cardiovascular health outcomes [4-6].Therefore, optimization of other cardiovascular risk factors, outside of glycemic control, may be as important, or in some individuals more important, for cardiovascular risk reduction [7]. Hypertension is one such modifiable risk factor with a reduction in blood pressure being considered capable of reducing the progression to cardiovascular disease[8]. Blood pressure lowering may be achieved by lowering dietary sodium intake [8]. Not surprisingly, population-wide lowering of sodium intake is recommended [8], with the current sodium intake targets set at less than 2300mg per day (100mmol/24h)[9] .

In spite of these and other preventative strategies, cardiovascular disease continues to rise [2]. Furthermore, our group[10] and others [11] have previously demonstrated that paradoxically, a lower habitual sodium intake was associated with higher all cause and cardiovascular mortality in individuals with diabetes[10, 11]. Studies aimed at underpinning the mechanisms responsible for the association between low sodium intake and poor cardiovascular health have been called for by the Institute of Medicine in order to improve overall cardiovascular health in high-risk individuals [12], such as those with diabetes.

Mechanistic studies exploring the sympathetic nervous system seems plausible given chronic sympathetic activation is associated with end organ damage and an adverse clinical prognosis [13]. Moreover, as autonomic dysfunction can be detected early on in the progression of diabetes [14], preferentially targeting the sympathetic nervous system may be valuable in lowering cardiovascular disease related risk in this population.

Additionally, studies investigating for the presence of underlying endothelial dysfunction, the precursor to atherosclerosis and cardiovascular events [15], are needed, given the potential for earlier intervention strategies. Importantly, as an impaired autonomic reflex is associated with a higher incidence of endothelial dysfunction and mortality in individuals with diabetes [16], targeting both these systems simultaneously may have added benefit in potentially reducing cardio-metabolic risk.

However, whilst sodium is known to have pleotropic effects on sympathetic activity [17] and endothelial function [15], there is paucity in data exploring the effect of dietary sodium intake on these systems along the glucose continuum. Therefore, the aim of this study was to compare sympathetic nervous system activity and endothelial function in the setting of low sodium intake along the glucose continuum in individuals who had either (i) normal glucose tolerance, (ii) impaired glucose tolerance, (iii) treatment naïve, diabetes or (iv) treated type 2 diabetes with established cardio-metabolic risk factors. We hypothesized that low sodium intake is associated with (i) poorer endothelial function and (ii) higher SNS activity in individuals with higher metabolic risk across the glucose continuum.

Methods

Study Design and Recruitment of Participants

In this prospective observational study, individuals who participated in previous studies at two centers (Department of Endocrinology at Austin Health and Human Neurotransmitters Laboratory at the Baker Heart and Diabetes Institute[18]) with similar experimental methodologies, were selected if they fulfilled the following criteria: age 50-75 years, Body Mass Index (BMI) 25-35 kg/m^2, systolic blood pressure <140mmHg and/or diastolic blood pressure <80mmHg, an absence of acute systemic illness. Additionally, individuals with type 2 diabetes with a history of cardiovascular or cerebrovascular disease were included given these individuals represent the population at highest cardiovascular risk. A low dietary sodium intake was defined as 24-hour urinary sodium excretion (24hUNa) measuring <150mmol/24h from a single collection

during the relevant study visit. This cut off was chosen as we have previously demonstrated sodium intake in the lowest tertile (24hUNa <150mmol/24h) was associated with adverse cardiovascular outcomes in people with type 2 diabetes [10]. Given that approximately 90% of sodium intake is excreted by the kidney [19], 24hour urine sodium excretion was chosen to provide more accurate estimates of sodium intake. Furthermore, we have previously demonstrated that a single measurement of 24hUNa is able to predict habitual dietary sodium intake in people with type 2 diabetes [20].

Exclusion criteria comprised a history of peripheral or autonomic neuropathy; cardiovascular, cerebrovascular, liver or thyroid disease; cancer within the past five years; an estimated glomerular filtration rate (eGFR) <45ml/min/1.73m^2, two or more anti-hypertensive agents, hormone replacement therapy and sodium-glucose linked transporter 2 inhibitors.

Participants were categorized into four groups; normal glucose tolerance (NGT), impaired glucose tolerance (IGT), treatment naïve type 2 diabetes (T2D-) and well established, treated type 2 diabetes (T2D+), based on biochemical results (oral glucose tolerance test (OGTT) or glycosylated hemoglobin A1C (HbA1c)) [21] or a known history of type 2 diabetes.

Participants in the NGT, IGT or T2D- groups were not taking any medications or dietary supplements. The individuals with T2D+ were expected to be on antihypertensive agents. Agents that could affect the Renin-Angiotensin-Aldosterone System (RAAS) and sympathetic nervous system such as angiotensin converting enzyme inhibitors, angiotensin II receptor blockers, mineralocorticoid receptor antagonists, centrally acting sympatholytic agents or beta-blockers, required a washout period of six weeks prior to the participant entering the study for which they were enrolled. These agents were substituted so that participants received calcium channel blockers such as lercanidipine or diltiazem as they are less likely to interfere with the sympathetic nervous system and RAAS[22].

The study protocol was approved by the Human Research Ethics Committee at the Austin Health as well the Baker Heart and Diabetes Institute and Alfred Health. All participants provided written informed consent. The previous aforementioned studies were registered as follows: The Department of Endocrinology at Austin Health study was registered with the Australian and New Zealand Clinical Trials Registry, identifier ACTRN12613000127707. The Human Neurotransmitters Laboratory at the Baker Heart and Diabetes Institute[18] study was registered with Clinical trials.gov, identifier NCT00408850.

Endpoints

The primary endpoints were to characterize the differences in (i) endothelial function, using pulse amplitude tonometry (PAT) derived measures of reactive hyperemic index and PAT ratio, and (ii) sympathetic nervous activity, using microneurography to assess muscle sympathetic nerve activity (MSNA), in individuals with a low sodium intake along the glucose continuum. The secondary endpoints were to assess cardiac baroreflex sensitivity, arterial stiffness as measured by the augmentation index, heart rate and blood pressure.

Plasma and 24hour urine biochemical measurements

Participants without an established diagnosis of diabetes had an OGTT using a 75gram glucose load. A diagnosis of normal glycemic control (fasting ≤ 6.0 mmol/L or 2 hour < 7.8 mmol/ L), impaired glucose tolerance (fasting plasma glucose 6.1-6.9 mmol/L or 2 hour plasma glucose 7.8 – 11.0 mmol/L) or diabetes (fasting ≥ 7.0 mmol/L or 2hour ≥ 11.1 mmol/ L) was made according to established criteria [21]. During baseline study visits, fasting blood samples were analyzed for serum haematological parameters, electrolytes, glucose, creatinine, HbA1c, high sensitivity C-reactive protein, and lipid profile. The 24-hour urine specimens were collected by participants the day prior to clinical investigations and returned on the day of testing. Participants were given prior instructions and advice on the importance of accuracy of 24-hour urine collection. The 24-hour urine specimens were analyzed for sodium, potassium and creatinine excretion.

Clinical investigations

All clinical investigations were performed at the Human Neurotransmitter Laboratory, Baker Heart & Diabetes Institute and were carried out without the influence of caffeine, exercise and alcohol 18 to 36 hours prior to eliminate any acute effects on investigations. Participants were assessed in the fasting state in a quiet room with an ambient of temperature 22°C. Anthropometric measurements comprised: body weight using a digital scale with bare feet and light clothing, height and BMI. Supine blood pressure was measured after five minutes of rest using a Dinamap monitor (Model 1846SX, Critikon, Tampa, FL) and recorded as the average of five consecutive measurements.

Assessment of MSNA was performed using microneurography and was performed and analyzed by one investigator (EL). A tungsten microelectrode was inserted directly into the right peroneal nerve at the fibular head [23]. A subcutaneous reference electrode was positioned three centimetres away from the recording site. The nerve signal was amplified (x 50,000), filtered (bandpass: 700 to 2000Hz), and integrated. Simultaneous blood pressure measurements from a finger cuff (Finometer; Finapres Medical System, Amsterdam, the Netherlands) and heart rate via an ECG were made. The blood pressure, ECG and MSNA measurements were digitalized with a sampling frequency of 1000Hz (PowerLab recording system, model ML 785/8SP, ADI Instruments) [23]. When a satisfactory nerve-recording site was attained, resting measurements were recorded over a minimum period of 15 minutes [23]. LabChart software (ADInstruments, Sydney, Australia) extracted the beat-to-beat values for MSNA, as well as for R-R interval, systolic and diastolic pressure. Results are expressed as burst frequency (bursts per minute), and burst incidence (bursts per 100 heartbeats)[18].

Endothelial function was assessed using the EndoPAT 2000 device (Itamar Medical, Israel) that can identify individuals at increased risk of cardiovascular events[24] by measuring the reactive hyperemic index and PAT ratio. Additionally, augmentation index was used to measure arterial stiffness. The pulse amplitude tonometry (PAT) device, was placed on the top of each index finger. Measurements were obtained for five minutes at baseline, followed by five minutes of occlusion of one arm by inflating a cuff on the upper arm to supra systolic blood pressure (60mmHg above systolic blood

pressure or 200mmHg), and then, after releasing the cuff to induce reactive flow-mediated hyperemia, measurements were recorded for a further five to ten minutes [23] which yielded the reactive hyperemic index. The PAT ratio was then calculated with the calculation used to assess endothelial function in the Framingham Heart Study [23,25].

Baroreflex function was determined by measuring the baroreflex slope (ms/mmHg) and baroreflex effectiveness index using the sequence method of Parati[26]. Whilst there is no gold standard technique for the quantification of baroreflex function, the Oxford Method, has been considered as "gold standard" which involves the infusion of vasoactive drugs such as phenylephrine or sodium nitroprusside to alter blood pressure. The reasons for not adopting this technique for the present study was that apart from it being invasive in nature, it may alter autonomic responses during the procedure with an increased risk of profound hypotension[27]. For these reasons, our study focused on using the sequence method of Parati. In brief, in this non-invasive technique, sequences were based upon the identification of three or more cardiac cycles for which there was a consecutive rise or fall in systolic blood pressure followed by consecutive lengthening or shortening of the R-R interval. Three or more sequences were required for analysis. The slope between cardiac interval and systolic blood pressure was calculated for each validated sequence and averaged over 15 minutes for the resting recording.

Statistics and data analysis

A one-way analysis of variance (ANOVA) was conducted to compare the differences in EndoPAT, MSNA, cardiac baroreflex sensitivity and biochemical characteristics in individuals with low sodium intake and the various metabolic risk conditions of NGT, IGT, T2D- and T2D+. A Tukey post-hoc test was performed if there was a statistically significant difference between groups. Data is represented as mean ± standard deviation (SD) as indicated. Statistical analyses were performed using STATA version 14.2. Correlations were assessed using Pearson's correlation coefficient ® for measuring statistical dependence between clinical investigations and biochemical parameters. A P value of <0.05 was regarded as statistically significant.

Results

Participants

Screening of databases for suitability to enter the study identified 99 eligible participants. As 45 participants had incomplete clinical investigations, a total of 54 participants were therefore included in the final analysis. Participants were then categorized accordingly into the following groups: NGT (n = 10), IGT (n = 15), T2D- (n=12) and T2D+ (n = 17) (Supplementary figure 1).

Anthropometric, hemodynamic, and biochemical characteristics of the participants

Participants were similar in age and anthropometric measurements (Table 1). Hemodynamic measurements revealed that the T2D+ group had higher systolic blood pressure (P = 0.04) and heart rate (p=0.002) and demonstrated a trend for higher diastolic blood pressure (P = 0.05) compared to the other groups. The mean (SD) sodium excretion for the entire cohort was 110.6 (26) mmol/24hr with no significant differences between groups (Table 1). As expected, the HDL was lower in the T2D+ group as compared to the other groups (p=0.001). Triglyceride levels were similar across groups (p=0.6). Serum cholesterol and LDL were lower in the T2D+ group compared to all other groups (p=0.0001 and p=0.002 respectively). These differences in total cholesterol and LDL are likely to be reflective of the high percentage of statin use (82%) in the T2D+ cohort (Table 2). The detailed characteristics of the T2D+ group are summarized in Table 2. These participants were predominately on metformin (94%) or sulfonylurea (23%) agents and achieved good glycemic control as evidenced by an HbA1c of 7.2 % (55mmol/mol) (Table 2).

Assessment of sympathetic activity, endothelial and cardiac baroreflex function, along the glucose continuum.

The analysis of MSNA demonstrated that burst incidence was lower in the T2D+ group (p = 0.005) (Figure 1A) as compared to other groups. As shown in the Supplementary Table 1, a Tukey post-hoc test revealed that the T2D+ group had lower burst incidence

as compared to the IGT (p=0.03) and T2D- group (p=0.006) and trended towards having lower burst incidence as compared to the NGT group (p=0.05). The burst frequency however was not statistically different amongst groups (p=0.6) (Supplementary Table 1).

The endothelial function derived measure of PAT ratio (p=0.04)(Figure 1C) was lower in the T2D+ group and the reactive hyperemic index trended towards being lower in the T2D+ group (p=0.08) (Figure 1D). There was no difference in arterial stiffness across the glucose continuum (p=0.12) as assessed by the augmentation index (Supplementary Table 1).

Despite the lower burst incidence, the baroreflex effectiveness index was shown to be lower in the T2D+ group as compared to all the other groups (p=0.0002) (Figure 1B). The baroreflex mean slope however was not different amongst groups (p=0.4) (Supplementary Table 1).

Discussion

Key findings

The most important finding in the current study was that despite adhering to a low sodium diet, being appropriately managed for cardio-metabolic risk factors and demonstrating a better sympathetic profile, individuals with T2D+ presented with worse endothelial and baroreflex function compared to the other individuals along the glucose continuum.

Individuals with treated type 2 diabetes with low sodium intake demonstrated lower sympathetic activity:

In individuals with a low sodium intake, MSNA levels were lower in those with T2D+ as compared to those with NGT, IGT and T2D-. These findings were surprising given we have previously demonstrated that individuals in the early phase of type 2 diabetes had an augmented muscle sympathetic drive characterized by higher MSNA levels[18]. These differences in MSNA levels between the current and previous study[18] are unlikely

to be due to differences in the duration of diabetes as in a separate study, MSNA was demonstrated to be higher in individuals, with established type 2 diabetes [28], with a duration of 22 years, regardless of blood pressure status [29], compared to the non-diabetes population. Sodium intake was however different between the current and previous studies[18, 28]. Sodium intake in the present study was lower, in order to be in line with current dietary recommendations [9], in comparison to the previous studies [18, 29]. Differences in insulin levels or other unmeasured factors such as fitness levels may have played a role.

Overall, the role of sodium on the sympathetic nervous system remains largely unresolved. Previously, in the non-diabetes population, a low sodium intake has been associated with an increase in MSNA [30, 31]. Additionally, in studies utilizing other methodologies to assess sympathetic activity, such as catecholamines, results are again inconsistent with the demonstration of low sodium intake either increasing plasma catecholamines [17] and renal norepinephrine spillover to plasma[32] or being associated with no significant alterations in plasma noradrenaline [33]. The observation from this study, of MSNA being lower in the more established diabetes group as compared to the other groups in the setting of a low sodium intake, adds to the uncertainty surrounding the role of sodium on sympathetic activity and deserves further evaluation. Using a combination of the MSNA and urinary or plasma catecholamines, to assess cause and effect would be prudent to further validate our findings.

Endothelial, baroreflex and heart rate function were impaired in the treated diabetes group in the setting of low sodium intake:

Despite the low sodium intake and the enhanced sympathetic activity as aforementioned, the T2D+ group demonstrated a lower PAT ratio and a trend for a lower reactive hyperemic index, suggestive of endothelial dysfunction[24]. Additionally, a lower baroreflex effectiveness index was observed in the T2D+ group, suggestive of an impaired baroreflex function. This is consistent with previous reports demonstrating a reduced baroreflex function in individuals with type 2 diabetes[18]. However the impaired baroreflex function comes as a surprise as physiologically baroreflex function usually decreases in the presence of sympathetic hyperactivity, yet in the present study, we demonstrated lower sympathetic activity in the T2D+ group. Likewise, whilst

expecting a lower heart rate in the T2D+ group given the lower sympathetic activity, in the present study, heart rate was higher in the T2D+ group which is consistent with previous reports of individuals with higher fasting glucose demonstrating higher resting heart rate [34]. As possible adverse health outcomes may be associated with low sodium intake in individuals with diabetes [10, 11] and given an impaired endothelium [15] and baroreflex [35] as well as an increased heart rate [34] are known to be independent risk factors for increased cardiovascular and all-cause mortality, the observations from the present study are important to further characterize in future studies.

Unraveling the answers to the above mechanistic concerns remains difficult. Previously, Dickinson et al suggested a low sodium diet increased flow-mediated dilatation [36] in the non-diabetes population. However, reactive hyperemic index was unaffected [37]. Similarly, the role of dietary sodium is unclear on baroreflex function. Whilst previous findings have demonstrated reduced baroreflex sensitivity occurred in those with a high sodium intake, based on dietary recall [38], Couruzi et al [39] demonstrated the opposite, in that a high-sodium diet improved spontaneous arterial baroreflex sensitivity. Concerning heart rate, former studies, in individuals without diabetes, have demonstrated similar findings of a low sodium intake leading to higher rate[40] and a high sodium intake was found to lower heart rate in hypertensive [39] individuals. However, other studies have demonstrated no significant increase in heart rate with lowering of sodium intake [30]. Overall, the differences in methodology and assessment of sodium intake as well as paucity of studies evaluating the high-risk diabetes population, need to be addressed in larger cross-sectional or interventional trials to further elucidate the role of sodium on endothelial and baroreflex function.

The impact of pharmacological agents used in the treated type 2 diabetes group on the clinical investigations

As we have previously demonstrated an association between circulating lipid species and central sympathetic outflow[41] and others have shown that statin therapy is capable of reducing sympathetic nervous activity[42], the high use of statin therapy in the T2D+ group, as compared to no use of statin therapy in the other groups, may have contributed to their lowered MSNA levels observed in this study[42]. Additionally, the use of anti-diabetic therapy in the T2D+ group, which was an unavoidable difference, may have

impacted the findings. However, participants in the study by Huggett et al [29] were also mainly treated with metformin yet no differences in MSNA were observed in those with or without metformin therapy. Additionally given and metformin [43] and statin therapy [44] may be associated with improved endothelial function, it would therefore be expected for the T2D+ group to have had better endothelial function parameters than were observed. Thus, overall, in the present study, it is deemed unlikely that statin therapy or metformin use played a significant role in lowering sympathetic activity.

Strengths and limitations of the study

The study being cross-sectional in design limits insight into causality. As sodium intake was required to meet the public health guidelines criteria of less than 2300mg per day (100mmol/24h)[9], many people did not meet these criteria. This is not surprising given the challenges faced in achieving population-wide sodium restriction targets, especially in people with type 2 diabetes [20]. We acknowledge that we did not have an average or a high sodium intake group as a comparator. However, this study was conducted to help understand endothelial function and sympathetic nervous activity in individuals with low sodium intake along the glucose continuum given we have previously demonstrated the association of lower sodium intake and adverse cardiovascular outcomes when 24hUNa excretion was <150mmol/24h in people with type 2 diabetes [10]. Measurement of aldosterone and plasma renin levels to further assess the effect of RAAS and plasma catecholamines to assess sympathetic nervous system activity would have been advantageous. Other important factors that may influence the sympathetic nervous system activity and heart rate, such as fitness levels and fasting and postprandial insulin levels, would have been ideal to have available for all groups. Whilst these measurements were available from our previous study[18] in the NGT, IGT and T2D- group, they were not available in the T2D+ group. These aforementioned factors could be incorporated into future studies in this area.

Whilst autonomic dysfunction can be estimated from indirect measurements such as heart rate variability, we utilized microneurography in the present study, which is considered to be the reference method for assessing sympathetic activity [45]. Only a few other studies, such as those from our group[18] and others[28,29], have utilized this technique

in people with diabetes. However, as we only examined sympathetic outflow to skeletal muscle, we cannot translate these findings to other organs. Utilizing 24hUNa is considered a further strength given it is considered the most reliable method for estimating sodium intake [46]. Endothelial function was measured in the peripheral digits rather than the coronary circulation, however EndoPAT has been shown to provide a high degree of sensitivity and specificity when compared to the assessment of coronary artery endothelial function cardiovascular events[24].

Conclusions

The present study makes noteworthy contributions, demonstrating that despite the lower sympathetic activity in individuals with treated type 2 diabetes, endothelial function, baroreflex function and heart rate were impaired as compared to those with NGT, IGT and treatment naïve type 2 diabetes. This occurred even though individuals with treated type 2 diabetes were appropriately managed for their cardio-metabolic risk factors and importantly, adhered to recommended sodium intake guidelines. Interventional studies aimed at looking at the association between the sympatho-vascular systems are required, if the debate surrounding the role of sodium on cardiovascular health is to be moved forward.

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Author Contributions

SB, EIE and EL conceived the study and selected participants from the Department of Endocrinology, Austin Health and the Human Neurotransmitter Laboratory, Baker IDI databases respectively. SB, NS and YWK collected clinical data. EL performed microneurographic recordings and analyses. SB performed the statistical analyses, wrote and revised the manuscript. SB, EIE and EL interpreted the data. All authors critically read and approved the final content of the manuscript. We thank our study participants, the Endocrinology Department at Austin Health and the Human Neurotransmitter Laboratory, Baker IDI for access to the databases and the respective pathology laboratories for blood and urine biochemical analysis. We are grateful to Ms

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List of abbreviations

24hUNa: 24-hour urinary sodium excretion

BEI: Baroreflex effectiveness index

BMI: Body mass index

eGFR: Estimated glomerular filtration rate

EndoPAT: Endothelial Pulse Amplitude Tonometry

HbA1c: Glycosylated hemoglobin A1c

HDL: High-density lipoprotein

LDL: Low-density lipoprotein

MSNA: Muscle sympathetic nerve activity

OGTT: Oral glucose tolerance test

PAT: Pulse amplitude tonometry

RAAS: Renin-angiotensin-aldosterone system

SD: Standard deviation

SEM: Standard error of the mean

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Tables

Table 1: Demographic and biochemical characteristics between groups (n=54)

Variable	NGT (*n*=10)	IGT (*n*=15)	T2D- (*n*=12)	T2D+ (*n*=17)	p-value [a]	Tukey post hoc analysis
Age, years	59 (8)	57 (5)	58 (3)	63 (8)	0.08	
Male sex	3 (30%)	6 (40%)	3 (25%)	8 (47%)	0.65	
Anthropometric measurements						
Weight, kg	84.3 (15)	91.6 (14)	91.5 (18)	85.6 (11)	0.4	
Body mass index, kg/m^2	29.6 (5)	31.6 (2)	32 (4)	30 (5)	0.3	
Clinic Hemodynamic measurements						
Systolic blood pressure, mmHg	135 (14)	122 (14)	133 (20)	136 (13)	**0.04**	††
Diastolic blood pressure, mmHg	76 (7)	69 (8)	71 (11)	78 (12)	0.05	
Heart Rate, beats per minute	64 (7)	63 (9)	60 (10)	74 (12)	**0.002**	††, ‡
Fasting lipid profile, mmol/L						
Total cholesterol	5.9 (1)	5.3 (0.9)	5.6 (0.7)	4.2 (1)	**0.0001**	***,††, ‡
High-density lipoprotein	1.5 (0.3)	2.5 (1)	1.9 (1)	1.3 (0.3)	**0.001**	*, ††
Low-density lipoprotein	3.8 (0.9)	2.1 (1)	2.9 (1.5)	2.2 (1)	**0.002**	*, ***
Triglyceride	1.4 (0.5)	1.5 (0.8)	1.7 (0.7)	1.7 (0.9)	0.6	
Fasting serum glucose mmol/L	5.0 (0.5)	5.4 (0.5)	5.98 (1.6)	6.9 (1.9)	**0.02**	††
HbA1c % [mmol/mol]				7.2[55] (1.72)		
CRP, mg/ L	2.1 (1.6)	2.3 (1.8)	2.8 (1.6)	1.9 (1)	0.4	
24 hour urine biochemistry excretion						
Sodium, mmol/24hr [range]	111(20) [71-148]	122(23) [73-150]	98(27) [50-133]	110(26) [36-145]	0.1	
Potassium, mmol/24hr [range]	70(21) [36-98.5]	87(24) [49-144]	74(21) [48-118]	72 (30) [35-134]	0.3	

Data is expressed as mean (standard deviation) or number (percentage) as indicated. ([a]) indicates the p values are derived from one-way-ANOVA. Bold values indicate statistical significance. The following symbols indicate Tukey *post-hoc* significance: (*) NGT vs IGT; (**) NGT vs T2D-; (***) NGT vs T2D+; (†) IGT vs T2D-; (††) IGT vs T2D+; (‡) T2D- vs T2D+. Abbreviations: CRP, c-reactive protein; IGT, impaired glucose tolerance; OGTT, oral glucose tolerance test; T2D-, treatment naïve type 2 diabetes; T2D+, treated type 2 diabetes.

Table 2: Detailed demographic characteristics of treated diabetes participants (n=17)

Variable	Total participants
Pre-existing comorbidities	
Hypertension	15 (88%)
Dyslipidaemia	16 (94%(
Ischaemic heart disease	4 (23 %)
Atrial fibrillation	0 (0 %)
Stroke	2 (11.8 %)
Transient ischaemic attack	1 (6%)
Peripheral vascular disease	1 (6 %)
Diabetic microvascular complications	
Diabetic retinopathy	5 (29 %)
Diabetic kidney disease	7 (41 %)
Types of dyslipidaemia medication	
Statin	14 (82 %)
Fibrate	1 (6 %)
Ezetimibe	1 (6 %)
Antihypertensive medication use during trial	
Beta Blocker	2 (11.8%)
Calcium Channel Blocker	5 (29 %)
Thiazide diuretic	1 (6 %)
Types of diabetes medication	
Metformin	16 (94%)
Sulfonylurea	4 (23 %)
Dipeptidyl peptidase 4 inhibitor	1 (6 %)
Glucagon-like peptide 1 receptor agonist	2 (11.8 %)
Thiazoledinedione	0 (0%)
Acarbose	1 (6 %)
Insulin Basal	2 (11.8 %)
Insulin Bolus	2 (11.8 %)
Insulin Premix	2 (11.8 %)
Smoking Status	
Currently not smoking	10 (58 %)
Currently smoking	3 (17.6%)
Previous smoking	5 (29 %)

Data are expressed as number (percentage)

Figures

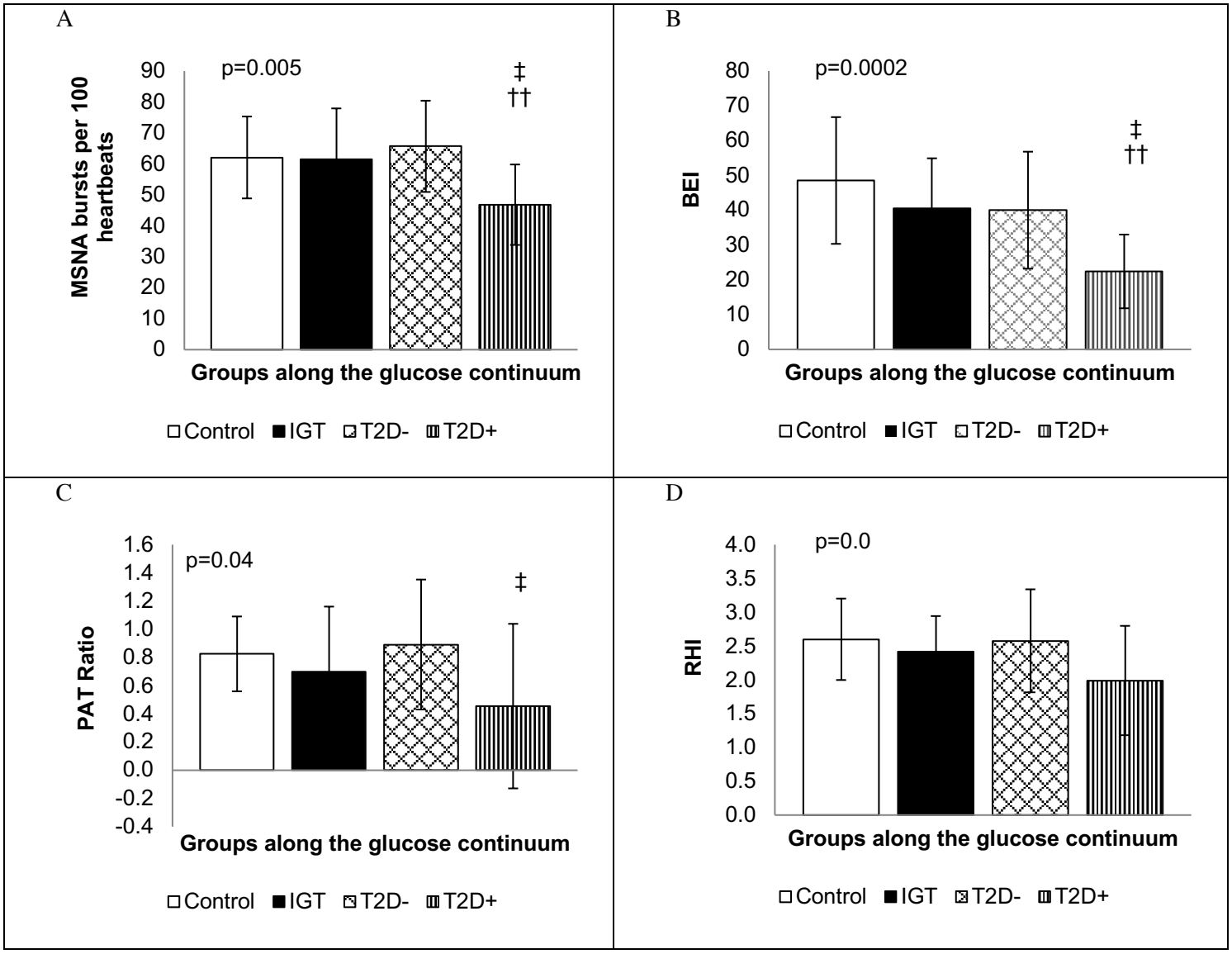
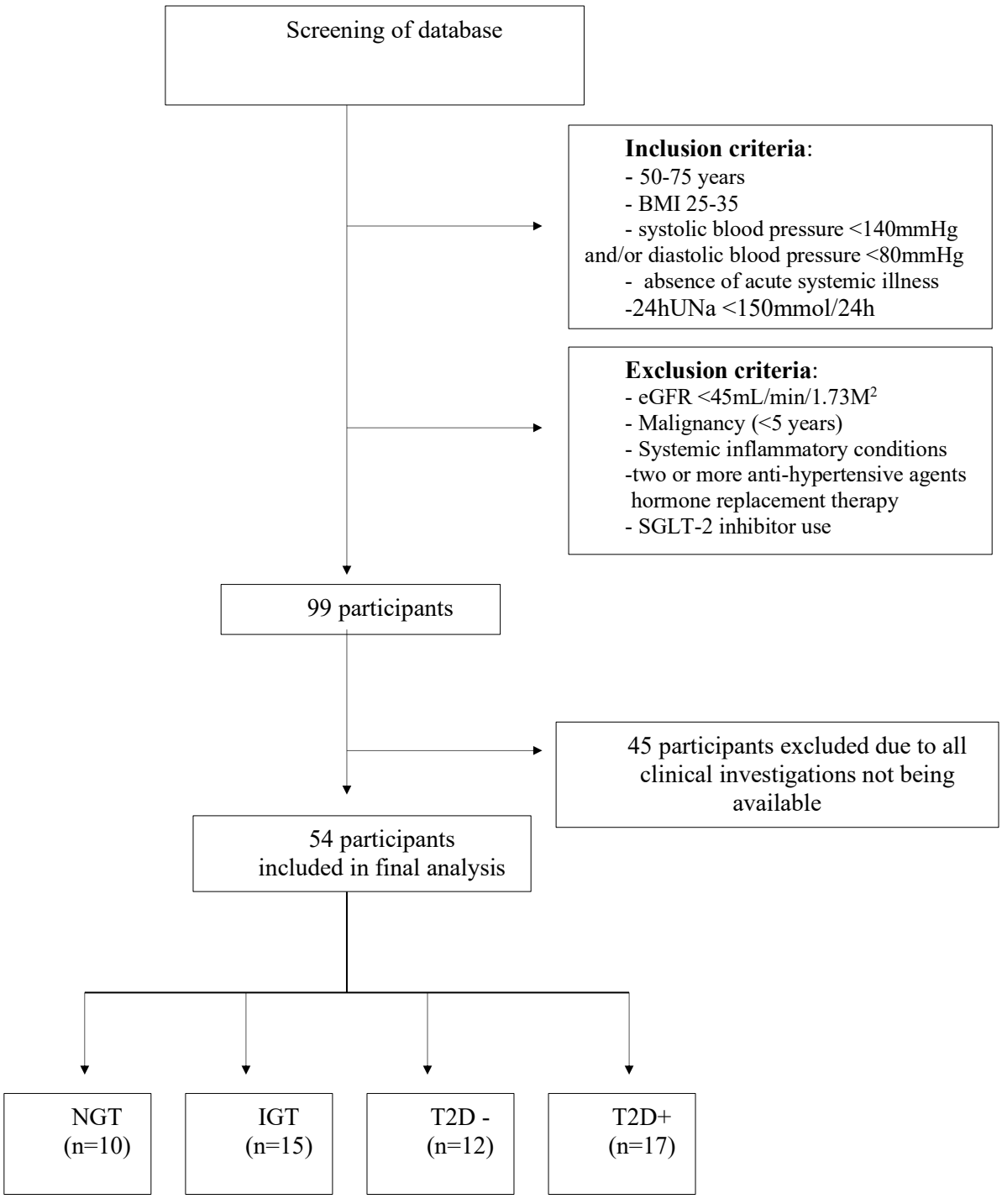


Figure 1: Comparison of sympathetic activity, endothelial function and baroreflex sensitivity between differing metabolic risk categories (n=54)

Data is expressed as the mean (SEM). A: MSNA incidence. B: Baroreflex function. C: Endothelial function PAT ratio. D: Endothelial function RHI. The p values are derived from one-way-ANOVA. Bold values indicate statistical significance. The following symbols indicate Tukey *post-hoc* significance: (*) NGT vs IGT; (**) NGT vs T2D-; (***) NGT vs T2D+; (†) IGT vs T2D-; (††) IGT vs T2D+; (‡)T2D-vs T2D+. Abbreviations: IGT, impaired glucose tolerance; MSNA, muscle sympathetic nerve activity; BEI, Baroreflex Effectiveness Index; RHI, Reactive Hyperemic Index; PAT, Pulse Amplitude Tonometry; T2D-, treatment naïve type 2 diabetes; T2D+, treated type 2 diabetes

Supplementary Information



Supplementary Figure 1: Participant recruitment flowchart

Abbreviations: T2D-, treatment naïve type 2 diabetes; T2D+, treated type 2 diabetes BMI, body mass index; eGFR, estimated glomerular filtration rate; IGT, impaired glucose tolerance; 24hUNa, 24hour urine sodium excretion; SGLT-2, sodium glucose co-transporter 2 inhibitor.

Supplementary Table 1: Comparison of raw values of MSNA, Cardiac Baroreflex and EndoPAT between groups (n=54)

Variable	NGT (*n*=10)	IGT (*n*=15)	T2D- (*n*=12)	T2D+ (*n*=17)	p-value [a]	Tukey post hoc analysis
MSNA						
Bursts per minute	39.6 (10)	36.4 (8.9)	36.96 (10)	34 (10)	0.6	
Bursts per 100 heartbeats	62 (13)	61 (15.7)	65.7 (14)	46.8 (11)	**0.005**	***,††,‡
Units per minute	2041 (513)	1922 (497)	1876.5(667.8	1733.46(667.	0.7	
Units per 100 heartbeat	3193.6 (639)	3249.9 (834)	)	8)	**0.02**	†
Median Amplitude	50 (3.6)	51 (7.5)	3314 (1222)	2360 (758)	0.5	
			47 (11.6)	46.81(8)		
Cardiac baroreflex						
Slope	13.9 (10.8)	16.5 (8.4)	17.7 (9.18)	12.8 (7.9)	0.4	
BEI	48.5 (18.3)	40.5 (14.6)	40 (17.72)	22.4 (9.8)	**0.0002**	**,††, ‡
Endothelial PAT						
RHI	2.6 (0.5)	2.4 (0.8)	2.6 (0.8)	1.99 (0.7)	0.08	
Augmentation Index	29 (19.6)	26 (21.9)	25.8 (13.8)	14 (17.8)	0.15	
PAT Ratio	0.8 (0.3)	0.7 (0.4)	0.9 (0.4)	0.5 (0.5)	**0.04**	‡

Data is expressed as mean (standard deviation). ([a])indicates p values are derived from one-way-ANOVA . Bold values indicate statistical significance. The following symbols indicate Tukey *post-hoc* significance: (*) NGT vs IGT; (**) NGT vs T2D-; (***) NGT vs T2D+; (†) IGT vs T2D-; (††) IGT vs T2D+; (‡) T2D- vs T2D+. Abbreviations: IGT, impaired glucose tolerance; T2D, type 2 diabetes; MSNA, muscle sympathetic nerve activity; BEI, Baroreflex Effectiveness Index; RHI, Reactive Hyperemic Index; PAT, Pulse Amplitude Tonometry, T2D-, treatment naïve type 2 diabetes; T2D+, treated type 2 diabetes.

Chapter 4: Effect of salt supplementation on sympathetic activity and endothelial function in salt sensitive type 2 diabetes

This chapter will be presented as the author-accepted manuscript of a peer-reviewed article published in *The Journal of Clinical Endocrinology & Metabolism*, 2020; 105(4):e1187-1200.

Abstract

Context: Lower sodium intake is paradoxically associated with higher mortality in type 2 diabetes (T2D).

Objective: To determine if sympathetic nervous system (SNS) activation and endothelial dysfunction contribute to these observations, we examined the effect of salt supplementation on these systems in people with T2D with habitual low sodium. We hypothesized that salt supplementation will lower SNS activity and improve endothelial function compared to placebo.

Design: Randomized, double blinded, placebo-controlled, cross-over trial.

Setting: Tertiary referral diabetes outpatients clinic.

Participants: Twenty-two people with T2D with habitual low sodium intake (24hour-urine sodium <150mmol/24h) were included.

Intervention: Salt supplementation (100mmol NaCl/24h) or placebo for three weeks was administered.

Main outcome measures: The primary outcome of SNS activity and endothelial function was assessed as follows: microneurography assessed muscle sympathetic nerve activity (MSNA), pulse Amplitude Tonometry assessed endothelial function via reactive hyperemic index (RHI) and arterial stiffness via augmentation index (AI). Secondary outcomes included: Cardiac baroreflex, serum aldosterone, ambulatory blood pressure monitoring (ABPM), heart rate variability (HRV) and salt-sensitivity.

Results: Compared to placebo, salt supplementation increased MSNA (burst frequency p= 0.047, burst incidence p=0.016) however RHI (p=0.24), AI (p=0.201), ABPM (systolic p=0.09, diastolic p=0.14) and HRV were unaffected. Salt supplementation improved baroreflex (slope p=0.026), lowered aldosterone (p=0.004) and in salt-resistant individuals there was a trend towards improved RHI (p=0.07).

Conclusions: In people with T2D and low habitual sodium intake, salt supplementation increased SNS activity without altering endothelial function or blood pressure but improved baroreflex function, a predictor of cardiac mortality. Salt-resistant individuals trended towards improved endothelial function with salt supplementation.

Precis

In people with type 2 diabetes with low sodium intake, salt supplementation increased sympathetic activity without altering endothelial function or blood pressure and improved baroreflex function.

Introduction

Ischemic heart disease is the leading cause of death [1] and is highly prevalent in those with type 2 diabetes [2]. Modifying risk factors, such as hypertension, helps reduce the progression of cardiovascular disease [1]. Lowering dietary sodium intake lowers blood pressure[3]. Public health bodies therefore recommend low dietary sodium intake (2300mg/ day (100mmol/24h))[4] to reduce blood pressure with the assumption it will improve cardiovascular outcomes. However, our group and others have demonstrated that lower sodium intake is paradoxically associated with higher cardiovascular and all-cause mortality in people with diabetes [5, 6]. Additionally, at the community-level, inverse associations have been demonstrated between low sodium intake and myocardial infarction and mortality [7]. The physiological basis for these observations, however, are not fully understood.

In people without diabetes, low sodium intake may be associated with endothelial dysfunction [8] and activation of the sympathetic nervous system (SNS) [9-11] and renin-angiotensin-aldosterone system (RAAS) [12]. These pleiotropic effects may negate blood pressure lowering benefits of low sodium. Furthermore, chronic sympathetic nervous system activation and endothelial dysfunction independently contribute to cardiovascular disease related morbidity and mortality[9]. The effect of sodium on these systems in people with type 2 diabetes, however, is largely unknown.

The Institute of Medicine has called for randomized control trials [13] given insufficient evidence to suggest either harm or benefit from sodium lowering in high-risk subgroups. To determine optimal sodium intake specific to high-risk subgroups, such as those with type 2 diabetes, underpinning the mechanistic links between sodium intake and cardiovascular outcomes is essential[13]. Individualizing sodium

recommendation targets for people with diabetes will in turn become increasingly important [14].

We therefore conducted this randomized trial in individuals with type 2 diabetes with low habitual sodium intake. We aimed to determine the effects of salt supplementation (100mmol/24h NaCl over three weeks) on cardiovascular health by measuring SNS activity and endothelial function as surrogate cardiovascular endpoints. As low sodium intake may be associated with SNS activation [9-11] and endothelial dysfunction [8], we hypothesized that administering salt supplementation will lower SNS activity and improve endothelial function as compared to placebo.

Research Design and Methods

Study Design and Recruitment of Participants

In this double-blinded, randomized crossover study, participants were recruited from a university teaching hospital's diabetes clinic if they fulfilled the following inclusion criteria: type 2 diabetes diagnosis, age 50-75 years, BMI 25-35, seated systolic blood pressure <140mmHg and diastolic blood pressure <80mmHg and an absence of serious or acute systemic illness. Habitual sodium intake was estimated from the mean of at least two out of three consecutive 24-hour urinary sodium excretions (24hUNa) within the previous 12 months and defined as 24hUNa <150mmol/24h. We have previously reported 24hUNa <150mmol/24h was associated with higher all-cause mortality and was representative of the lowest tertile of 24hUNa for people with type 2 diabetes [6]. Furthermore, whilst public health bodies advocate for low sodium intake (2300mg/day (100mmol/24h))[4], we have previously demonstrated that only 3% of males and 14% of females with type 2 diabetes meet these targets [15]. Thus, 24hUNa <150mmol/24h was deemed appropriate to (i) capture individuals at the highest cardiovascular risk and (ii) to ensure feasibility of recruitment.

Exclusion criteria included documentation of prior history of peripheral or autonomic neuropathy. Investigators further conducted a history and foot examination to exclude peripheral neuropathy and assessed for orthostatic hypotension to exclude autonomic

neuropathy in all eligible participants. Documented liver and thyroid disease; cancer within the past five years; estimated glomerular filtration rate (eGFR) <45mL/min/1.73m^2; congestive cardiac failure and/or coronary artery bypass grafting; hypertension (resting supine blood pressure >140/ 85mmHg or two or more antihypertensives prescribed) and medications, such as hormone replacement therapy and sodium-glucose linked transporter 2 (SGLT-2) inhibitors, were further exclusion criteria.

Participants prescribed antihypertensive agents capable of affecting the RAAS and SNS (ACE inhibitors, angiotensin II receptor blockers (ARB) and beta blockers) and those able to increase sodium excretion (loop or thiazide diuretics) required six weeks washout prior to trial entry. Substitution was with calcium channel blockers lercanidipine or diltiazem given these drugs are less likely to interfere with the SNS and RAAS[16]. The maximum dose required during the washout period was kept stable throughout the trial.

Trial capsules were manufactured by an external pharmacy, which has participated in similar trials[6]. Investigators and participants were blinded to treatment allocation. Salt supplementation (NaCl 100mmol/24h) or matched placebo capsules were taken daily for three weeks. Following a three-week washout period, the protocol was repeated in reverse.

Anthropometric measurements comprised of weight, height and BMI. The 24-hour ambulatory blood pressure and Holter monitor, urinary electrolytes and biochemical analyses were determined at baseline, weeks 3, 6 and 9. Primary endpoints of MSNA and EndoPAT were measured at weeks 3 and 9. The study protocol is outlined in Figure 1.

Participants received dietary education on sources of sodium in commonly consumed foods, a shopping list of high sodium foods and were advised to remain on their usual low sodium diet. After each trial visit, the Accredited Practicing Dietitian conducted a 24-hour dietary recall, to ensure adherence to a low sodium diet. Data was analyzed

using a computerized database of Australian foods (FoodWorks Professional, Xyris Software, Highgate Hill, Australia, version 8).

The study protocol and associated written materials were approved by the Human Research Ethics Committee at Austin Health and at participating sites, Baker Heart and Diabetes Institute and Alfred Health. All participants provided written informed consent. Ethics and Therapeutics Goods Association approval was obtained for slow release NaCl capsules and placebo capsules. The trial was registered with the Australian and New Zealand Clinical Trials Registry (ACTRN12613000127707).

Endpoints

The primary endpoints were the mean changes in SNS activity (determined by muscle sympathetic nerve activity (MSNA)) and endothelial function (measured by pulse amplitude tonometry (PAT)) following salt supplementation compared to placebo. The secondary endpoints were the mean changes in arterial stiffness (measured by augmentation index (AI)), cardiac baroreflex; RAAS activity (assessed by serum aldosterone and plasma renin), blood pressure, heart rate and heart rate variability (HRV). Additionally, salt sensitivity or salt resistant status was assessed for each participant.

Biochemical serum and urinary analyses

Fasting blood samples were analyzed for hematological parameters, electrolytes, glucose, creatinine, HbA1c, plasma renin activity, plasma aldosterone and lipids. Urinary electrolytes (sodium and potassium) were assessed and creatinine excretion measurements verified completeness of the collections. Participants attending our clinic are routinely advised on the accurate method of 24-hour urine collection.

24-hour Ambulatory blood pressure monitor and Holter monitor

The card(X)plore-device, Meditech Ltd, measured 24-hour ambulatory blood pressure (ABPM) and simultaneous electrocardiography (ECG) and HRV. Low sodium intake has been shown to be associated with HRV in healthy adults with responses consistent with sympathetic activation[17]. However, to our knowledge, the association between

sodium intake and HRV in those with diabetes has not been studied. We analyzed the following for HRV: RR intervals; SD of all normal RR Intervals (SDNN); proportion of the number of pairs of successive beats that differ by more than 50 ms (pNN50); HRV triangular index (HRVti); root-mean-square successive difference (rMSDD).

Clinical investigations of microneurography, EndoPAT and baroreflex.

Participants refrained from caffeine (18h), exercise and alcohol (36 h). On the testing day, participants were fasting and assessed in a quiet room in the supine position.

Microneurography, measured MSNA and was performed and analyzed by one investigator (EL) throughout the trial. A tungsten microelectrode was inserted into the right peroneal nerve at the fibular head [18]. The nerve signal was amplified (x 50, 000), filtered (bandpass: 700 to 2000Hz), and integrated. Simultaneous blood pressure measurements from a finger cuff (Finometer; Finapres Medical System, Amsterdam, the Netherlands) and heart rate via an ECG were made. Measurements were digitalized with a sampling frequency of 1000Hz (PowerLab recording system, model ML 785/8SP, ADI Instruments) [18]. When a satisfactory nerve-recording site was attained, resting measurements were recorded for 15 minutes [18]. LabChart software (ADInstruments, Sydney, Australia) extracted beat-to-beat values for MSNA, as well as for R-R interval, systolic and diastolic pressure. The MSNA was manually calculated for each participant. Results were expressed as burst frequency (bursts per minute) and burst incidence (bursts per 100 heartbeats). Total MSNA was calculated by multiplying the mean burst amplitude per minute by burst rate and expressed as either units per minute or units per 100 heartbeats**.**

Pulse amplitude tonometry was assessed using the EndoPAT 2000 device (Itamar Medical, Israel) derived measures of endothelial function through the reactive hyperemic index (RHI) and of arterial stiffness through the augmentation index (AI) [19]. The PAT device was placed on the top of each index finger. Measurements were recorded at baseline, post-occlusion (inflating the cuff to supra systolic blood pressure (60mmHg above systolic blood pressure or 200mmHg)) and after releasing the cuff to

induce reactive flow-mediated hyperemia [18]. The PAT ratio was calculated using the calculation used to assess endothelial function in the Framingham Heart Study [18, 20].

Cardiac baroreflex sensitivity was determined using the sequence method of Parati [21]. This identifies sequences comprised of three or more cardiac cycles for which there is either a consecutive rise in systolic blood pressure followed by a progressive lengthening of the R-R interval (type 1 sequence) or a progressive fall in systolic blood pressure followed by progressive shortening of the R-R interval (type 2 sequence). Three or more sequences were required for analysis. For each sequence, the linear correlation coefficient between the R-R interval and systolic blood pressure was computed and validated when the correlation coefficient was ≥ 0.85. The slope of the regression line was then calculated for each validated sequence and averaged over 15 min for the resting recording. The sensitivity of the baroreflex gain was represented as baroreflex slope (ms/mmHg) and baroreflex effectiveness index (BEI).

Assessment for Salt-Sensitivity

The use of ABPM and dietary sodium intake has previously been utilized for the diagnosis of salt-sensitivity [22]. In the present study, an increase in mean 24 hour arterial blood pressure (MAP) of at least 4mmHg [22] defined salt-sensitivity after salt supplementation. The MAP increment was the difference in the MAP from the ABPM recordings on the final visit of both placebo and salt supplementation periods. Participants who met the MAP increment criteria, were defined salt-sensitive if there was an associated rise in sodium excretion during salt supplementation as compared to placebo.

Statistics and data analysis

Baseline demographics were reported as mean, standard deviation (SD) or as percent frequency as appropriate. The paired Student t test compared the differences between salt and placebo for primary and secondary endpoints. These results are reported as mean, standard error of the mean (SEM). A two-tail probability value of <0.05 was regarded as statistically significant. Statistical analyses were performed using STATA version 14.2. As this was an exploratory study, power calculations for the primary endpoints were determined from previous studies [23, 24]. For this study, using a minimum

of within-group differences of 10 bursts per 100 heartbeats for MSNA (SD of difference 14) required 20 participants and a minimum change in the RHI of 0.2 (SD=0.295) required 22 participants [90% power, a-value of 0.05 (two-tailed)]. Statistical support for the MSNA power calculation is based on a previous study of 11 participants where dietary salt restriction to 80mmol/24h (from 210mmol/24h) increased MSNA from 36 to 45 bursts per minute where the SD of difference was 10 [23]. The EndoPAT power calculations were based on a study that demonstrated for a crossover study, designed to detect changes in RHI by 0.25 and PAT ratio by 0.2, 22 and 19 participants respectively were required [80% power, a-value of 0.05 (two-tailed)][24].

Results

Participants

We screened 1035 people with type 2 diabetes who had 24hUNa measurements for eligibility from June 2013 to June 2016. Sixty people met the 24hUNa criteria. Thirty-two people were excluded as they did not fulfill the inclusion criteria, were not contactable or declined to participate. Twenty-eight participants were randomized. Post randomization, six participants declined and exited before intervention. Thus 22 participants completed the trial (supplementary figure 1)[25].

Baseline characteristics

Table 1 demonstrates participants had a mean (SD) diabetes duration of 13 (9) years. The mean (SD) blood pressure (systolic pressure 133 (9) mmHg and diastolic pressure 70.5 (8) mmHg) and the mean (SD) glycemic control as assessed by a glycosylated hemoglobin A1C (HbA1c) of 7.3% (1.5) or 56.4 mmol/mol (3.6) was considered acceptable.

Biochemical characteristics

Changes in biochemical characteristics following intervention are presented in Table 2. Mean (SD) 24hUNa was 153.95 (61) mmol/24hr which increased after salt supplementation to 205 (63) mmol/24hr. The mean difference in 24hUNa of 51mmol/24h (p=0.001) is a comparable increase to previous studies[10]. There was no

statistically significant difference in the two baseline measurements (weeks zero and six) of 24hUNa (p=0.5) (supplementary figure 2)[26]. Serum aldosterone was reduced after salt supplementation (p=0.004).

Physical characteristics

As shown in Table 2, whilst the ABPM data demonstrated no significant differences in overall mean blood pressure between the intervention phases. A trend for an increase in mean systolic blood pressure was observed (p=0.09). Night-time systolic and diastolic blood pressures were higher post salt supplementation (p=0.04 respectively). Measures of HRV were not altered after salt supplementation.

Clinical measurements

The MSNA burst incidence (Figure 2A, p=0.016) and burst frequency (Figure 2B, p=0.047) increased post salt supplementation compared to placebo. The PAT ratio (Figure 2C, p=0.25) and RHI (Figure 2D, p=0.24) were not altered. An increase in cardiac baroreflex slope (Figure 3A, p= 0.026) and BEI (Figure 3B, p = 0.03) was observed post salt supplementation. Arterial stiffness (Figure 3C, p=0.2) did not change following salt supplementation. Detailed values are provided in supplementary table 1[27].

Effect of salt-sensitive status on hemodynamic and clinical measurements

Eight participants demonstrated an increase in MAP of 4mmHg post salt supplementation as compared to post placebo. One participant failed to have a corresponding increase in 24UNa post salt supplementation. Thus, seven participants were deemed to be salt-sensitive.

Table 3 summarizes the analysis of these salt-sensitive individuals. All measures of ABPM increased significantly post salt supplementation. The MSNA total activity (units per 100 heartbeats) significantly increased post salt supplementation ($p = 0.04$). A trend for an increase in MSNA burst frequency (p=0.08), incidence (p=0.05) was observed. Whilst cardiac baroreflex slope increased following salt supplementation

($p = 0.023$) the BEI was not significantly altered ($p = 0.5$). All measures of endothelial function were unchanged. Individual responses are shown in supplementary figure 3[28]. The mean difference in 24UNa between post placebo and post salt supplementation phases was 60 mmol/24h (p= 0.02) (supplementary figure 4)[29].

Effect of salt-resistant status on hemodynamic and clinical measurements

Of the remaining 14 participants who were categorized as salt-resistant, Table 3 highlights that in these individuals, ABPM measurements did not increase post salt supplementation, moreover there was reduction in diastolic pressure (-3mmHg, p=0.02) and MAP (-3.8 mmHg, p=0.03) and a trend towards a reduction in systolic blood pressure (-6mmHg, p=0.05). Only MSNA total activity (p=0.03) increased post salt supplementation. All measures of endothelial function trended towards an improvement post salt supplementation. Individual responses are shown in supplementary figure 3[28]. The mean difference in 24hUNa post placebo and post salt supplementation was 52.5 mmol/24h (p=0.008) (supplementary figure 4) [29].

Salt-sensitive individuals had higher MSNA activity as compared to salt-resistant individuals

Interestingly, we observed that MSNA total activity levels were lower in the salt-resistant individuals compared to the salt-sensitive individuals (supplementary figure 3B) [28]. We conducted a one-way ANOVA to determine if these observed differences were significant. Total MSNA activity was significantly higher post salt supplementation in salt-sensitive individuals as compared to salt-resistant individuals (p=0.03). Furthermore, the mean difference in total MSNA post salt supplementation was also significantly higher in salt-sensitive individuals (p=0.04).

Discussion

Key Findings

This is the first trial that has assessed the effect of sodium intake on cardiovascular parameters in people with type 2 diabetes. The main findings were that salt supplementation increased sympathetic activity but without evidence of endothelial dysfunction or blood pressure alteration. Moreover, cardiac baroreflex function improved and RAAS activity, as measured by serum aldosterone, was reduced. Salt-resistant individuals demonstrated a trend towards improved endothelial function after salt supplementation. These findings suggest the role of stringent sodium lowering in people with type 2 diabetes, especially those who are salt-resistant, may be unnecessary.

The effect of salt supplementation on sympathetic activity.

Chronic SNS activation is associated with an adverse clinical prognosis [9]. The effect of sodium intake on sympathetic activity is unclear with conflicting results. We demonstrated salt supplementation led to higher MSNA activity as compared to placebo. Experimental studies have demonstrated a high sodium diet increases sympathetic activity by disturbing the balance between the sympatho-excitatory and sympatho-inhibitory influences in the pressor area of the rostral ventrolateral medulla [30]. Conversely, other studies have demonstrated a high sodium intake leads to lower serum norepinephrine and epinephrine [31]. As sodium impacts tissue clearance of catecholamines [23] this confounds the use of catecholamines in assessing SNS responses to sodium intake. Measurement of sympathetic activity via MSNA is a more direct and reliable method of assessing sympathetic outflow in humans with strong intra-individual reproducibility [32], forming the basis for its use in the present study.

We, and others, have previously demonstrated that individuals with type 2 diabetes have higher baseline MSNA levels compared to those without diabetes [33-35]. Although these studies [33-35] were not designed to assess the effect of sodium intake on MSNA, participants in these former studies [33-35] did have a sodium intake that was comparable to the participants in the present study post salt supplementation.

Defining a "normal" range of MSNA is difficult given the large inter-individual variability, [32]. An accepted range is wide, 1 to 60 bursts per minute [32]. In healthy individuals, 30 to 50 bursts per minute is common [36]. Similar levels of MSNA were seen in our study post salt supplementation. In contrast, in heart failure, MSNA levels are around 90 bursts per minute [36]. Thus, in the present study, whilst MSNA levels increased following salt supplementation, they were at levels comparable to those seen in healthy individuals [36] and did not reach levels seen in pathological disease states [36].

Individuals with diabetes and salt-sensitivity may be more susceptible to hypertensive effects of sodium as compared to salt-resistant individuals [37]. Furthermore, as salt-sensitivity itself is capable of increasing the risk of renovascular and cerebrovascular disease, independent of its hypertensive effects[38], it is important to understand the salt-sensitive phenotype in people with diabetes. Whilst we did not observe changes in mean 24hour blood pressure post salt supplementation, after categorizing individuals based on salt-sensitivity, mean 24hour blood pressure increased post salt supplementation in salt-sensitive individuals. In contrast, blood pressure trended towards a decrease in salt-resistant individuals. Salt-sensitive hypertension may explain the higher sympathetic activity we observed in salt-sensitive individuals post salt supplementation as compared to placebo and in turn explain the higher MSNA in salt-sensitive individuals as compared to salt-resistant individuals. Similarly, previous reports demonstrated salt-sensitive hypertension was associated with higher sympathetic activity [39] and high sodium intake increased plasma norepinephrine in salt-sensitive but not salt-resistant individuals [40]. In contrast, others have shown that in people without diabetes, a low sodium intake increased MSNA in both salt-sensitive and salt-resistant states[10].

Overall, given the inconsistencies from the available literature, it is not possible at present to answer, with confidence, whether a low or high sodium intake increases sympathetic activity. Salt-sensitive hypertension should be assessed for in any future trials of this nature given we and others have demonstrated salt-sensitive individuals are more likely to experience the hypertensive effects of sodium intake [37] and in turn higher sympathetic activity[39].

The effect of salt supplementation on endothelial function

Despite the MSNA increase, we did not observe poorer endothelial function following salt supplementation. Interestingly, endothelial function trended towards improvement, with higher RHI and PAT ratio, in salt-resistant individuals as compared to salt-sensitive individuals. Limited studies have assessed the effects of sodium intake on endothelial function [41] with inconsistent conclusions. Our findings differ from other human studies in the non-diabetes population [42], where high sodium intake has been associated with endothelial dysfunction. As systolic blood pressure increased post salt supplementation in the previous study[42], it becomes difficult to differentiate whether the endothelial dysfunction observed was a consequence of increased sodium intake or from increased blood pressure. In contrast, in the present study, mean 24hour blood pressure was not significantly altered. The methodological differences in endothelial function assessment could account for discrepancies. Others [42], used an invasive approach allowing direct measurement of endothelial function, whilst our study utilized pulse amplitude tonometry [19] a non-invasive, reproducible approach, capable of evaluating the presence of endothelial function [19].

Influence of sodium on baroreflex function

The baroreflex is essential for cardiovascular homeostasis. Depressed baroreflex sensitivity (values less than 3.0ms/mmHg) is a significant predictor of cardiac mortality, independent of well-established cardiovascular risk factors [43]. Individuals with type 2 diabetes have reduced baroreflex function [33]. Interestingly, we observed improved cardiac baroreflex function with a mean increase in baroreflex sensitivity of 3.15ms/mmHg post salt supplementation which is above the value associated with significant multivariate risk of cardiac mortality [43]. Previous studies likewise demonstrated a high sodium intake led to improved spontaneous arterial baroreflex sensitivity, as assessed from continuous blood pressure recordings [44]. Furthermore, in healthy adults with salt-resistant blood pressure, seven days of high dietary sodium increased cardiovagal baroreflex sensitivity as assessed using the sequence method [45], a finding and methodological approach which was similar to the present study. Whether this improvement in cardiac baroreflex post salt supplementation translates to longer-term improvement in cardiovascular outcomes requires further analyses given the simultaneous elevation in mean night-time systolic blood pressures we observed.

Importantly however, despite this increase in night-time systolic blood pressure of 4.6mmHg post salt supplementation, previous studies demonstrated an increase in night-time systolic blood pressure of 10mmHg was associated with a 25% increase in myocardial infarction and stroke[46]. On balance, as both baroreflex function[43] and night-time blood pressure[47] are independent predictors of future cardiovascular events, larger interventional trials to evaluate our findings further are needed.

Effect of sodium on RAAS

Sodium lowering leads to compensatory activation of the RAAS [48]. Activation of RAAS increases cardiovascular morbidity and mortality [12] through stimulation of the SNS [49] and endothelial dysfunction [50]. In the present study, we observed reduced serum aldosterone levels following salt supplementation, in keeping with previous findings from our group [51]. We have previously shown that in individuals with diabetes, lower 24hUNa was associated with higher serum aldosterone, both in the presence and in the absence of RAAS blockade [52]. Similarly, plasma aldosterone and renin have been shown to increase following low dietary salt intake as compared to high dietary salt intake [31] [53]. However, despite expecting renin to decrease during salt supplementation [51], we did not observe a statistical reduction in plasma renin in the present study. We have, however, previously reported no associations between renin and urine sodium excretion [52]. Overall in people with diabetes, reducing dietary sodium intake may be associated with RAAS activation and an increased risk of developing aldosterone escape [54]. This is turn may be associated with increased cardiovascular mortality [12]. Additionally, whilst a high sodium intake may potentially increase blood pressure, it may in turn improve endothelial function via down regulation of local tissue RAAS [55].

Study limitations and strengths

The small sample size is the main limitation. Despite high number of participant screening, many did not meet the 24hUNa criteria. Whilst the current study is of short duration, two weeks have been previously demonstrated to be sufficient to assess the effect of dietary sodium restriction on vascular function [41], the SNS[56] and RAAS activity [57]. Nevertheless, longer-term salt supplementation studies are needed to assess for adaptive responses that may have occurred in the acute period. The main strength is the randomized, cross-over design which provides the advantage of each participant

serving as their own control. The use of 24hUNa to estimate dietary sodium intake provides a better estimate in contrast to dietary recall, which underestimates dietary sodium intake by up to 50% [58]. The use of ABPM rather than clinic measurements as compared to other studies[10] is considered advantageous. Furthermore, the use of MSNA and endoPAT as relatively non-invasive methods to assess our outcome measures are considered further strengths.

Future Directions

The present study excluded the use of medications that interfere with the RAAS and SNS to enable understanding of the effects of sodium alone on these systems. In future, concomitant use with these pharmacological agents as well as evaluating the effects of SGLT-2 inhibitors on sympatho-vascular outcomes would be useful. Assessing healthy volunteers as controls subjected to the same study protocol would be advantageous.

Conclusion

This randomized trial is timely given the recent call for mechanistic studies to underpin the role of sodium on cardiovascular health outcomes in high-risk subgroups. We demonstrated that in people with type 2 diabetes with low habitual sodium intake, despite the increase in sympathetic activity, no significant changes in blood pressure or endothelial function were seen. Interestingly, improvements in cardiac baroreflex function and RAAS activity were observed. On balance, our findings suggest that relaxing sodium targets in people with type 2 diabetes may possibly have no overall detrimental effect on the sympatho-vascular system. This may be especially true for salt-resistant individuals. Lowering dietary sodium intake to current general population recommendations in people with type 2 diabetes must therefore be carefully considered as its blood pressure lowering effects on cardiovascular outcomes may benefit only select individuals, such as those with salt-sensitivity. Our study therefore highlights the need of individualized dietary sodium intake targets for those with diabetes. In particular, establishing an individual's salt-sensitivity status may become necessary for future studies in this field and for determining individualized sodium intake targets for optimal cardiovascular outcomes.

Author Contributions

SB, EL, GJ and EIE were involved in the study conception, design and data interpretation. RJM, GL, AXC and CO contributed to the interpretation of the study results. SB and YWK carried out participant recruitment and supervised all clinical visits. EEL performed microneurography and EndoPAT measurements and analysis. SB prepared the food list, which was reviewed by MB. MB conducted the dietary recalls and provided one-on-one dietary education to trial participants using trial education materials. SB performed the statistical analyses and graphical representation of the data. All authors were involved in the drafting and critical editing of this manuscript. EEL and EIE approved the study design and the final content of the manuscript.

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Disclosure Summary

SB, YWK, AXC, CO, RJM, MB and GJ have nothing to declare. EIE is an investigator in separate studies sponsored by Novo Nordisk, Bayer, Sanofi and Mobius Medical. GL and EL are investigators in recent studies sponsored by Medtronic. The laboratory of GL recently received research funding from Medtronic. GL has acted as a consultant for Medtronic and has received honoraria or travel support for presentations from Pfizer, Wyeth Pharmaceuticals, Servier and Medtronic.

Data Availability

All data generated or analyzed during this study are included in this published article

List of abbreviations

24hUCr: 24-hour urinary creatinine excretion

24hUNa: 24-hour urinary sodium excretion

ABPM: Ambulatory blood pressure monitor

AI: Augmentation index

ARB: Angiotensin II receptor blocker

BMI: Body mass index

CCB: Calcium channel blockers

CVD: Cardiovascular disease

eGFR: Estimated glomerular filtration rate

EndoPAT: Endothelial Pulse Amplitude Tonometry

HbA1c: Glycosylated hemoglobin A1c

HDL: High-density lipoprotein

HRV: Heart rate variability

LDL: Low-density lipoprotein

LVH: Left ventricular hypertrophy

MSNA: Muscle sympathetic nerve activity

NHMRC: National Health and Medical Research Council

PAT: Pulse amplitude tonometry

PRA: Plasma renin activity

RAAS: Renin-angiotensin-aldosterone system

RCT: Randomized controlled trial

SD: Standard deviation

SEM: Standard error of the mean

SNS: Sympathetic nervous system

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Tables

Table 1: Baseline Demographic Characteristics

Variable	Total participants (n=22)	95 % Confidence Interval
Age, years (mean, SD)	64 (8)	60 – 67.5
Male sex	10 (45.5%)	
Diabetes duration, years (median, IQR)	13 (9)	9 – 17
Body mass index, kg/m² (mean, SD)	29.9 (5.7)	27 - 32.5
Ambulatory Blood pressure, mmHg (mean, SD)		
Systolic Blood Pressure	133 (9)	129.6 – 137.8
Diastolic Blood Pressure	70.5 (8)	67 – 74
Fasting serum lipid profile, mmol/L (mean, SD)		
Total cholesterol	4 (0.95)	3.7– 4.6
High-density lipoprotein cholesterol	1.4 (0.4)	1.2 – 1.5
Low-density lipoprotein cholesterol	2 (0.9)	1.7 – 2.5
Triglyceride	1.5 (0.9)	1.2 – 1.9
Fasting serum glucose level, mmol/L (mean, SD)	6.9 (2)	5.9 – 7.8
HbA1c % (mean, SD)	7.3 (1.5)	6.6 – 8
HbA1c **mmol/mol (mean (SD)**	56 (3.6)	49-64
C-peptide nmol/L (mean, SD)	0.98 (0.6)	0.7 – 1.2
eGFR, mL/min/1.73m² (CKD-EPI) (mean, SD)	74 (3)	67 – 81
Creatinine μmol/L (mean, SD)	84 (20)	75 – 93
CRP, mg/ L(mean, SD)	2.2 (1.4)	1.5 – 2.8
Renin $\log_{10}$ mIU/L(mean, SD)	1.4 (0.5)	1.2 – 1.6
Aldosterone $\log_{10}$ pmol/ L(mean, SD)	2.5 (0.2)	2.4 – 2.6
Renin: aldosterone ratio $\log_{10}$ (mean, SD)	1.2 (0.4)	1 – 1.4
24 hour urine biochemistry mmol/24h (mean, SD)		
Sodium excretion	134 (49)	112.5– 156
Potassium excretion	71 (26)	59.5 – 83
Creatinine excretion	11 (3.6)	9.6 – 12.8
Pre-existing comorbidities (n (%))		

Hypertension	19 (86.36%)	
Dyslipidaemia	19 (86.36%)	
Ischaemic heart disease	5 (22.73%)	
Atrial fibrillation	1 (4.55%)	
Stroke	2 (9.09%)	
Transient ischaemic attack	1 (4.55%)	
Peripheral vascular disease	1 (4.55%)	
Diabetic microvascular complications (n (%))		
Diabetic retinopathy	6 (27.7%)	
Diabetic neuropathy	0 (0%)	
Diabetic kidney disease	9 (40.91%)	
Gastroparesis	1 (4.55%)	
Types of dyslipidaemia medication (n (%))		
Statin	17 (77.27%)	
Fibrate	1 (4.55%)	
Ezetimibe	1 (4.55%)	
Types of diabetic medication (n (%))		
Metformin	20 (90.91%)	
Sulfonylurea	6 (27.27%)	
Dipeptidyl peptidase 4 inhibitor	1 (4.55%)	
Glucagon-like peptide 1 receptor agonist	2 (9.09%)	
Thiazolidinedione	0 (0%)	
Acarbose	1 (4.55%)	
Insulin Basal	4 (18.18%)	
Insulin Bolus	2 (9.09%)	
Insulin Premix	4 (18.18%)	
Sodium Glucose Transport 2 Inhibitors	0 (0%)	
Smoking Status (n (%))		
Currently not smoking	13 (81.25%)	
Currently smoking	3 (18.75%)	
Previous smoking	6 (27.27%)	

Data are expressed as mean (Standard Deviation), or number (percentage), or median (interquartile range). Abbreviations: eGFR, estimated glomerular filtration rate estimated using the CKD-EPI equation; CRP, C-Reactive protein

Table 2: Comparison of biochemical, physical and hemodynamic characteristics post placebo and post salt intervention (n=22)

Variable	Post Placebo	Post Salt	Difference between Salt and Placebo	95 % Confidence Interval [a]	p-value [b]
Serum Biochemistry					
Fasting serum lipid profile, mmol/L					
Total cholesterol	4.09 (0.80)	4.12 (0.95)	0.03 (0.5)	-0.2 – 0.3	0.4
High-density lipoprotein	1.36 (0.29)	1.37 (0.31)	0.01 (0.18)	-0.07 – 0.08	0.5
Low-density lipoprotein	2.05 (0.73)	2.07 (0.87)	0.02 (0.4)	-0.19 – 0.22	0.4
Triglyceride	1.6 (0.79)	1.57 (0.93)	-0.03 (0.4)	-0.20 – 0.14	0.3
Fasting serum glucose mmol/L	7.24 (2.49)	6.7 (2.01)	-0.54 (2.3)	-1.6 – 0.5	0.2
HbA1c % (mean, SD)	7.14 (1.4)	7.2 (1.5)	0.06 (0.4)	-0.12 – 0.2	0.3
HbA1c [mmol/mol mean (SD)]	51 (22)	55 (16)	2 (16.5)	-3.3 - 11	0.1
C-peptide nmol/L	1.04 (0.53)	0.97 (0.57)	-0.07 (0.3)	-0.2 – 0.08	0.2
eGFR, mL/min/1.73m^2 (CKD-EPI)	76.5 (13)	78.2 (15)	1.7 (7)	-1.7 – 5	0.2
Creatinine μmol/L	81 (17.8)	78.5 (19)	-2.5 (8.6)	-6.6 – 1	0.1
Renin $\log_{10}$ mIU/L	1.32 (0.3)	1.24 (0.5)	-0.08 (0.3)	-0.2 – 0.07	0.1
Aldosterone $\log_{10}$ pmol/ L	2.5 (0.15)	2.4 (0.2)	-0.1 (0.2)	-0.2 - -0.04	**0.004**
Renin: aldosterone ratio $\log_{10}$	1.19 (0.4)	1.13 (0.5)	-0.06 (0.4)	-0.3 – 0.1	0.2
24 hour urine biochemistry					

Sodium excretion, mmol/24hr	153.95 (61)	205 (63)	51.05 (62.5)	21 – 81	**0.001**
Potassium excretion, mmol/24hr	79.4 (34)	77.6 (22)	-1.8 (29)	-16 – 12	0.4
Albumin excretion, mg/24hr rate	107 (260)	94 (207)	-13 (54)	-42 – 16	0.17
Anthropometric measurements					
Weight, kg	85.48 (13.2)	86.06 (12.8)	0.58 (2)	-0.4 – 1.5	0.1
Body mass index, kg/m^2	30.44 (5.7)	30.62 (5.4)	0.18 (0.8)	-0.2 – 0.5	0.2
24 hour Ambulatory Blood Pressure					
Mean Systolic Blood Pressure, mmHg	134 (12)	138 (12)	4 (12)	-1.9 – 10	0.09
Mean Diastolic Blood Pressure, mmHg	72 (7)	73.6 (7.6)	1.6 (6)	-1.4 – 4.6	0.1
Daytime Systolic Blood Pressure,mmHg	138 (12.4)	141.4 (12.5)	3.4 (12.6)	-2.7 – 9.5	0.13
Daytime Diastolic Blood Pressure,mmHg	75.5 (9.2)	76.1 (8.1)	0.6 (6.6)	-2.6 – 3.8	0.4
Night-time Systolic Blood Pressure,mmHg	123.8 (13)	128.4 (11.7)	4.6 (9.9)	-0.5 – 9.7	**0.04**
Night-time Diastolic Blood Pressure,mmHg	64.5 (6.4)	67.5 (6.6)	3 (6.8)	-0.5 – 6.6	**0.04**
Heart Rate, beats per minute	76.95 (8)	77.95 (8)	1 (5)	-1.6 – 3.6	0.2
24hour Heart Rate Variability					
SDNN, ms	118 (32)	127 (39.8)	9 (28.5)	-8.8 – 25.6	0.2
pNN50	8.8 (11.6)	7.2 (11)	-1.6 (8)	-7 – 4	0.3
HRVti	29.15 (10)	28.62 (8.5)	-0.53 (13.7)	-8.8 – 7.8	0.5
r MSDD, ms	33 (28.5)	31 (29)	-2 (13)	-11.6 – 7.6	0.3

Data are expressed as mean (Standard Deviation) or as percentage. [a] 95 % confidence interval is derived from the difference between groups, [b] p values are derived from paired t-test. Abbreviations: eGFR, estimated glomerular filtration rate estimated using the CKD-EPI equation ; SDNN, Standard Deviation of all normal RR Intervals; pNN50, proportion of the number of pairs of successive beats that differ by more than 50 ms; HRVti, Heart Rate Variability Triangular Index; rMSDD, Root-Mean-Square Successive Difference; ms, milliseconds.

Table 3: The effect of salt-sensitive and salt-resistant status on hemodynamic and clinical measurements post placebo and post salt intervention.

	Salt-Sensitive (n=7)					Salt-Resistant (n=14)				
Variable	**Post Placebo**	**Post Salt**	**Mean Difference (salt vs placebo)**	**95 % Confidence Interval [a]**	**p-value [b]**	**Post Placebo**	**Post Salt**	**Mean Difference (salt vs placebo)**	**95 % Confidence Interval [a]**	**p-value [b]**
24 hour Ambulatory Blood Pressure										
Mean systolic blood pressure, mmHg	126 (9.7)	138.9 (14)	12.7	0.6 – 25	**0.02**	141 (11.5)	135(13)	-6	-13 – 1	0.05
Mean diastolic blood pressure, mmHg	67.6 (7)	74.6 (8.8)	7	2 – 12	**0.007**	72.5 (8)	69 (9.5)	-3	-6 – - 0.3	**0.02**
Mean arterial blood pressure, mmHg	87 (5.6)	96 (10.4)	8.9	-2 – 16	**0.012**	88.4(26)	84.6 (26)	-3.8	-8 – 0.8	**0.03**
MSNA										
Bursts per minute	36 (11)	49.8 (14.5)	13.8	-8 – 36	0.08	30 (11.7)	32 (9)	2	-3.7 – 8	0.2
Bursts per 100 heartbeats	47 (16)	66.5 (14)	19.5	-6.8 – 46	0.05	42 (14)	47 (15)	5	-2.6 – 12	0.09
Units per minute	1945 (962)	2649 (1108.5)	704	-292 – 1701	0.06	1477 (541)	1607 (495)	130	-95 – 356	0.1
Units per 100 heartbeat	2508 (1183)	3515 (1282)	1007	-242– 2256	**0.04**	2075(396)	2318 (764)	243	-23 – 510	**0.03**
Cardiac baroreflex										
Slope	10.86 (8.78)	17 (13.59)	6.14	0.19 – 12.10	**0.023**	11 (7.6)	13 (9)	2	-1.5 – 6	0.09
BEI	21.71 (12.37)	21.86 (6.09)	0.14	-0.35 -12.95	0.5	22 (7)	32 (14.5)	10	-0.35 -12.95	**0.012**
Endothelial PAT										

Reactive Hyperemic Index	2.11 (0.67)	1.78 (0.67)	-0.33	-1.35 – 0.69	0.2	1.8 (0.6)	2.3 (1)	0.5	-0.2 – 1	0.07
Augmentation Index	15.71 (19.44)	21.29 (21.31)	5.57	-10.33 -21.47	0.2	13.8 (18)	6 (21.31)	-7.8	-19 – 4	0.09
PAT Ratio	0.51(0.73)	0.42 (0.35)	-0.09	-0.76 – 0.586	0.4	0.4 (0.5)	0.6 (0.35)	0.2	-0.1 – 0.58	0.09

Data are expressed as mean (Standard Deviation) or as percentage. [a] 95 % confidence interval is derived from the difference between groups, [b] p values are derived from paired t-test. Bold values indicate statistical significance. Abbreviations: MSNA, muscle sympathetic nerve activity; BEI, Baroreflex Effectiveness Index; PAT, Pulse Amplitude Tonometry.

Figures

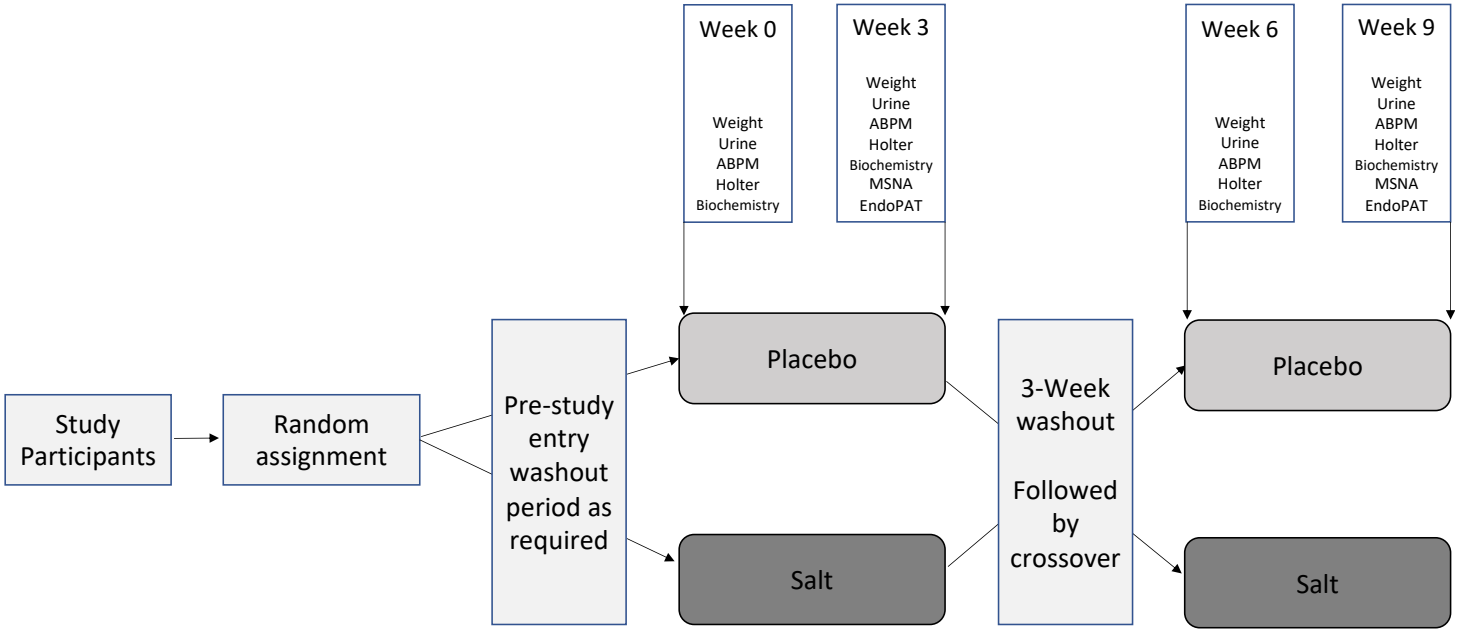


Figure 1: Study design and protocol

Abbreviations: ABPM, ambulatory blood pressure monitor; MSNA, muscle sympathetic nerve activity; PAT, Pulse Amplitude Tonometry

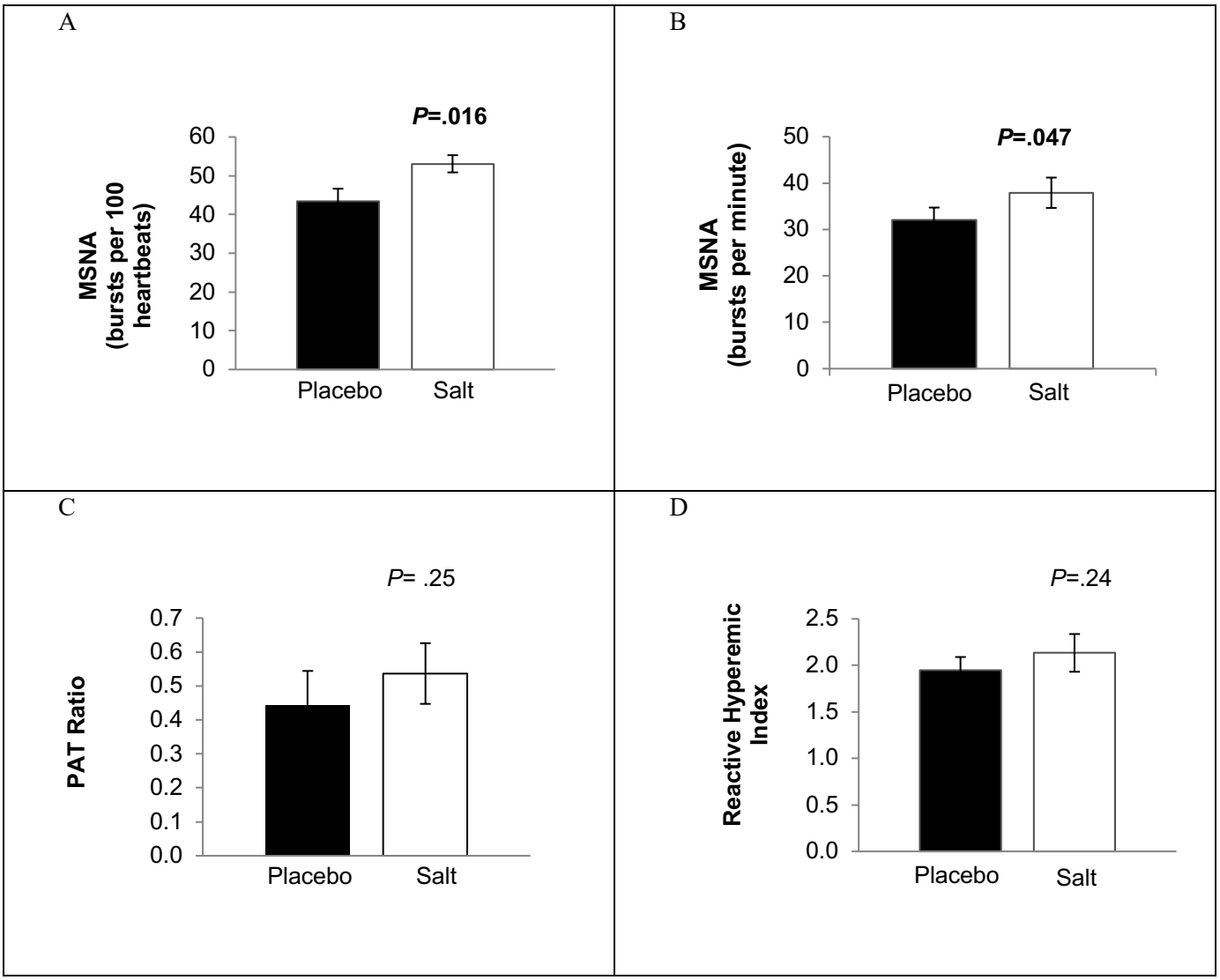


Figure 2: Comparison of the primary endpoints of sympathetic activity and endothelial function post intervention (n=22)

Data is expressed as the mean (+/-SEM). A: MSNA burst incidence. B: MSNA burst frequency. C: Endothelial function PAT ratio. D: Endothelial function reactive hyperemic index. The p values are derived from paired t-test. Bold values indicate statistical significance. Abbreviations: MSNA, muscle sympathetic nerve activity; PAT, Pulse Amplitude Tonometry

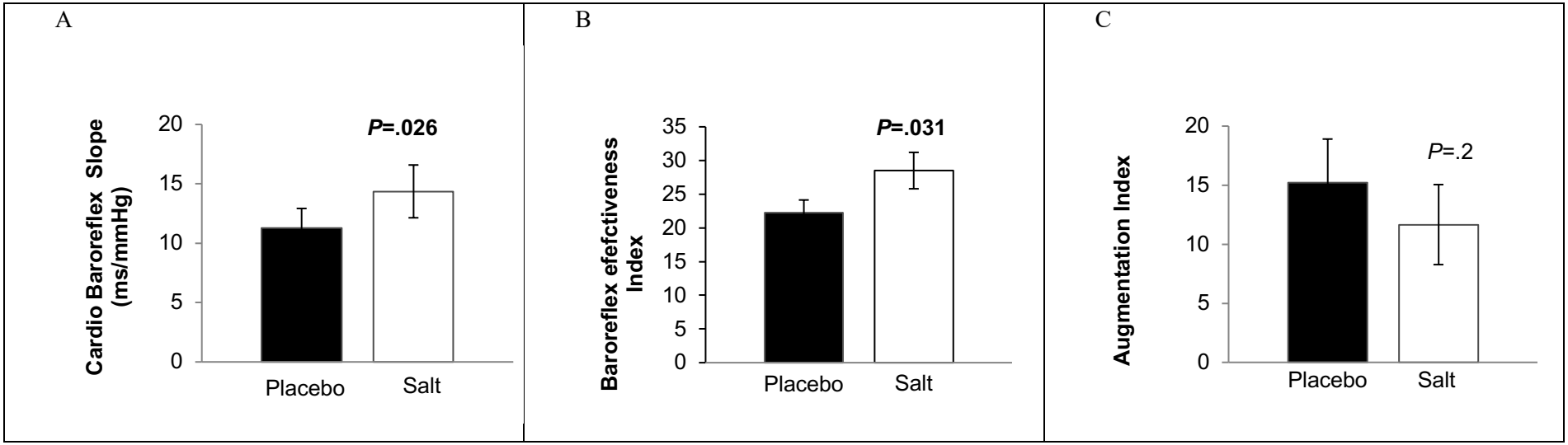


Figure 3: Comparison of the secondary endpoints of cardiac baroreflex function and arterial stiffness post intervention (n=22)

Data is expressed as the mean (+/-SEM). A: Cardiac baroreflex sensitivity (slope). B: Baroreflex effectiveness index. C: Arterial stiffness measured by augmentation index. The p values are derived from paired t-test. Bold values indicate statistical significance. Abbreviations: ms, milliseconds.

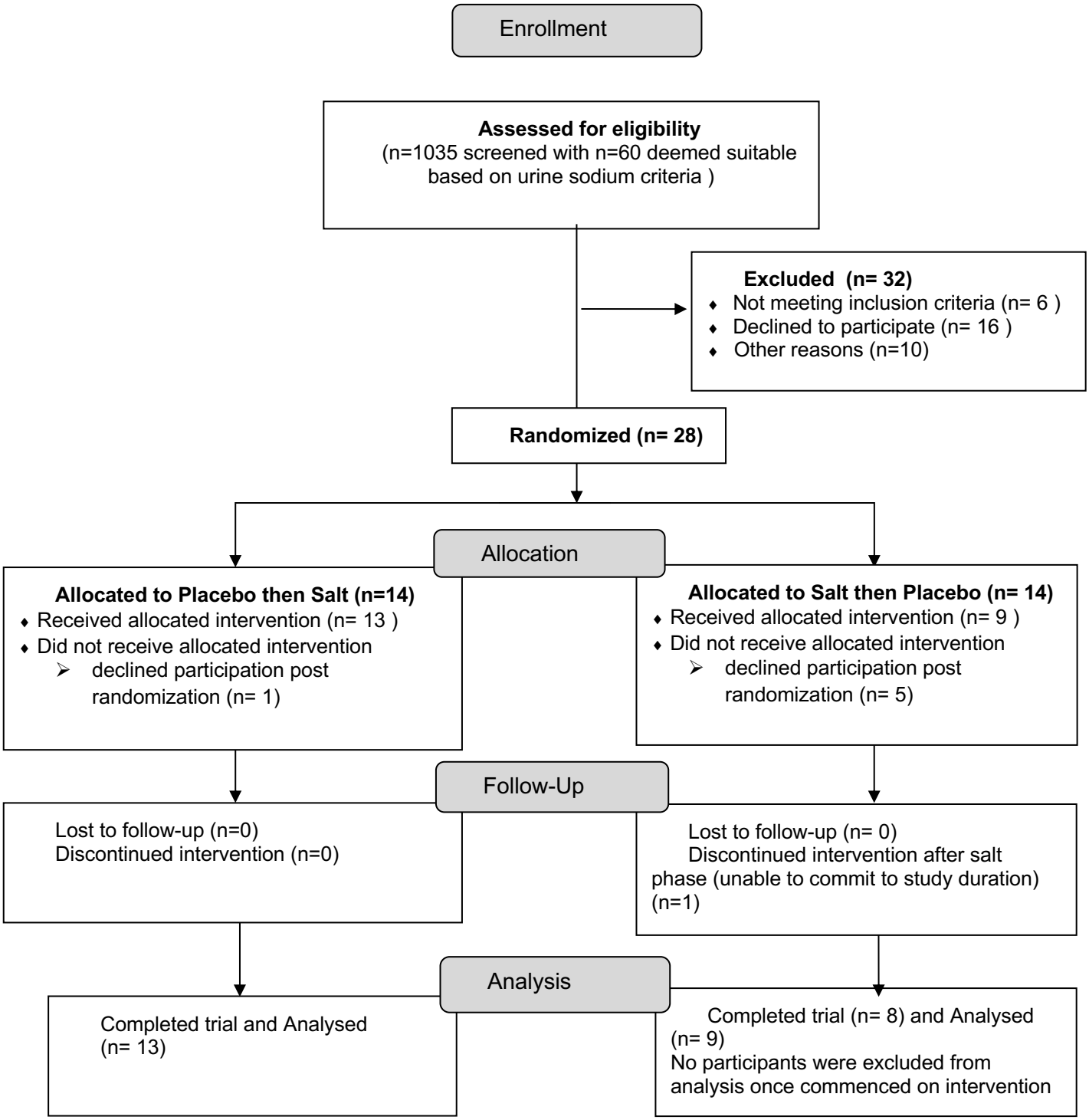
Supplementary Information

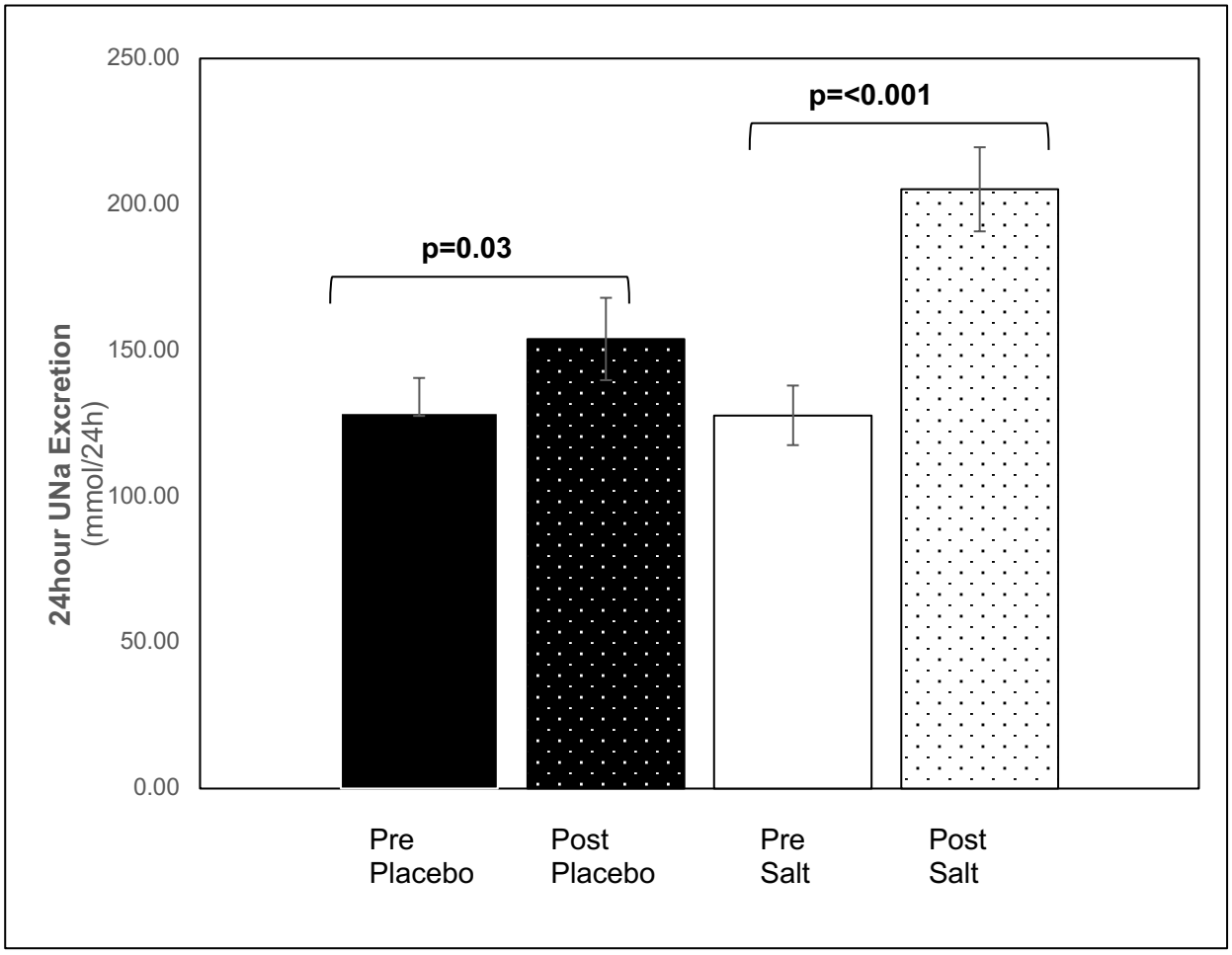
Supplementary Table 1: Comparison of raw values of MSNA, EndoPAT and cardiac baroreflex post placebo and post salt intervention (n=22).

Variable	Post Placebo	Post Salt	Mean Difference (salt vs placebo)	Mean Difference as percentage change (%)	95 % Confidence Interval [a]	p-value [b]
MSNA						
Bursts per minute	32.2 (11.5)	37.9 (13.5)	5.7 (12.8)	17.7	-1.1 – 12.6	**0.047**
Bursts per 100 heartbeats	43.8 (14)	53 (17)	9.2 (15.8)	21	0.90 – 18	**0.016**
Units per minute	1623 (702)	1933 (860)	310 (568)	19	7 – 612	**0.023**
Units per 100 heartbeat	2210 (848)	2692 (1075)	482 (713)	22	102 – 862	**0.008**
Cardiac baroreflex						
Slope	11.25 (7.8)	14.4 (10)	3.15 (7)	28	-0.27 – 6.32	**0.026**
BEI	22.2 (9)	28.5 (12.8)	6.3 (15)	28	-0.35 -12.95	**0.031**
Endothelial PAT						
Reactive Hyperemic Index	1.95 (0.7)	2.13 (0.97)	0.18 (1.2)	9	-0.35 – 0.71	0.24
Augmentation Index	15.23 (18)	11.68 (16)	-3.55 (19)	-23	-12.17- 5.08	0.2
PAT Ratio	0.44 (0.5)	0.54 (0.4)	0.1	22.7	-0.19 – 0.38	0.25

Data are expressed as mean (Standard Deviation) or as percentage as indicated. [a] 95 % confidence interval is derived from the difference between groups, [b] P values are derived from paired t-test. Bold P values indicate statistical significance. Abbreviations: MSNA, muscle sympathetic nerve activity; BEI, Baroreflex Effectiveness Index; PAT, Pulse Amplitude Tonometry; BEI, Baroreflex Effectiveness Index.

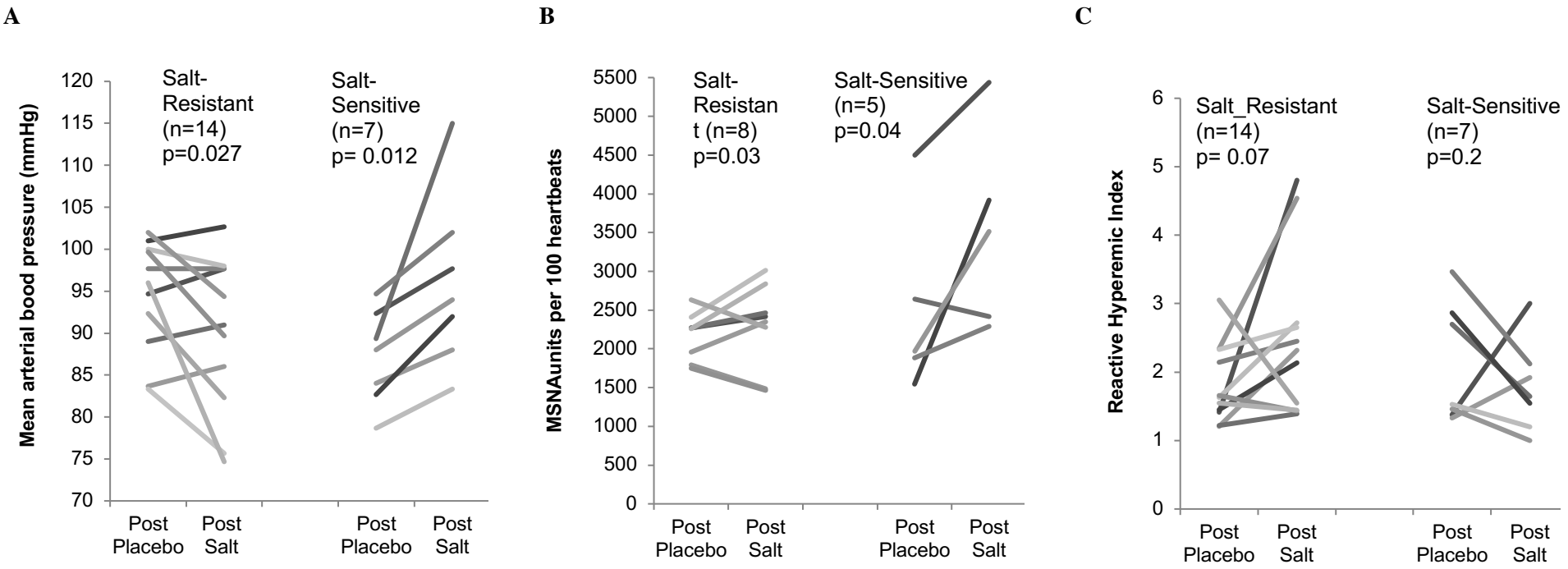
Supplementary Figure 1: Enrollment, Randomization and Retention of Study Participants





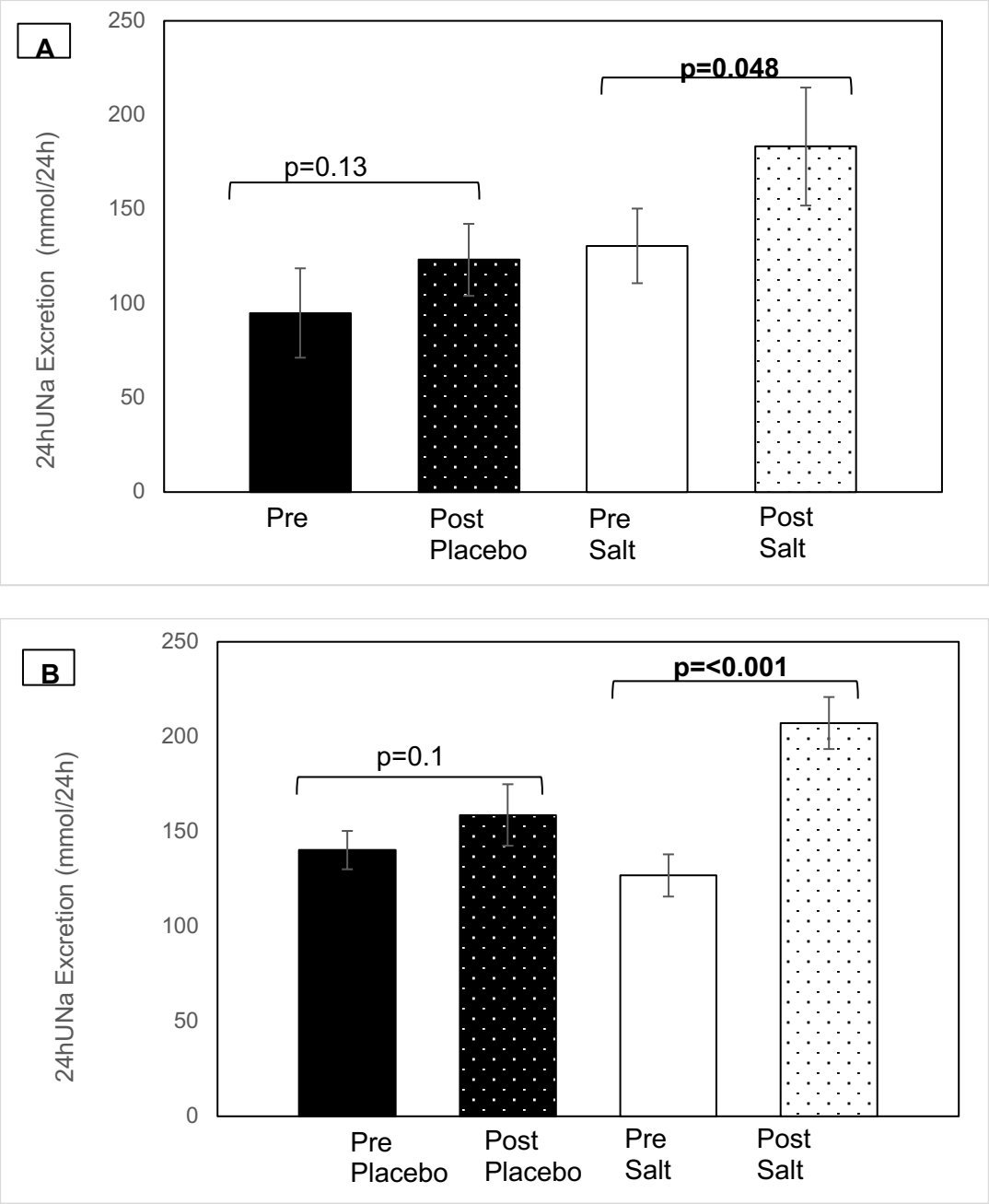
Supplementary Figure 2: 24hour Urine Sodium Excretion values pre and post each intervention phase.

Data is expressed as the mean (+/-SEM). The p values are derived from paired t-test. Bold values indicate statistical significance. Abbreviations: 24hUNa, 24hour urine sodium excretion (mmol/24h). Sodium excretion mean (SEM) values (mmol/24h): Pre Placebo 128.6 (12), Post Placebo 153.95 (14.1); Pre Salt 127.8 (10.2), Post Salt 205.2 (14.4)



Supplementary Figure 3: Individual responses to blood pressure, sympathetic activity and endothelial function based on salt-resistant or salt-sensitive status

Data is expressed as the mean (+/-SEM). A: Mean arterial blood pressure. B: MSNA total activity. C: Endothelial function assessed by reactive hyperemic index. The p values are derived from paired t-test. Bold values indicate statistical significance. Abbreviations: MSNA, muscle sympathetic nerve activity; PAT, Pulse Amplitude Tonometry



Supplementary Figure 4: 24hour Urine Sodium Excretion values pre and post each intervention phase for salt-sensitive and salt-resistant individuals

Data is expressed as the mean (+/-SEM). The p values are derived from paired t-test. Bold values indicate statistical significance. Abbreviations: 24hUNa, 24hour urine sodium excretion (mmol/24h). Figure 4A: Salt-sensitive individuals, Sodium excretion mean (SEM) values (mmol/24h): Pre Placebo 95 (23.7), Post Placebo 123.2 (19.1); Pre Salt 130.6 (19.8), Post Salt 183.3 (31.3). Figure 4BA: Salt-resistant individuals, Sodium excretion mean (SEM) values (mmol/24h): Pre Placebo 140.3 (10), Post Placebo 158.75 (16.2); Pre Salt 126.9 (40), Post Salt 207.15 (49).

Chapter 5: The relationship between habitual dietary sodium intake and RAAS blockade on circulating microparticle levels in type two diabetes.

This chapter will be presented as the author-accepted manuscript of a peer-reviewed article published in *Clinical Science*. 2018;132(20):2207-20.

Abstract

Objective: Low sodium intake is paradoxically associated with adverse cardiovascular outcomes in individuals with type 2 diabetes (T2D), possibly from renin-angiotensin-aldosterone system (RAAS) activation, leading to endothelial dysfunction. In this study, we investigated the associations between habitual sodium intake and RAAS blockade on endothelial function by measuring circulating microparticles (MPs) in individuals with T2D.

Methods: We conducted a prospective, cross-sectional study in 74 individuals with T2D. Habitual dietary sodium intake was estimated using the mean of three corrected 24-hour urine sodium excretion measurements (24hUNa). MP subtypes in platelet-free plasma were quantified using flow cytometry.

Results: No associations between 24hUNa with levels of endothelial MPs were observed. Instead, a trend towards higher diabetes related CD36+/CD235a+ MP levels was associated with lower 24hUNa (rho = -0.23, p= 0.05). When stratified according to tertiles of 24hUNa, platelet- derived CD42b+/CD41+ and CD42+/CD41+/Annexin V+ MPs were higher in the lowest tertile (24hUNa <157mmol/24h) (p=0.02 respectively). Despite RAAS blockade being associated with lower levels of most MP subsets, it was not associated with lower MPs, in the setting of low sodium intake.

Conclusion: Lower sodium intake is associated with higher circulating procoagulant MPs but not with evidence of endothelial dysfunction in individuals with T2D.

Key Terms

Microparticles, endothelial dysfunction, sodium intake, cardiovascular disease, type 2 diabetes, urinary excretion, RAAS.

Clinical Perspective

- **Background:** In an effort to underpin the mechanistic links behind the association of low sodium intake and higher mortality in people with diabetes, we assessed the effect

of habitual sodium intake and renin-angiotensin-aldosterone system (RAAS) blockade on circulating microparticles (MPs).

- **Results:** Endothelial MP levels were not associated with sodium intake, yet procoagulant, platelet-derived MP levels were higher in those individuals with the lowest tertile of sodium intake (<157mmol/24h) (p=0.02 respectively). Whilst RAAS blockade was associated with lower levels of most MP subsets it was not associated with lower MPs in the setting of low sodium intake.
- **Significance of results:** Our findings warrant investigation in larger trials to further explore the role of sodium on microparticle levels and question whether the therapeutic benefits of RAAS blockade may be attenuated during lower sodium intake.

Introduction

Cardiovascular related disease is the leading cause of morbidity and mortality in people with diabetes1. Reduction in blood pressure lowers cardiovascular risk[2]. As high dietary sodium intake is associated with hypertension [3], public health bodies recommend lowering dietary sodium intake to less than 2300mg/day (100mmol/24h) for the general population[4]. Previous targets were more stringent for those with diabetes, set at less than 1600mg/day or 70mmol/24h[4]. In light of observational studies demonstrating the paradoxical association between low sodium intake and higher all-cause and cardiovascular related mortality in people with diabetes [5, 6], the American Diabetes Association has revised recommendations suggesting people with diabetes follow general population sodium intake guidelines[7].

Whilst a paradoxical association of higher mortality with lower sodium intake has been replicated in other studies [5, 8], findings of either a lower mortality [9-11], U-/J-shaped relationships [8, 12], uncertain associations [13, 14] and no associations[15] have also been demonstrated. Methodological differences are likely to account for these discrepancies. The role of sodium on cardiovascular health nevertheless appears complex requiring further understanding. As such, mechanistic studies have been called for by health bodies to further investigate these associations [16].

Experimental diabetes models have suggested that low sodium intake may lead to endothelial dysfunction [17], possibly via activation of neuro-hormonal pathways such as the renin-

angiotensin-aldosterone system (RAAS) and the sympathetic nervous system [18]. This may in turn increase circulating adrenaline, noradrenaline, cholesterol, triglycerides, insulin resistance and lipids [18]. These pleiotropic effects may trigger endothelial dysfunction, in turn negating any blood pressure lowering benefits. Given endothelial dysfunction is the earliest identifiable precursor to atherosclerosis with subsequent progression to cardiovascular disease [19], timelier identification and intervention may improve outcomes.

Underpinning the molecular mechanisms of endothelial dysfunction may be possible through the use of biomarkers, such as circulating microparticles [20, 21]. Microparticles are cell-derived vesicles (0.1 – 1.0 μm), formed from the outward budding of the plasma membrane, and are released into the extracellular space during cellular activation, injury or death (Supplementary figure 1) [22]. They retain surface proteins of their cell of origin, allowing isolation and quantification of specific endothelial, platelet, erythrocyte or leucocyte -derived microparticles[22]. Present in physiological states at low concentrations [23], elevated levels are associated with cardiovascular related diseases, such as type 2 diabetes [24-27]. Reflective of an injured endothelium[21], microparticles are considered non-invasive surrogate markers of vascular dysfunction[20]. The cell surface marker CD36, for example, involved in insulin resistance and pro-atherogenic processes [28], is considered a biomarker for type 2 diabetes and atherosclerotic plaque instability [28], making it ideal to study in this population.

Furthermore, RAAS activation observed during a low sodium intake may lead to endothelial dysfunction, which could be reflected in higher microparticle counts. This concept is plausible given studies have demonstrated RAAS activation occurs when sodium intake falls below 3000mg/ day (130mmol/day) [29] and RAAS activation has been shown to induce generation of microparticles which in turn creates a pro-thrombotic state [30]. Moreover, RAAS blockade has been shown to lower microparticle levels [31, 32]. However, to our knowledge, whilst a single study has assessed the effect of RAAS blockade on microparticle levels people with type 2 diabetes [33], there has been no investigation into the association between RAAS blockade, sodium intake and microparticles in those with or without diabetes.

Accordingly, we conducted this study to determine the associations between habitual dietary sodium intake and RAAS blockade on circulating microparticle levels in people with type 2 diabetes. We hypothesized that those with lower habitual sodium intake will have higher

circulating endothelial microparticle levels, reflective of a damaged endothelium, and use of RAAS blockade will be associated with lower circulating endothelial microparticle levels.

Methods

Study design and participant recruitment

In this proof of concept, prospective, cross-sectional study we recruited individuals from our university teaching hospital diabetes outpatient clinics who met the following inclusion criteria: type 2 diabetes, aged 50-75 years, body mass index between 25 and 35, systolic BP < 140mmHg and/or diastolic BP < 80mmHg, at least two previous 24hUNa and an absence of serious systemic illness. Participants were excluded if they had a history of peripheral or autonomic neuropathy, liver or thyroid disease; cancer within the past five years; an estimated glomerular filtration rate (eGFR) <45ml/min/1.73m^2, taking greater than three anti-hypertensive agents, hormone replacement therapy or sodium-glucose linked transporter 2 (SGLT2) inhibitors. Written consent was obtained from all participants. The study was approved by the Austin Health and Hunter New England Area Human Research Ethics Committees.

Endpoints

The primary endpoints were to assess the association between habitual sodium intake and endothelial microparticles, considered surrogate biomarkers of endothelial function. The secondary endpoints were to (i) assess the association between RAAS blockade and circulating microparticle counts and (ii) to determine if the presence of RAAS blockade altered the association between circulating microparticles and 24hUNa.

Estimation of habitual dietary salt intake using 24-hour urine sodium excretion

Utilizing 24-hour urine sodium excretion (24hUNa) is considered the most reliable method for estimating sodium intake [34]. Patients who attend our diabetes clinics perform a 24-hour urine collection before each visit with written and verbal instructions provided to ensure the accuracy of the collection. Standardized methods used by our Department of Laboratory Medicine measured 24hUNa and 24hU creatinine (24hUCr) excretions. We have previously

demonstrated that a single measurement of 24hUNa is able to predict habitual dietary sodium intake in people with type 2 diabetes, with an intra-individual coefficient of variation (21 ±1%) suggesting day-to-day variation of sodium intake is approximately 20% [35]. To improve accuracy in determining habitual sodium intake, we obtained results of the three most recent 24hUNa collections for each participant prior to study entry within a three year period. For each of the participant's 24hUNa collection, we corrected for any changes in renal function over time and for the possibility of incomplete urine collections by calculating the mean 24hUCr measurements and then utilizing the following formula:

$$Corrected\ 24hUNa = \frac{24hUNa\ x\ overall\ mean\ 24hUCr}{24hUCr}$$

The mean of three corrected 24hUNa collections was used to estimate habitual dietary sodium intake and was then correlated with microparticle levels.

Preparation of platelet-free plasma for analysis of circulating microparticles

Fasting peripheral whole blood samples were collected in 4ml 3.2% sodium citrate tubes (BD Vacutainer) using a 21-gauge needle. A serial double centrifugation protocol (Thermo Scientific Heraeus Pico 21 centrifuge) was used to spin whole blood at 1500xg for 20 minutes to obtain platelet-rich plasma and then at 13000xg for 2 minutes to obtain platelet-free plasma [36]. The supernatant was carefully removed. Aliquoted samples were stored at -80°C in Eppendorf tubes within 120 minutes and later thawed at 37°C immediately prior to flow cytometry.

Antibody staining of platelet-free plasma for microparticle analysis

Fluorescent antibodies against the most commonly cited cell surface markers were used to identify microparticle subtypes. Platelet microparticles were identified to ensure final endothelial microparticle counts were not contaminated with other microparticle subtypes. As previously described [37], a 10 microliter aliquot of platelet-free plasma was combined with the various combinations of following antibodies: CD41-PE (clone PL2-49, Biocytex; platelet marker), CD42b-FITC (clone HIP1, BD Pharmingen, San Diego, CA, USA; platelet marker), CD235a-APC (clone GA-R2 (HIR2), BD Pharmingen; erythrocyte marker), CD 54-BB515

(clone HA58, BD Horizon; endothelial marker), CD105-PE (clone 1G2, Beckman Coulter, Brea, CA, USA; endothelial marker), CD31-PE (clone WM59, BD Pharmingen; against endothelial marker PECAM-1), CD62e PE-Cy5 (clone 68-5H11, BD Pharmingen, against activated endothelial marker E-selectin) and Annexin V-APC (Bioscience, San Diego, CA, USA; phosphatidylserine), CD36 (clone 11H5) conjugated to DyLight-488. Careful consideration was given to the microparticles detected for this study to reflect endothelial function. In people with type 2 diabetes, CD31+/CD42b- endothelial microparticle levels positively correlated with brachial artery pulse wave velocity (baPWV), but negatively correlated with flow-mediated dilation [26] and were identified as an independent risk factor for both baPWV and flow-mediated dilation [26]. A subsequent study demonstrated CD31+/CD42b- endothelial microparticles to be the most significant contributor to severe endothelial dysfunction, defined as baPWV above the 75th percentile for their cohort [27]. The strong evidence supporting the use of the aforementioned endothelial microparticles as biomarkers of endothelial dysfunction and cardiovascular risk in clinical research was the basis of their use in the present study. Additionally, CD36 was included for analysis as a biomarker of type 2 diabetes and atherosclerotic plaque instability [38].

Analysis of microparticles by flow cytometry

Analysis of microparticles was performed using a FACS Canto Flow Cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) as previously described [37]. The cytometer was calibrated for forward scatter resolution using Megamix beads (Biocytex, Marseille, France) using a blend of 2:1:1 0.5, 0.9 and 3-micrometer diameter fluorescent beads. The lower microparticle detection limits were set according to the manufacturer's instructions. Events were collected for 60 seconds at a low flow rate and subsequently analyzed using FACS Diva software (BD Biosciences). All assays were diluted to a final volume of 500 □l in PBS (without Ca^{+2} and Mg^{+2}) or calcium-rich binding buffer (for those stained with Annexin V) with the addition of a known quantity of CountBright (Molecular Probes, Eugene, OR, USA) counting beads and 15 □M PPAK (D-Phe-Pro-Arg-CMK-HCl) to inhibit clumping.The absolute number of microparticles for each sample was calculated using the formula:

$$\frac{Microparticles}{microliter} = \left(\frac{microparticle\ count}{bead\ \ count}\right) x \left(\frac{total\ number\ of\ beads}{test\ volume}\right)$$

Statistical analysis

For this exploratory study, the sample size estimation was based on the statistical power from a previous study which required 41 participants to detect a 20% difference in the levels of platelet derived microparticles (80% power, two-sided alpha 0.05) [32]. Baseline characteristics data are presented using mean (standard deviation) if data was normally distributed and as median (interquartile range) if the data was not normally distributed. Categorical data was expressed as number of participants (%). Associations between each microparticle subset and 24hUNa were assessed separately using Spearman rank analyses rho. An independent t-test was run to assess the relationship between RAAS blockade use on microparticle counts. Statistical analysis was performed with STATA version 14.1 software (StataCorp. 2015. *Stata Statistical Software: Release 14*. College Station, TX: StataCorp LP). A p value of <0.05 was considered statistically significant.

Results

Approximately 695 people with diabetes who attended our outpatient clinics were screened for eligibility from June 2013 to June 2016. A total of 81 eligible participants were enrolled. As seven participants had marked differences in the pre-analytical processing phase, which is known to affect microparticle levels, these participants were excluded. A total of 74 participants were therefore included for final analysis (Supplementary figure 2).

Baseline cohort characteristics

Baseline characteristics are summarized in Table 1. The mean (SD) corrected 24hUNa for the cohort was 194.6 mmol/24h (75.2), in accordance with previous epidemiological studies demonstrating high dietary sodium intake across populations 39. The median (SD) duration between collections was 423.5 (257) days with an interquartile range of 267-673. Diabetes median duration was 16.6 years (IQR 11-21 years). The mean glycosylated hemoglobin A1C (HbA1c) was 7.7% (61mmol/mol), reflecting the tertiary referral nature of our clinic. Other cardiovascular risk factors were adequately controlled as assessed by appropriate mean total cholesterol, LDL and blood pressure findings. A majority of participants were on RAAS blockade (64%). These participants (n=47) had a longer duration of diabetes (p=0.01), poorer glycemic control as measured by HbA1c (p=0.03), higher mean 24hUNa excretion (p=0.014) and lower HDL (p=0.02) compared to participants not on RAAS blockade (n=27).

Correlation between 24-hour urine sodium excretion and microparticle levels

As demonstrated in Figure 1A, a trend towards higher CD36+/CD235a+ erythrocyte microparticles was associated with lower 24UNa (rho = -0.23, P = 0.05). No statistical significance between 24hUNa excretion, total CD36+ microparticles (Figure 1B) or other levels of microparticles (Table 2) were seen. Neither the endothelial nor platelet subtypes correlated with urine sodium excretion (Table 2).

Association between sodium intake, stratified according to tertiles of 24hUNa, and mean microparticle counts.

Whilst not the primary aim of the study, we assessed microparticle levels according to tertiles of 24hUNa (Figure 2). Similar to our previous findings[5], approximately one-third of participants had a sodium excretion <157 mmol/24 h (lowest tertile), one-third had an excretion >217 mmol/24 h (highest tertile) with the middle tertile falling between 157 and 217 mmol/24 h. Therefore, in the present study, 24hUNa <157mmol/24h represents the lowest tertile or Tertile 1(n=25); 24hUNa 157- 217 mmol/24h represents the middle tertile or Tertile 2(n=24) and 24hUNa >217 mmol/24h represents the highest tertile, or Tertile 3(n=25). Furthermore, only 7 participants in this study had a 24hUNa <100mmol/24h suggesting a majority of this cohort were not adhering to dietary sodium guidelines, which is frequently observed in clinical practice[4]. By stratifying individuals according the 24hUNa tertiles, we observed an association between higher levels of total platelet-derived CD42b+/CD41+ and CD42+/CD41+/Annexin V+ (p=0.02 respectively) microparticles as well as a trend towards higher CD36+ (p=0.07) and CD105+ (p=0.06) microparticle levels with sodium excretion from the lowest tertile group (24hUNa <157mmol/24h) (Figure 2).

Association between RAAS blockade and circulating microparticles

In the 47 participants on RAAS blockade, other than CD54+/CD105+ and CD105+/CD62e+, all the other microparticle counts, including endothelial derived microparticles, were significantly lower compared to the 27 participants who were not on RAAS blockade (Figure 3).

Association between RAAS blockade, microparticles and habitual sodium intake.

Interestingly, RAAS blockade was not associated with lower microparticle levels in the setting of low sodium intake (Table 3). We were particularly interested in CD36+/235a+, as this demonstrated a trend for higher levels in the setting of lower sodium intake. Despite expecting CD36+/235a+ levels to be lower in those individuals on RAAS blockade, we did not observe any meaningful relationship between RAAS blockade and CD36+/235a+ levels in the setting of low sodium intake ($\varrho = -0.19$, $P = 0.21$) (Figure 4A). In individuals who were not on RAAS blockade, there was no association between CD36+/235a+ erythrocyte microparticles and 24hUNa excretion ($\varrho = 0.08$, $P = 0.69$) (Figure 4B).

A regression was run to determine the association between microparticle subsets with 24hUNa adjusting for plasma renin activity in the 15 participants for whom plasma renin levels were available. None of these participants were on any form of RAAS blockade. There was no relationship between 24hUNa and any of the microparticle subsets assessed (Supplementary Table 1).

Discussion

The key findings in this study were that lower habitual sodium intake in individuals with type 2 diabetes is (i) associated with higher procoagulant platelet-derived microparticles (CD42b+/CD41+ and CD42+/CD41+/Annexin V+) and a trend towards higher CD36+/CD235a+ erythrocyte-derived microparticle levels but without evidence of endothelial dysfunction and (ii) the beneficial effects of RAAS blockade on endothelial function may be offset in the context of low sodium intake.

The association between sodium intake and endothelial microparticles in type 2 diabetes is largely undetermined.

Endothelial microparticles are elevated in type 2 diabetes [24-27] and correlate with arterial stiffness and impaired endothelium-mediated vasodilation [25] making them ideal vascular dysfunction biomarkers [20] to study the vascular effects of sodium intake in this population. Yet, to our knowledge, no studies have examined the association between habitual dietary sodium intake and microparticle levels, especially in type 2 diabetes. Contrary to expectations, this

study did not demonstrate an inverse correlation between endothelial microparticles and 24hUNa, which may be explained by our cohort characteristics. Our participants were taking medications to reduce vascular complications and as such were appropriately treated for cardio-metabolic risk factors as evidenced by well-controlled diabetes, blood pressure and lipids. Furthermore, endothelial microparticles vary according to the underlying pre-existing vascular complications. Previously, endothelial microparticle CD31+/CD42b– and CD31+/Annexin V+ levels have been shown to be higher in individuals with macrovascular complications compared to those with microvascular or no underlying complications [40]. Whilst 26% of our participants had underlying ischemic heart disease, there was a low prevalence of other macrovascular complications such as stroke (4%) and peripheral vascular disease (10%). Additionally, whilst smoking is associated with higher endothelial microparticle levels [41], only 12% of our cohort were current smokers. Overall these factors may have led to a more healthy vascular function and may in turn explain the lack of correlation seen between endothelial microparticles and sodium excretion.

A trend towards higher CD36+/CD235a+ erythrocyte microparticle levels was associated with lower habitual sodium intake.

Whilst no significant associations between endothelial microparticles and habitual sodium intake were demonstrated, the present study did observe a trend towards higher CD36+/CD235a+ erythrocyte-derived microparticles being associated with lower sodium intake. Although a few studies have examined CD36 and erythrocyte-derived CD36+ microparticles in people with type 2 diabetes [37, 38], these have not examined the association between habitual sodium intake and microparticles, making it difficult to compare and comment on the overall significance of our findings. We were fundamentally interested in CD36+ given its involvement in pro-atherogenic processes [28] and its association with diabetes and insulin resistance [38]. CD36 is expressed in various cells and tissue such as erythrocytes, platelets and endothelial cells, with its circulating form being entirely associated with microparticles [37]. Previous studies have demonstrated that CD36+ microparticles in people with type 2 diabetes are primarily derived from erythrocytes [38], in contrast to individuals without diabetes where CD36+ microparticles are primarily derived from endothelial cells[38]. Thus the combination of CD36+ and CD 235a, which is of erythrocyte origin[42], was deemed important to analyze in our study. Perhaps this predilection of CD36+ microparticles being primarily erythrocyte in origin in people with type 2 diabetes serves as an explanation as to why we did

not see a significant correlation between total CD36 and 24hUNa, yet observed a trend towards higher CD36+/CD235a+ erythrocyte derived microparticle levels being associated with lower sodium intake in our study. Interestingly, glycemic control did not correlate with levels of CD36+/235a+ either alone or in association with 24hUNa excretion suggesting that sodium intake alone may have contributed to the inverse trend observed.

Platelet-derived microparticles were higher in the lowest tertile of sodium intake.

When stratified according to the tertiles of 24hUNa, we were able to demonstrate an association between platelet-derived microparticles (Total CD42b+/CD41+ and CD41+/CD42b+) being higher in the lowest tertile of 24hUNa (<157mmol/24h) group. Although out of the scope of this paper, acknowledging this observation is important given platelet-derived microparticles are known to be higher in the presence of cardiovascular risk factors, such as diabetes, and contribute to the development of atherosclerosis, thrombus formation and vascular damage [42]. Furthermore, coupled with the trend observed towards higher CD36+ and CD105+ microparticle levels being associated with sodium excretion from the lowest tertile of 24hUNa, the significance of our findings warrant further evaluation, as compared to traditional vascular risk factors, elevated microparticle levels have been suggested as the leading risk factor for macroangiopathy in individuals with type 2 diabetes [25, 27], and are regarded as a more comprehensive index of endothelial homeostasis [25].

RAAS blockade was associated with an overall lower level of microparticle counts.

As 64% of our patients were on RAAS blockade with either angiotensin converting enzyme inhibitors (ACEI) or Angiotensin II receptor blockers (ARBs), we aimed to ascertain the association between RAAS blockade and microparticles in the setting of habitual sodium intake, as this remains largely undetermined. We observed RAAS blockade use was associated with lower microparticle levels, for most of the microparticle subtypes, as compared to no RAAS blockade use. This is in keeping with previous findings where ARBs have been associated with lower monocyte, platelet, and endothelial cell-derived microparticles in hypertensive people with diabetes, suggesting in addition to its anti-hypertensive effects, ARBs may have anti-atherosclerotic effects[33] (Supplementary table 2). Studies in people without diabetes have likewise demonstrated RAAS blockade is associated with lowering of

microparticle levels [31, 32] (Supplementary table 2). Thus, the role of RAAS blockade on microparticle levels, and therefore endothelial health, appears promising. Future studies could examine whether there is a dose-dependent effect of RAAS blockade on microparticle count lowering.

There was no relationship between RAAS blockade and microparticle counts in the setting of low sodium intake.

Contrary to the beneficial effects of RAAS blockade on microparticle counts seen in this and other studies [32], we found no association between RAAS blockade and circulating microparticles in the setting of low sodium intake. A possible explanation could be that ongoing RAAS activation in the setting of low sodium intake, which occurs in spite of the RAAS blockade, possibly attenuates the endothelial benefits of RAAS blockade [43, 44]. Our findings are in line with previous studies demonstrating the pleiotropic effects of sodium[17, 43]. Low sodium intake has been associated with RAAS activation and increased plaque accumulation in experimental models of diabetes [17] possibly through mechanisms related to Angiotensin II formation [45]. Animal models have demonstrated a threefold increase in plasma Angiotensin II, associated with a 60% reduction in flow-induced vasodilation in coronary arterioles of dogs fed the low sodium (0.05% NaCl) as compared to a normal sodium (0.65% NaCl) diet[46]. Certainly, in people with diabetes, RAAS blockade in the setting of low dietary sodium intake has been associated with a higher risk of developing 'aldosterone breakthrough'[47] with a subsequent increase in cardiovascular morbidity[48]. Randomized controlled studies from our group have previously shown that salt supplementation was associated with a decrease in plasma renin activity and a trend towards reduction in aldosterone ($p=0.05$) in the setting of telmisartan use in hypertensive patients with type 2 diabetes[44]. Based on the findings from the present study and from previous reports [43, 44], a low sodium intake may therefore counteract the beneficial effects of RAAS blockade. Collectively, these findings have important therapeutic implications to consider given the significant proportion of people with diabetes who are administered these agents.

Furthermore, our findings question the role of reviewing sodium intake as assessed by 24hUNa, before administering RAAS blockade in people with type 2 diabetes. In those individuals found to have a habitual low sodium intake, liberating sodium intake in order to achieve the maximal therapeutic benefit of RAAS blockade on endothelial health may be necessary. Previous studies

have suggested a similar approach to liberate sodium intake in order to reduce reactive hyper-reninaema in those with elevated plasma renin activity[49]. This approach may be met with concerns of inducing increases in blood pressure. However given the recent updated standards of care from the American Diabetes Association have revised blood pressure targets in people with diabetes to 140 mmHg systolic and less than 90mmHg diastolic[7], a more liberal sodium intake in order to prioritize the potential improvements in endothelial health from RAAS blockade appears justified. These findings and the questions presented from this study require further evaluation in larger scaled, randomized controlled trials.

Strengths and Limitations

We recognize the small sample size as a limitation, however, based on previous studies of a similar nature[32], our sample size was comparable. Due to the cross-sectional nature of the study design, a causal relationship between habitual dietary sodium intake and microparticle levels cannot be determined. We acknowledge a control group is lacking, however for this exploratory study, we were interested in assessing the associations between sodium intake and endothelial function in individuals with type 2 diabetes at high cardiovascular risk given we previously demonstrated sodium intake in the lowest tertile (24hUNa <150mmol/24h) is associated with poorer cardiovascular outcomes in this population[5]. Whilst only seven out of 74 participants, i.e. 9.5% of this cohort, had sodium intake as per recommended dietary sodium guidelines, this is in accordance with our previous findings, highlighting the difficulty people with type 2 diabetes have in achieving sodium restriction targets [35]. Angiotensin II and plasma renin levels would have been advantageous to further assess the effect of RAAS blockade, however this could be incorporated into future studies of this nature. Using multiple corrected 24hUNa measurements to estimate habitual dietary sodium intake, preparing platelet-free plasma by two consistent investigators, as well as using flow cytometry, currently considered to be the method of choice for analysis of microparticles [50], strengthens the methodology of the present study.

Conclusion

This study has for the first time examined the association between habitual sodium intake, RAAS blockade and endothelial function as assessed by circulating microparticles. Despite expecting higher endothelial microparticles being associated with lower sodium intake, we did not demonstrate any significant association between sodium intake and endothelial

microparticles. However, we observed a trend towards higher CD36+/CD235a+ erythrocyte-derived microparticle levels being associated with lower sodium excretion and higher platelet-derived microparticles being associated with the lowest tertile of 24hUNa excretion, Overall, given the pro-atherogenic roles of CD36+/CD235a+ microparticles and the procoagulant effects of platelet-derived microparticles, our findings warrant investigation in larger trials to further explore the role of sodium on microparticle levels. This is required if we are to improve knowledge on the mechanisms underlying the paradoxical association between sodium intake and cardiovascular health in people with diabetes. Additionally as RAAS blockade was not associated with lower microparticles counts in the setting of a low sodium intake, these findings question whether the therapeutic benefits of RAAS blockade may be attenuated during lower sodium intake. Given the proportion of individuals with diabetes who receive these agents, exploring this further is of considerable public health importance.

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Author Contributions:

SB, GJ, EIE and DL were involved in the study conception, design and data interpretation. SB, DL and YWK were involved in participant recruitment and the pre-analytical processing of participant samples. LL was the lead scientist involved in microparticle characterization and analysis. All authors were involved in the drafting and editing of the manuscript. EIE and LL approved the study design and the final content of the manuscript. We thank Professor Leonid Churilov for statistical guidance.

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List of Abbreviations

175 MDRD: 175 Modification of Diet in Renal Disease

24hUCr: 24-hour urinary creatinine excretion

24hUNa: 24-hour urinary sodium excretion

ACEI: Angiotensin converting enzyme inhibitor

ARB: Angiotensin II receptor blocker

baPWV: Brachial artery pulse wave velocity

BMI: Body mass index

CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration

DKD: Diabetic kidney disease

eGFR: Estimated glomerular filtration rate

EMPs: Endothelial microparticles

FMD: Flow-mediated dilation

IOM: Institute of Medicine

MPs: Microparticles

NHMRC: National Health Medical Research Council

PMPs: Platelet microparticles

RAAS: Renin angiotensin aldosterone system

SGLT-2: Sodium glucose co-transporter 2 inhibitor

SNS: Sympathetic nervous system

T2DM: Type Two Diabetes Mellitus

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Tables

Table 1: Baseline characteristics of study participants

Variable	Total cohort (n = 74)	RAAS blockade use (n=47)	No RAAS blockade use (n=27)	P-value *
Age, years (mean, SD)	65 (9)	66 (10)	64 (6.8)	0.1
Male sex	47 (64%)	33 (70%)	14 (52%)	
Body mass index, kg/m^2 (mean, SD)	32.5 (4.7)	32.5 (4.2)	32.5 (5.5)	0.5
Blood pressure, mmHg (mean, SD)				
Systolic blood pressure	133.6 (14)	135.4 (14.6)	130.4 (13.2)	0.07
Diastolic blood pressure	75 (10)	74.6 (10.5)	76 (9)	0.3
Corrected 24hUNa, mmol/24h (mean, SD)	194.6 (75)	209 (74.7)	169 (70)	**0.01**
24hUCr, mmol/24h (mean, SD)	12.8 (3.8)	12.9 (3.9)	12.5 (3.7)	0.3
Brochner-Mortensen corrected GFR, mL/min/1.73m^2 (mean, SD)	78.7 (29)	77.2 (29)	82 (28.8)	0.3
CKD-EPI, mL/min/1.73m^2 (mean, SD)	70.5 (22.6)	68.4 (23)	74 (21)	0.2
Serum creatinine, µmol/L (mean, SD)	98.6 (45)	102 (41)	93 (51)	0.2
Fasting serum lipid levels, mmol/L				
Total cholesterol (median, IQR)	3.8 (3.4-4.6)	3.7 (3.4 -4.6)	4.2 (3.4 -4.7)	0.2
LDL cholesterol (median, IQR)	1.8 (1.5-2.5)	1.7 (1.5 -2.1)	2 (1.5 -2.5)	0.3
HDL cholesterol (mean, SD)	1.2 (1-1.5)	1.1 (0.9-1.4)	1.4 (1 -1.7)	**0.02**
Triglycerides (median IQR)	1.6 (1.2-2.4)	1.8 (1.2 -2.6)	1.5 (1-1.9)	0.06
Fasting serum glucose level, mmol/L (median, IQR)	8.1 (5.7-9.8)	8.2 (5.9 -9.8)	7.3 (5.6 -9.6)	0.2
HbA1c, % (median, IQR), (mmol/mol)	7.7 (7.0-8.9) (61)	8.05 (7.2 - 9.1) (64)	7.4 (6.6-7.9) (57)	**0.03**
C-peptide, nmol/L (median, IQR)	1.0 (0.7-1.3)	1.1 (0.6-1.4)	0.8 (0.7-1.2)	0.6
C-reactive protein, mg/L (median, IQR)	2.1 (1.2-5.5)	2.1 (0.9-5.6)	1.8 (1.4-5)	0.6
Diabetes duration, years (median, IQR)	16 (11- 21)	19 (14-23)	15 (7-17)	**0.01**
Pre-existing microvascular complications				
Diabetic retinopathy	28 (38%)	19 (40%)	9 (33%)	
Diabetic neuropathy	13 (18%)	11 (23%)	2 (7%)	
Diabetic kidney disease (DKD)	44 (60%)	33 (70%)	11 (41%)	
DKD Stage 1	8 (11%)	6 (13%)	2 (7%)	
DKD Stage 2	14 (19%)	11 (23%)	3 (11%)	
DKD Stage 3A	12 (16%)	8 (17%)	4 (15%)	

DKD Stage 3B	5 (7%)	4 (9%)	1 (4 %)	
DKD Stage 4	5 (7%)	4 (9%)	1 (4%)	
DKD Stage 5	0 (0%)	0 (0%)	0 (0%)	
Pre-existing macrovascular complications				
Ischemic heart disease	19 (26%)	15 (32%)	4 (15%)	
Acute coronary syndrome	3 (4%)	3 (6%)	0 (0%)	
Coronary artery bypass graft	9 (12%)	7 (15%)	2 (7%)	
Congestive cardiac failure (systolic)	5 (7%)	3 (6%)	2 (7%)	
Congestive cardiac failure (diastolic)	10 (14%)	4 (9%)	6 (22%)	
Atrial fibrillation	4 (5%)	3 (6%)	1 (4%)	
Stroke	4 (5%)	3 (6%)	1 (4%)	
Transient ischemic attack	5 (7%)	3 (6%)	2 (7%)	
Peripheral vascular disease	7 (10%)	6 (13%)	1 (4%)	
Types of diabetes medication				
Metformin	60 (81%)	39 (83%)	21 (78%)	
Sulfonylurea	23 (31%)	17 (36%)	6 (22%)	
Dipeptidyl peptidase 4 inhibitor	9 (12%)	6 (13%)	3 (11%)	
Glucagon-like peptide 1 receptor agonist	12 (16%)	8 (17%)	4 (15%)	
Thiazolidinedione	1 (1%)	1 (2%)	0 (0%)	
Acarbose	2 (3%)	1 (2%)	1 (4%)	
Basal insulin	26 (35%)	19 (40%)	7 (26%)	
Bolus insulin	20 (27%)	14 (30%)	6 (22%)	
Pre-mixed insulin	15 (20%)	10 (21%)	5 (19%)	
Types of dyslipidemia medication				
Statin	62 (84%)	42 (89%)	20 (74%)	
Fibrate	6 (8%)	3 (6%)	3 (11%)	
Ezetimibe	7 (10%)	5 (11%)	2 (7%)	
Fish oil	3 (4%)	2 (4%)	1 (4%)	
Number of antihypertensive medication (median, IQR)	2 (1-3)	2 (2-3)	1 (0-1)	
Types of antihypertensive medication				
ß blocker	17 (23%)	12 (26%)	5 (19%)	
Calcium channel blocker	31 (42%)	23 (49%)	8 (30%)	
Angiotensin converting enzyme inhibitor (ACEI)	19 (26%)	19 (40%)	0 (0%)	
	28 (38%)	28 (60%)	0 (0%)	
Angiotensin II receptor blocker (ARB)	47 (64%)	47 (100%)	0 (0%)	
ACEI or ARB	12 (16%)	10 (21%)	2 (7%)	
Loop diuretic	18 (24%)	15 (32%)	3 (11%)	
Thiazide diuretic	5 (7%)	3 (6%)	2 (7 %)	
Potassium-sparing diuretic	6 (8%)	6 (13%)	0 (0%)	

Moxonidine	1 (1%)	1 (2%)	0 (0%)	
Methyldopa	4 (5%)	4 (9%)	0 (0%)	
Prazosin	5 (7%)	5 (11%)	0 (0%)	
Nitrates				
Smoker	9 (12%)	6 (12%)	3 (11%)	

Data are expressed as mean (Standard Deviation), or number (percentage), or median (interquartile range) as indicated. (*) P values are derived from the independent t-test for normally distributed data or from the Wilcoxon rank-sum test for data that is not normally distributed. Statistically significant values are in bold. Abbreviations: IQR, interquartile range; 24hUNa, 24-hour urinary sodium excretion; 24hUCr, 24-hour urinary creatinine excretion; GFR, glomerular filtration rate; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; eGFR, estimated glomerular filtration rate estimated using the CKD-EPI equation; CRP, c-reactive protein.

Table 2: Correlation between corrected 24hUNa excretion and Endothelial and Platelet Microparticle levels.

Microparticle subtype	Spearman's rank correlation value	P value
Endothelial microparticles		
Total CD31+/CD42b-	-0.17	0.14
CD31+/CD42b-/Annexin V+	-0.14	0.22
CD54+/CD105+	-0.08	0.50
CD105+/CD62e+	-0.07	0.54
Total CD105+	-0.17	0.16
Platelet microparticles		
CD42b+/CD41-	-0.14	0.23
Total CD42b+/CD41+	-0.08	0.48
CD41+/CD42b+/Annexin V+	-0.15	0.20
CD41+/CD42b-	-0.11	0.35

Data are expressed as Spearman's rank correlation value, p value.

Table 3: The relationship between RAAS blockade with the correlation between corrected 24hUNa excretion and microparticles.

Microparticle subtype	RAAS blockade use (n=47)		No RAAS blockade use (n=27)	
	Spearman's correlation	**P value**	**Spearman's correlation**	**P value**
CD 36 + microparticles	0.15	0.3	-0.4	**0.04**
Endothelial microparticles				
Total CD31+/CD42b-	0.15	0.3	-0.3	0.1
CD31+/CD42b-/Annexin V+	0.21	0.2	-0.3	0.1
CD54+/CD105+	-0.13	0.4	0.1	0.6
CD105+/CD62e+	-0.2	0.2	0.1	0.6
Total CD105+	-0.05	0.7	-0.2	0.4
Platelet microparticles				
CD42b+/CD41-	-0.05	0.8	-0.13	0.5
Total CD42b+/CD41+	0.27	0.07	-0.33	0.1
CD41+/CD42b+/Annexin V+	0.16	0.3	-0.32	0.1
CD41+/CD42b-	-0.02	0.9	-0.03	0.9

Data are expressed as Spearman's rank correlation value, p value. Bold values indicate statistical significance. Abbreviations: 24hUNa, 24-hour urinary sodium excretion; RAAS, Renin angiotensin aldosterone system.

Figures

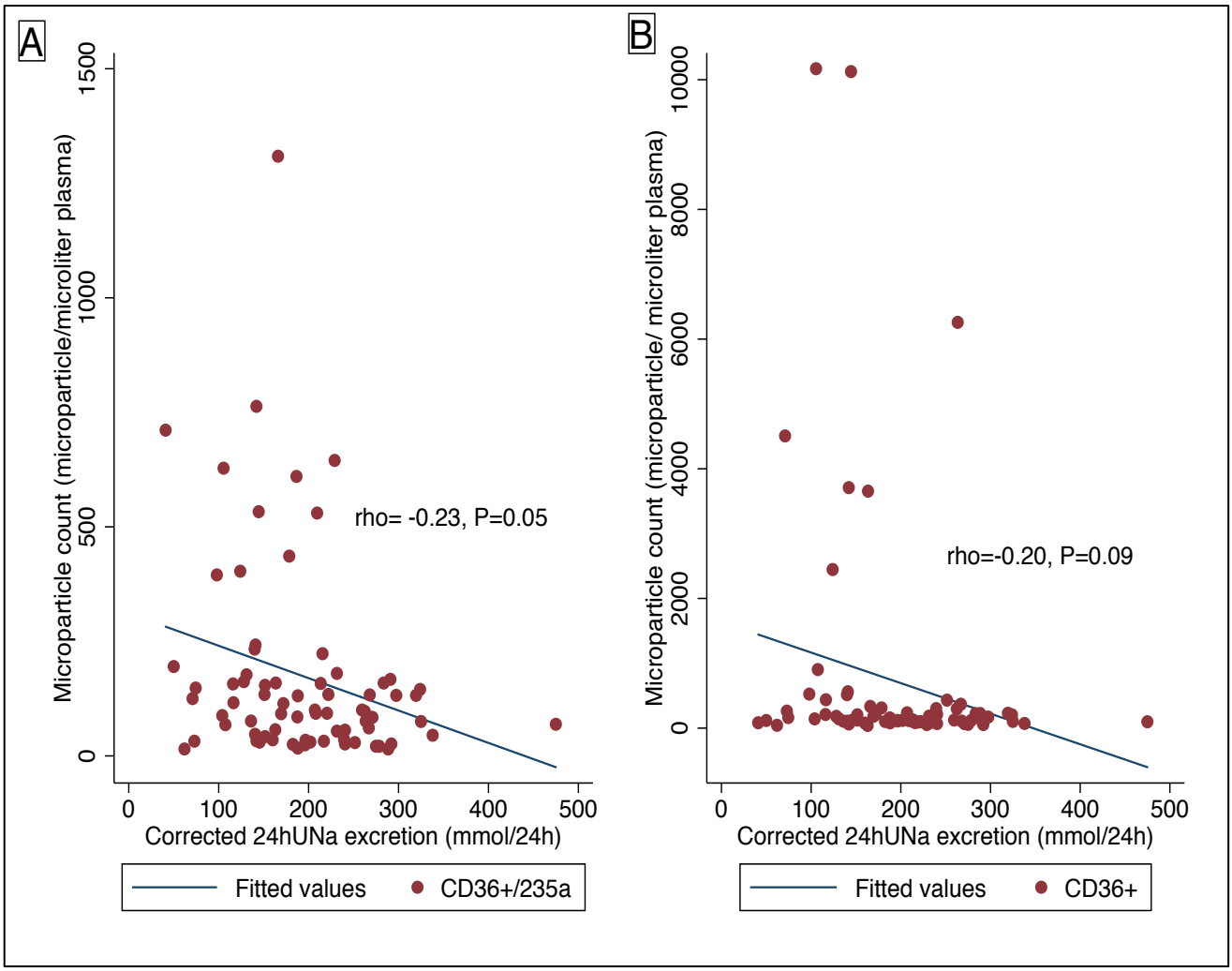


Figure 1: Correlation analysis between corrected 24hUNa excretion and CD36 microparticles

Data are expressed as Spearman's rank correlation value, p value. A: CD36+/235a+ erythrocyte microparticles. B: Total CD36+ microparticles. Abbreviations: 24hUNa, 24-hour urinary sodium excretion; MP, microparticles.

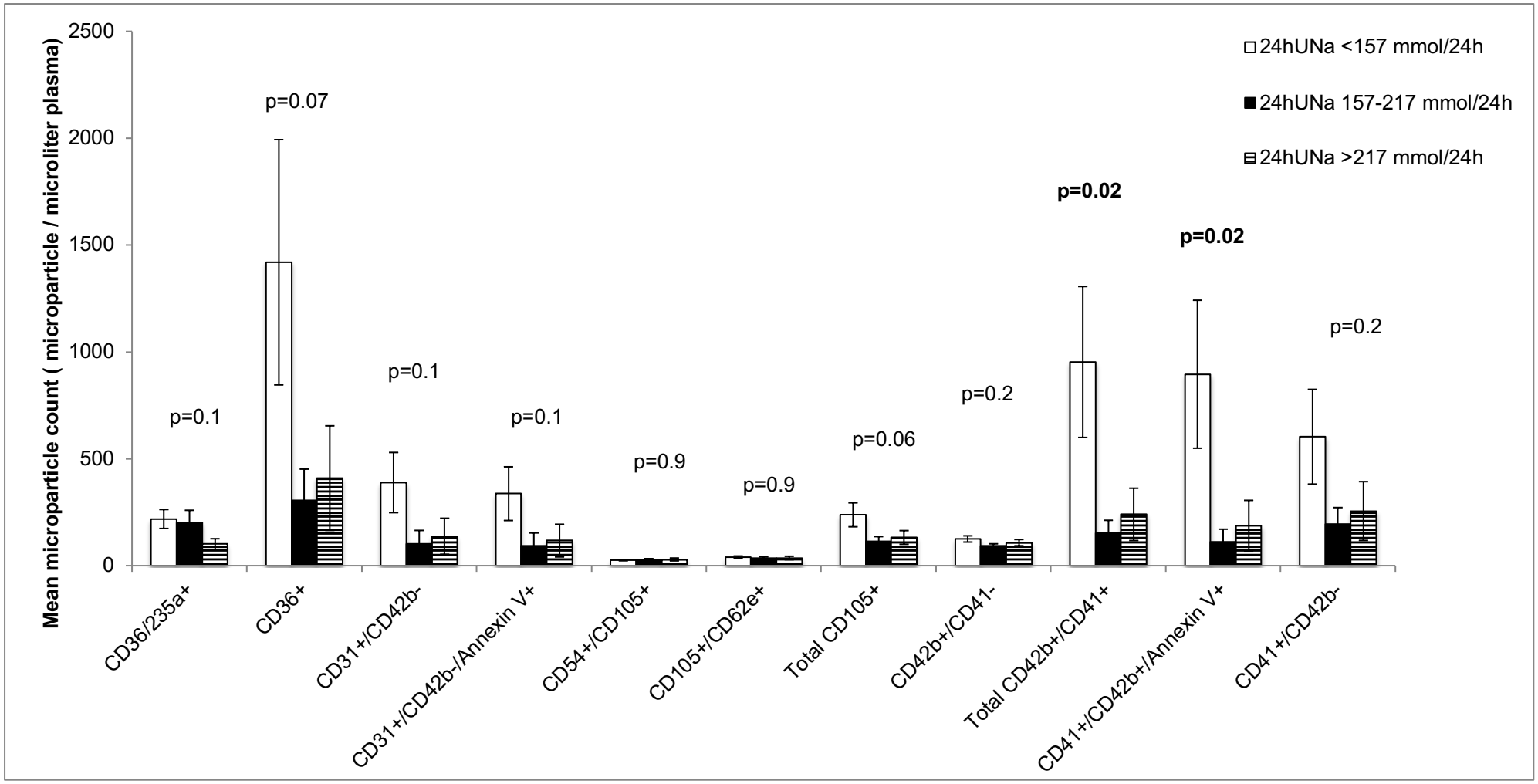

Mean microparticle count (microparticle / microliter plasma)
2500
2000
1500
1000
500
0
24hUNa <157 mmol/24h
24hUNa 157-217 mmol/24h
24hUNa >217 mmol/24h
p=0.1
p=0.07
p=0.1
p=0.1
p=0.9
p=0.9
p=0.06
p=0.2
p=0.02
p=0.02
p=0.2
CD36/235a+
CD36+
CD31+/CD42b-
CD31+/CD42b-/Annexin V+
CD54+/CD105+
CD105+/CD62e+
Total CD105+
CD42b+/CD41-
Total CD42b+/CD41+
CD41+/CD42b+/Annexin V+
CD41+/CD42b-

Figure 2: The association between sodium intake stratified according to tertiles of 24h urinary sodium excretion and mean microparticle counts

Data is expressed as mean (+/-SEM). Microparticle subsets: CD 36+/235a+, CD36+; *Endothelial microparticles*, Total CD31+/CD42b-, CD31+/CD42b-/Annexin V+, CD54+/CD105+, CD105+/CD62e+, Total CD105+; *Platelet microparticles*, CD42b+/CD41-, Total CD42b+/CD41+, CD41+/CD42b+/Annexin V+, CD41+/CD42b-. The p values are derived from One-way ANOVA. Bold values indicate statistical significance within the tertiles. 24hUNa <157mmol/24h represents Tertile 1(n=25), 157- 217 mmol/24h represents Tertile 2(n=24) and >217 mmol/24h represents Tertile 3(n=25). Abbreviations: 24hUNa, 24-hour urinary sodium excretion; RAAS, Renin angiotensin aldosterone system.

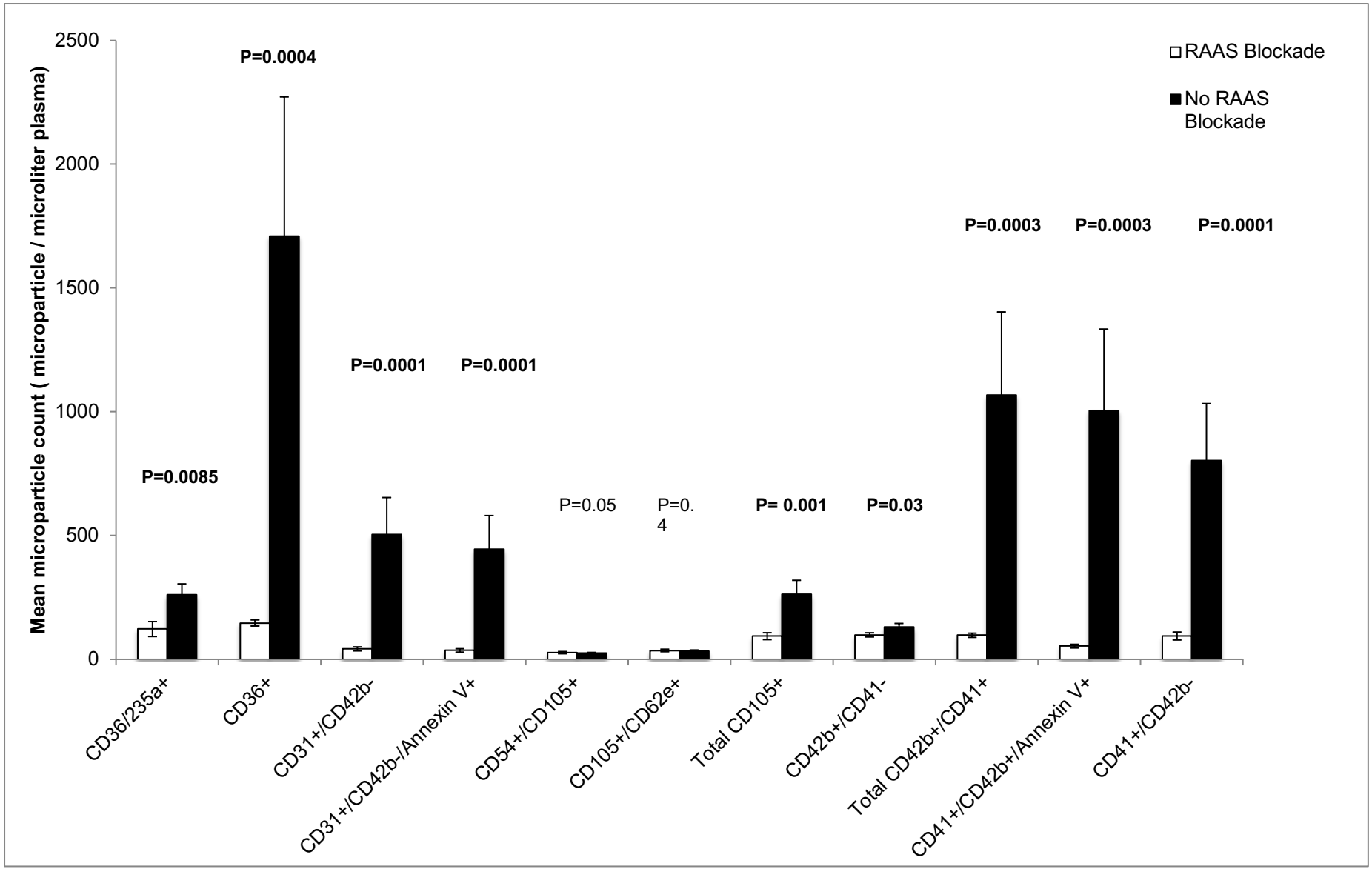

Mean microparticle count (microparticle / microliter plasma)
2500
2000
1500
1000
500
0
RAAS Blockade
No RAAS Blockade
P=0.0004
P=0.0085
P=0.0001
P=0.0001
P=0.05
P=0.4
P= 0.001
P=0.03
P=0.0003
P=0.0003
P=0.0001
CD36/235a+
CD36+
CD31+/CD42b-
CD31+/CD42b-/Annexin V+
CD54+/CD105+
CD105+/CD62e+
Total CD105+
CD42b+/CD41-
Total CD42b+/CD41+
CD41+/CD42b+/Annexin V+
CD41+/CD42b-

Figure 3: The relationship between RAAS blockade use with the mean microparticle counts

Data are expressed as mean (+/-SEM). Microparticle subsets: CD 36+/235a+, CD36+; *Endothelial microparticles*, Total CD31+/CD42b-, CD31+/CD42b-/Annexin V+, CD54+/CD105+, CD105+/CD62e+, Total CD105+; *Platelet microparticles*, CD42b+/CD41-, Total CD42b+/CD41+, CD41+/CD42b+/Annexin V+, CD41+/CD42b-. The p values are derived from the independent t-test. Bold values indicate statistical significance. Abbreviations: RAAS, Renin angiotensin aldosterone system.

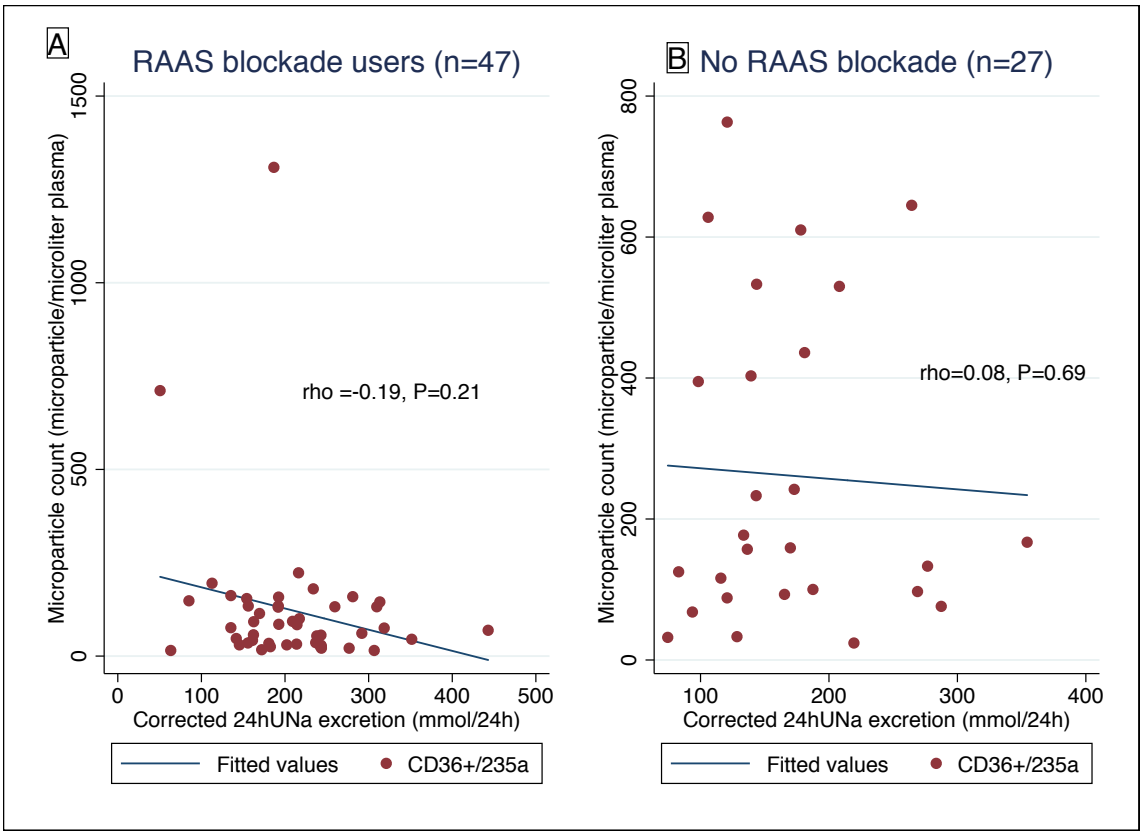


Figure 4: The relationship between RAAS blockade with the correlation between corrected 24hUNa excretion and CD36+/CD235a+ erythrocyte microparticles.

Data are expressed as Spearman's rank correlation value, p value. A: Effect of RAAS blockade on the correlation between CD36+/235a+ erythrocyte microparticles and 24hUNa excretion. B: The effect of having no RAAS blockade on CD36+/235a+ erythrocyte microparticles and 24hUNa excretion. Abbreviations: 24hUNa, 24-hour urinary sodium excretion; MP, microparticles; RAAS, Renin angiotensin aldosterone system.

Supplementary Information

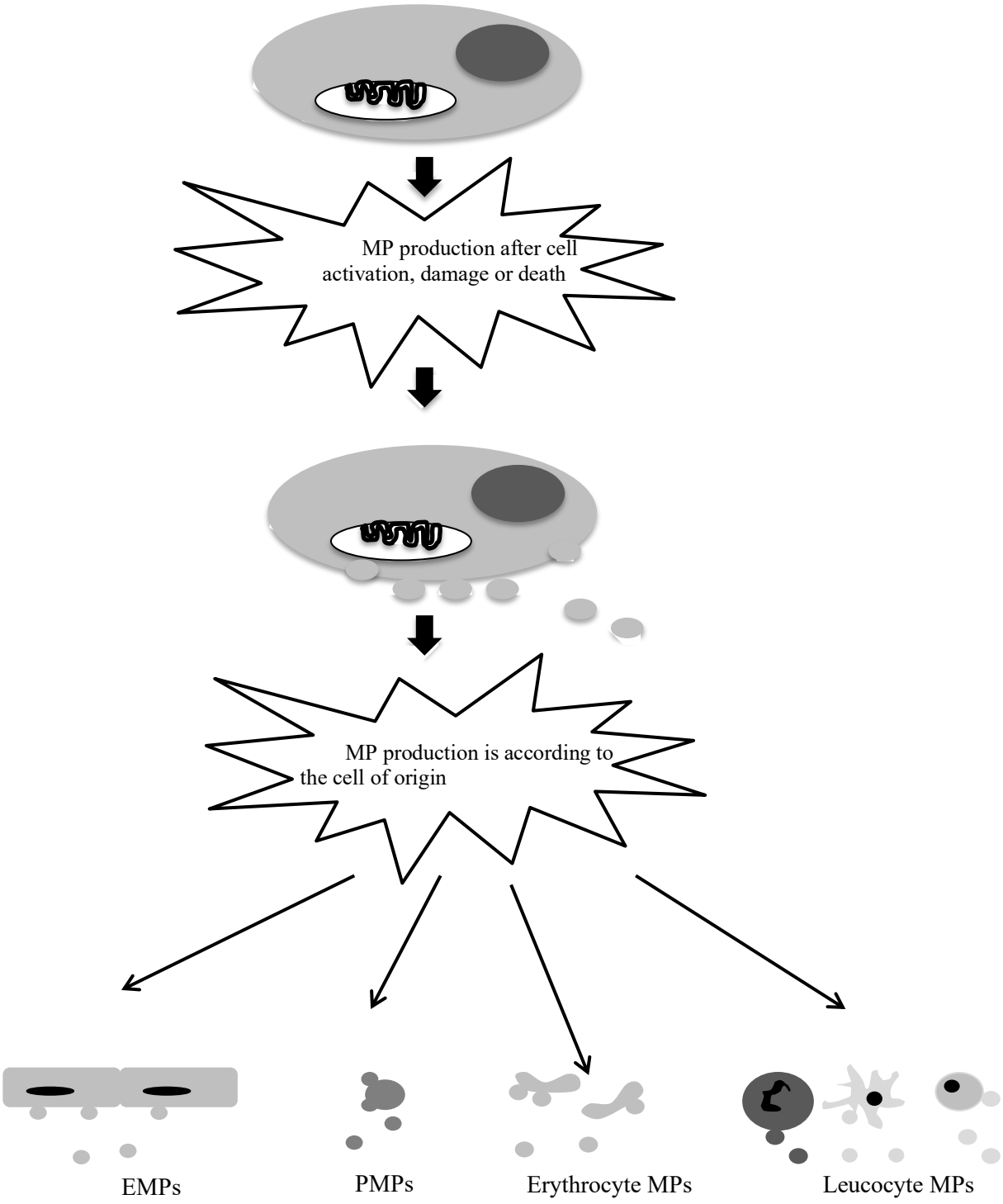
Supplementary Table 1: Multiple regression analysis between corrected microparticle subsets with 24hUNa excretion and plasma renin activity (n=15).

Microparticle subtype	Coefficient of determination value	P value
CD 36 Microparticles		
CD 36+	0.06	0.7
CD36+/235a+	0.05	0.7
Endothelial microparticles		
Total CD31+/CD42b	0.14	0.4
CD31+/CD42b-/Annexin V+	0.14	0.4
CD54+/CD105+	0.1	0.5
CD105+/CD62e+	0.02	0.9
Total CD105+	0.07	0.7
Platelet microparticles		
CD42b+/CD41-	0.3	0.1
Total CD42b+/CD41+	0.03	0.8
CD41+/CD42b+/Annexin V+	0.02	0.9
CD41+/CD42b-	0.12	0.5

Data are expressed as Coefficient of determinant value, p value.

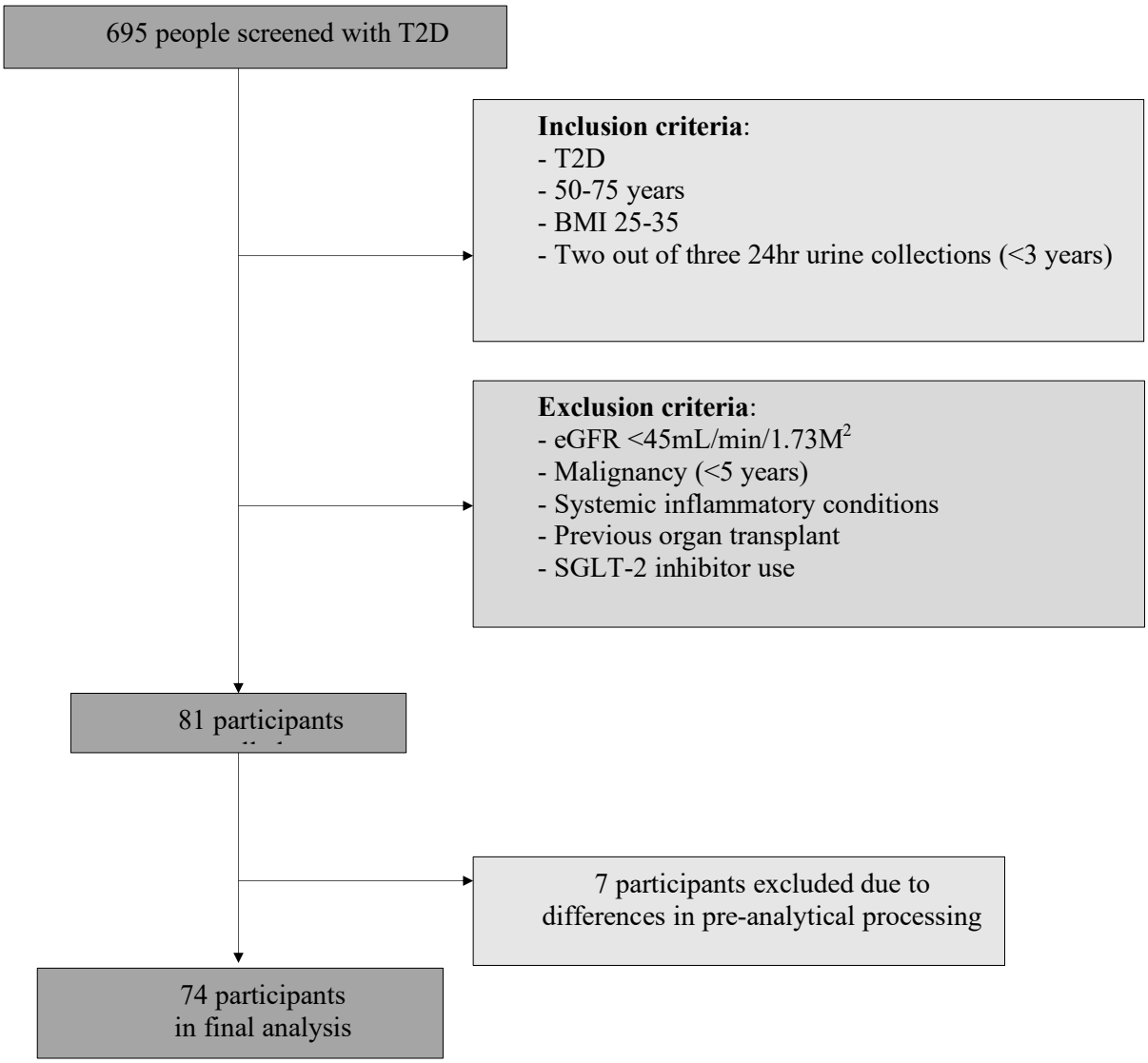
Supplementary Table 2: Studies investigating the effect of RAAS blockade on microparticles in those with or without diabetes

Study	Number of participants	Drug investigated	Findings
Moriya et al 2015 (32).	21 on hemodialysis with hypertension	Aliskiren 150mg once daily for 12 weeks	Reduction in platelet-derived microparticles and improved flow mediated dilation in patients on haemodialysis, independent of its antihypertensive effect
Nomura et al 2006 (33)	53 hypertensive with (n=28) or without type 2 diabetes (n=25) and compared to normotensive controls with (n=12) or without diabetes (n=20)	Valsartan 80mg daily for 8 weeks	Monocyte and endothelial cell activation marker levels were higher in hypertensive participants with type 2 diabetes compared to normotensive controls and were significantly reduced by valsartan in those with type 2 diabetes
Silvestre M 2005 (31)	30 with stage 1 or 2 hypertension compared to 31 controls.	Eprosartan 600mg daily for two months	Eprosartan reduced platelet microparticle formation in addition to its blood pressure lowering effect.



Supplementary Figure 1: Schematic representation of the production of microparticles

Microparticles are formed from the outward blebbing of the plasma membrane and released into the extracellular space, retaining cell-surface proteins and cytosolic contents from the cell of origin. Abbreviations: MPs, microparticles; EMPs, endothelial microparticles; PMPs, platelet microparticles.



Supplementary Figure 2: Patient recruitment flowchart

Abbreviations: T2D, Type 2 Diabetes; BMI, body mass index; eGFR, estimated glomerular filtration rate; SGLT-2, sodium glucose co-transporter 2 inhibitor.

Chapter 6: Dietary sodium and potassium intake in people with diabetes: are guidelines being met?

This chapter will be presented as the author-accepted manuscript of a peer-reviewed article published in *Nutrition and Diabetes*. 2020; 10(23).

Abstract

Objective: Despite public health bodies advocating for lowering dietary sodium and increasing potassium intake to improve cardiovascular outcomes, people with diabetes are not meeting these targets. We hypothesize that (i) both at an individual level and within the cohort, there will be a low adherence to the guidelines and (ii) sodium and potassium intake will remain stable over time.

Methods: We conducted this prospective study in a cohort of 904 participants with diabetes who provided 24-hour urine collections from 2009 – 2015. Dietary sodium and potassium intake were estimated from 24h urinary sodium (uNa) and potassium (uK) measurements. Additional data were collected for: 24h urinary volume (uVol), creatinine (uCr),; serum creatinine, urea, estimated glomerular filtration rate (eGFR), glycated haemoglobin (HbA1c), fasting glucose, lipids); clinical characteristics (age, blood pressure (BP), body mass index (BMI) and duration of diabetes). Adherence to recommended dietary sodium (uNa <2300mg/24h (100mmo/24h)) and potassium (uK > 4680mg/24h(120mmol/24)) intake were the main outcome measures.

Results: Participants (n=904) completed 3689 urine collections (average 4 collections/participant). The mean ±SD (mmol/24h) for uNa was 181 ±73 and uK was 76 ± 25. After correcting uNa for uCr, 7% and 5% of participants met dietary sodium and potassium guidelines respectively. Males were less likely to meet sodium guidelines (OR 0.40, $p<0.001$) but were more likely to meet potassium guidelines (OR 6.13, $p<0.001$). Longer duration of diabetes was associated with higher adherence to sodium and potassium guidelines (OR 1.04, $p<0.001$ and OR 0.96, $p = 0.006$ respectively). Increasing age was significantly associated with adherence to potassium guidelines (OR 0.97, $p = 0.007$).

Conclusions: People with diabetes do not follow current dietary sodium and potassium guidelines and are less likely to change their dietary intake of sodium and potassium over time.

Key Terms
sodium, salt, NaCl, endothelial dysfunction, type 2 diabetes,
cardiovascular disease, dietary sodium intake, dietary potassium intake

Condensed abstract
In this prospective study, we estimated dietary sodium and potassium intake in people with diabetes from 24hour urinary measurements. Of the 904 participants who provided 3689 urine collections, only 7% and 5% met the national guidelines for dietary sodium and potassium intake respectively. Dietary intake overall remained stable over time. Despite health bodies advocating for lowering dietary sodium and increasing potassium, these guidelines are not being met and intake is unlikely to change over time.

Introduction

Cardiovascular related diseases are the leading cause of morbidity and mortality [1], especially in those with diabetes [2]. Blood pressure is a modifiable cardiovascular risk factor [1]. Dietary sodium and potassium intake play a pivotal role in blood pressure regulation [1, 3]. High dietary salt intake can raise blood pressure [3, 4] whereas a diet low in sodium and high in potassium is associated with lower blood pressure [5, 6]. Not surprisingly, public health bodies such as the American Diabetes Association [7] and Institute of Medicine [8], recommend an upper limit of sodium intake at 2300mg per day (100mmol/24h) [7 8] and daily potassium intake of 4680mg per day (120mmol/24)[8].

However, worldwide mean sodium intake is almost double the recommended level of intake (3,950mg/24h (172mmol/24h)) [9]. A recent meta-analysis of the Australian population found that the mean sodium intake in people with type 1 or type 2 diabetes was 9.66g/24h (420 mmol/24h) [10]. Therefore, almost all of the population will have to implement major dietary changes in order to achieve the recommended targets [11]. Whether adhering to these guidelines are necessary and achievable [12] for people with diabetes has been called into question. Despite its blood pressure lowering effects, lower sodium intake has been paradoxically associated with higher cardiovascular and all-cause mortality in people with diabetes [13, 14]. Moreover, in a community-dwelling

population, inverse associations have been demonstrated between low sodium intake and myocardial infarction and mortality [15].

The association between low dietary sodium intake and total and cardiovascular mortality has been inconsistent. Study findings have suggested lower sodium intake is associated with either a higher risk of death [13-15], a lower risk of death [16-18], U-/J-shaped relationship [19,20], an uncertain association [21,22] or no association[23] between sodium intake and cardiovascular health outcomes. Methodological differences may account for these discrepancies. In earlier studies, sodium intake was estimated by dietary recall which underestimates intake by 50% [24]. Measurement of 24-hour urinary sodium excretion (uNa) [25] provides a more accurate estimation of dietary sodium intake.

Whilst four studies in Australia have utilized 24h urine collections to assess adherence to dietary sodium and potassium intake in people with diabetes [12, 26-28], only one study has assessed dietary sodium and potassium consumption in people with both type 1 and type 2 diabetes [26]. We, therefore, aimed to provide an updated estimate of whether people with diabetes of any type are adhering to current dietary sodium and potassium guidelines. We assessed our study cohort's sodium and potassium dietary intake over the study period (cohort level analysis). Additionally, we estimated the percentage of adherence to these dietary guidelines over the study period (individual-level analysis). We hypothesize that (i) there will be an overall low adherence to the guidelines and (ii) sodium and potassium intake will not change over time.

Research Design and Methods

Study Design and Recruitment of Participants

In this prospective study, we recruited 904 participants with a diagnosis of diabetes from our university teaching hospital diabetes outpatient clinics. We assessed adherence to dietary sodium and potassium guidelines using 24h urinary excretion values, as the best estimate for dietary intake, from 2009 to 2015 inclusive. The dietary guidelines used to define sodium and potassium intake for this study were based on the American Diabetes Association recommendations[7] of sodium intake < 2300mg per day

(100mmol/24h) [7, 8] and from the Institute of Medicine potassium intake recommendations at > 4680mg per day (120mmol/24)[8] as they are specifically intended for high-risk subgroups, such as those with diabetes. This study was approved by the Austin Health Human Research Ethics Committee. Written consent was obtained from all participants.

Endpoints

The primary endpoints were to (i) provide an estimate of an individuals' sodium intake over the seven-year period and to (ii) assess the adherence to the dietary sodium intake guidelines at a cohort level. Our secondary endpoints were to estimate the potassium intake, as above, at both the individual and cohort level.

Biochemical urinary and serum analyses

Twenty-four-hour urinary sodium (uNa), urinary potassium (uK), urinary volume (uVol), urinary creatinine (uCr), urinary urea (uUrea), and urinary glucose (uGlu) were recorded for each urine sample collected by the participants over the seven-year period. Biochemical parameters of serum creatinine, estimated glomerular filtration rate (eGFR) as calculated by Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula, serum urea, uric acid, glycated haemoglobin (HbA1c), fasting glucose, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides and c-peptide were collected.

Urinary volume was recorded by weighing the specimen. No preservative was used for the 24 h urine collection. From January 2009 to January 2012, uNa, uK and uGlu, uCr, fasting glucose, serum urea, total cholesterol, serum HDL and serum triglycerides were analyzed by Beckman Coulter UniCel DXC800⁄600 System(s) Analyzer. From January 2012 until December 2015 our institution's pathology department protocol was changed and the same parameters were analyzed on Roche Cobas 8000. The HbA1c was measured by immunoassay on Roche Integra and LDL was calculated using the Friedewald equation[29].

Given approximately 90% of ingested sodium and 80% of potassium is excreted in the urine [25], we used 24h urinary excretion to most accurately estimate dietary sodium and

potassium intake over other methods such as dietary recall [24]. We have previously demonstrated that a single measurement of 24hUNa can predict habitual dietary sodium intake in people with type 2 diabetes, with an intra-individual coefficient of variation 21 ±1% suggesting day-to-day variation of sodium intake is approximately 20% [12]. To improve accuracy, we analyzed multiple urine collections for each participant.

Ensuring the accuracy of urine collections

Patients attending the diabetes clinics routinely perform 24-hour urine collections. Written and verbal instructions are provided by clinicians to ensure the accuracy of urine collections. For each participant's uNa collection, we corrected for any changes in renal function over time and for the possibility of incomplete urine collections by calculating the mean 24hUCr measurements and then utilizing the following formula:

$$Corrected\ uNa = \frac{uNa\ x\ overall\ mean\ uCr\ for\ that\ individual}{uCr\ in\ that\ sample}$$

To remain comparable with previous regional surveys and blood pressure trials [13, 14, 19, 20], whereby urinary sodium levels were measured, we did not adjust dietary sodium and potassium intake excretion to compensate for insensible losses. The uNa and uK values were then used to determine the adherence to the dietary guidelines at both an individual and a population level and to determine the intra-individual variability of sodium and potassium intake over time.

Anthropometry and clinical characteristics

Where available, additional characteristics such as blood pressure, body mass index (BMI) and duration of diabetes were recorded.

Statistics and data analysis

A single investigator collected all the data. A 10% random validity check was done to assess for accuracy. Baseline characteristics are reported as mean ± standard deviation. Yearly averages of both 24h uNa and uK excretion were calculated and graphed accordingly to assess the cohort level adherence. Individual-level adherence was

assessed by estimating the percentage a participant would adhere to the dietary guidelines over time. The intra-individual change over time was defined as an average of the percentage that each participant met the guidelines. For example, if the participant had ten 24h uNa readings and met the guidelines once, their percentage would be 10%. We then calculated the mean of these percentages for the cohort and represented the data as median (interquartile range). The data were represented as a box-plot. This was deemed the most effective method of calculating the intra-individual change over the study period time. Univariate logistic regression analysis was used to describe the univariate relationships between explanatory variables such as age, sex, duration of diabetes, HbA1c, fasting glucose, eGFR, serum urea, and lipid profile and a patient's ability to meet the dietary sodium and potassium guidelines. The effect of these explanatory variables on an individual's ability to meet the guidelines was reported with an odds ratio and a 95% confidence interval. Multivariable logistic regression analysis was performed determining the effect of age, sex, duration of diabetes and HbA1c on the ability to meet the guidelines. Multiple regression results allow us to account for potential redundancy in the explanatory variables. Co-linearity tests were performed to account for any substantial correlation between any of the variables included in the multivariable analysis that may have affected the results. A p-value of <0.05 was considered statistically significant. Statistical analysis was performed with STATA version 14.1 software (StataCorp. 2015. *Stata Statistical Software: Release 14*. College Station, TX: StataCorp LP).

Results

Baseline cohort characteristics

This study included 904 participants of whom 732 (81%) had type 2 diabetes, 144 (15.9%) had type 1 diabetes and 10 (1.1%) had latent autoimmune diabetes in adults. Secondary causes such as corticosteroid-induced diabetes, new-onset diabetes after transplant, hemochromatosis, and pancreatitis induced pancreatic insufficiency were seen in 12 (1.3%) participants. Six participants had no documentation of their type of diabetes.

Participants completed a total of 3689 24h urine collections, ranging from one to sixteen collections per participant with an average of four 24h urine collections. Baseline characteristics are summarized in Table 1. Once adjusted for uCr excretion the corrected mean ± SD sodium excretion (mmol/24h) was 181 ± 73 and the mean± SD potassium excretion (mmol/24h) was 76 ± 25 (Table 1). After this correction, 63 of the 904 (7%) participants were adherent to the dietary sodium guidelines and 42 of the 904 participants (5%) participants were adherent to the dietary potassium guidelines. The mean ± SD sodium to potassium ratio was calculated separately and in our cohort was 2.5±0.9.

The following classes of medications, which are known to affect sodium and or potassium excretion, were taken at the time of the urine collections (n=3689) as follows: Sodium-glucose co-transporter-2 inhibitors in 13 (0.4 %) collections; Potassium Sparing Diuretics in 80 (2.2 %) collections; Loop diuretics in 273 (2.2%) collections and Thiazide diuretics in 663 (18%) collections.

Cohort level analysis

The mean yearly uNa from 2009 – 2015 remained above the recommended sodium intake target of <100mmol/24h (figure 1)[30]. Similarly, the mean yearly uK remained well below the potassium guideline recommendations of >120mmol/24h (figure 2)[31]. Over time, at a population level, sodium and potassium intake did not change (figures 1 and 2).

Individual-level analysis

The intra-individual variability and the percentage of participants adhering to the guidelines over time remained low. The percentage of adherence to the guidelines over time was (median (interquartile range) (number of times guidelines met / number of 24hUNa samples)) 0% (25%) for sodium guidelines and 0% (0%) for potassium guidelines.

Figure 3[32] represents the participants' percentage of adhering to the dietary sodium guidelines. The box-plot suggests that 50% of participants are non-adherent. Of the

remaining 50%, 50% adhere to the guidelines approximately 20% of the time and 50% approximately 60% of the times. Figure 4[33] represents the participants' likelihood of adhering to dietary potassium guidelines. The box-plot suggests that 100% of participants are unlikely to adhere to the potassium intake guidelines.

Univariate analysis

We examined the likelihood of participants meeting the guidelines in relation to eleven explanatory variables (age, sex, duration of diabetes, HbA1c, fasting glucose, eGFR, serum urea and lipid profile (cholesterol, HDL, LDL and triglycerides)) (Table 2).

Regarding sodium intake, males were less likely to meet the guidelines (OR 0.40, $p<0.001$). Participants were more likely to meet guidelines as duration of diabetes increased (OR 1.04, $p<0.001$). Age had no significant effect (OR 1.01, $p = 0.06$). A higher eGFR and triglyceride level was associated with participants being less likely to meet guidelines (OR 0.99, $p = 0.003$, and OR 0.86, $p = 0.04$ respectively). Higher LDL levels were associated with a trend towards a lower likelihood of participants meeting guidelines (OR 0.86, $p = 0.05$). No other parameters had any significant effect (Table 2).

For potassium intake, male participants were more likely to meet the guidelines (OR 6.13, $p<0.001$). Increasing duration of diabetes (OR 0.96, $p = 0.006$) and age had a higher likelihood of adhering to the guidelines (OR 0.97, $p = 0.007$). No other parameters had any significant effect (Table 2).

Multivariable analysis

The following variables: age, sex, duration of diabetes and HbA1c, were entered into a multivariable regression analysis as these showed the strongest association with a participant's ability to adhere to the dietary sodium and potassium guidelines from the univariate analysis. For dietary sodium, male sex (OR 0.38, $p<0.001$), duration of diabetes (OR 1.04, $p<0.001$) and HbA1c (OR 0.86, $p = 0.01$) were all significantly associated with a participant's ability to meet the sodium guidelines when controlled for each other (Table 2). For the dietary potassium guidelines, male sex (OR 5.73, $p<0.001$) and HbA1c (OR 0.77, $p = 0.02$) showed a significant association when controlled for the other explanatory variables (Table 2). The potential for collinearity

was accounted for when these four parameters were placed in the multiple regression analysis and no major correlations were found.

Discussion

Key findings

Public health bodies advocate for high sodium and low potassium intake to improve cardiovascular health outcomes. We demonstrated that people with diabetes are having difficulties adhering to these recommendations with 93 % and 95 % of participants not meeting sodium and potassium guidelines respectively. The likelihood of changing dietary intake over time to adhere to the guidelines is low.

People with diabetes are not meeting recommended dietary sodium and potassium guidelines and this is unlikely to change over time.

The World Health Organisation aims to reduce the mean population sodium intake by 30% by the year 2025 [34]. Many countries, including Australia, have signed up to these global targets. Despite strategies being implemented to reduce dietary salt intake over many years [35], progress has been slow [36, 37]. In the present study, we demonstrated that the yearly mean sodium and potassium intake in people with diabetes in Australia, who attended a university teaching hospital, exceeds recommendations made by public health bodies. Only 7% of participants achieved dietary sodium targets (<100mmol/24h) and 5% of participants achieved dietary potassium targets (>120 mmol/24h). These results are consistent with previous studies in people with diabetes[12] and comparable to other significant past [38, 39] and recent [40] large-scale population studies whereby the majority of people do not adhere to guidelines.

Additionally, the likelihood of each participant meeting the dietary guidelines over time was low. Our results are in keeping with a recent meta-analysis that demonstrated there had been no change in dietary sodium consumption in the Australian population since 1989 [10]. A limitation of that study [10], however, was that the analysis of change over time was not based on repeat representative samples of the population. An advantage of the current study is having the average of four urine samples per participant. This provides

a more accurate measure of temporal changes in dietary intake over time at an individual level.

In the present study, participants were more likely to adhere to the dietary sodium guidelines as the duration of diabetes increased. Previous studies have shown that people with poorer health and comorbidities have reduced appetites and consume less dietary sodium [41]. Interestingly, in our study, as age increased, there was no significant association in the ability to adhere to sodium guidelines. Similarly, in a meta-analysis, there was no relationship between age and sodium intake [10].

The HbA1c, as a single explanatory variable, had no association with the ability to adhere to sodium intake guidelines. However, in the multivariable analysis, after adjusting for age, sex, and duration of diabetes, lower HbA1c was significantly associated with the ability to meet guidelines. Given the observational nature of the study it is not possible to infer the nature of this relationship.

Males were less likely to adhere to sodium intake guidelines but were more likely to adhere to potassium intake guidelines. Males consume more food than women and as a result have higher sodium as well as higher potassium intakes overall. Our findings are consistent with the recent meta-analysis of Australian sodium consumption demonstrating a sex difference in sodium intake [10].

Salt appetite, salt taste perception and sodium content in foods may explain variations in sodium intake.

Overall, people with diabetes may have increased salt appetite and reduced salt taste perception to account for the inability to adhere to low sodium intake. It is not known how salt appetite is derived, however, evidence suggests that genetics [42], environment [43] and pre-existing disease states [41] may play a role. A gene polymorphism (C825T) has been associated with low sodium intake in people with type 2 diabetes [42]. People with type 2 diabetes with low sodium intake were found to have the genetic polymorphism, whereas those with high sodium intake did not [42]. Furthermore, people with diabetes and hypertension have reduced salt taste perception and are unlikely to recognize the amount of sodium consumed [44]. No previous study has examined salt appetite and salt

taste perception in conjunction with 24h uNa in people with diabetes. This presents an opportunity for future studies to investigate this concept further.

Sources of dietary sodium are also important to consider. The Japanese diet, for example, despite its high sodium intake, has been associated with lower risk of cardiovascular disease [45] as dietary sodium comes from soybean products, fish, seaweed and green tea which may have unique properties to benefit the cardiovascular system [45]. In Western countries, such as the United Kingdom, United States [43] and Australia [46], approximately 80% of sodium [46] is from processed foods. Strategies in Western countries aimed at modifying consumer behaviour, such as advising people not to add salt to their foods, may not reduce dietary sodium intake, as added salt is only a small proportion of their daily sodium intake[47]. Education on sodium content in food may have more bearing [36] on health outcomes.

However, sodium content in foods is poorly understood by people with type 2 diabetes [28, 48]. In a randomized controlled trial, a single session of food label education to help choose low sodium products (<120mg/100g) in people with type 2 diabetes did not reduce mean 24h uNa excretion as compared to placebo, despite high levels of self-reported adherence [28]. Furthermore, in a separate study, despite 80% of people reporting reading food labels, dietary sodium intake, as estimated from 24h uNa, remained high at 3,887 +/- 736mg/24h (169 +/- 32mmol/24h) in males and 2,645 +/- 621mg/24h (115 +/- 27mmol/24h) in females [48]. The major contributors to dietary sodium intake in people with type 2 diabetes [28] come from staple foods, such as bread and cereals. Most people are unaware these foods contain large amounts of sodium [48]. Therefore, whilst we did not formally assess the sources of dietary sodium in our study, the aforementioned studies [28, 48] provide insight into the dietary habits of people with diabetes in Australia and may, in part, explain why our cohort did not meet dietary sodium recommendations.

Potassium intake and the role of the sodium to potassium ratio

Only 5% of participants met dietary potassium intake guidelines. People with diabetes consume processed foods with high sodium contents [46] which are most likely depleted of potassium [41]. Males were more likely to meet guidelines consistent with previous

studies demonstrating that whilst potassium intake is low globally [6, 41], a sex difference for potassium exists [41]. Potassium is considered to play a crucial role in cardiovascular health outcomes and deserves similar attention to sodium intake. Potassium intake has been shown to have an inverse relationship with blood pressure [6], with those in the highest range of potassium intake having the lowest blood pressure [49].

The ratio of sodium to potassium intake is considered more important on cardiovascular health outcomes than each factor alone [41 50]. Lower sodium to potassium ratio is associated with lower blood pressure [41]. Higher sodium to potassium ratio[50], consistent with high sodium intake and low potassium intake, as was seen in our study, is associated with adverse cardiovascular health outcomes[50].

Is there a range for optimal sodium intake?

Optimal dietary sodium targets are a matter of ongoing debate. Observational studies demonstrate that sodium intakes below 2,645mg (115mmol/24h) and above 4,945mg (215mmol/24h) are associated with higher mortality [49, 51]. The results of a meta-analysis supports these findings by comparing three sodium groups; high (> 5000mg/24h or 217mmol/24h), moderate (2700-5000mg/24h or 117-217mmol/24h) and low (< 2700mg/24h or 117mmol/24h), with the risk of a cardiovascular event[52]. Whilst the high sodium group had the highest risk of an event and all-cause mortality, the moderate sodium group was found to be at lower risk of an event and all-cause mortality as compared to the low sodium group [52]. Multiple observational studies report sodium intake between 115mmol/24h and 215mmol/24h may provide the best cardiovascular outcomes [53]. It is reassuring that the majority of participants in the present study were consuming sodium within this range. Although the Institute of Medicine [8] and the American Diabetes Association [7] no longer recommend very low sodium intakes of < 70mmol/24h in people with diabetes and recommend sodium intake of < 100mmol/24h consistent with the general population, in light of the aforementioned evidence and the demonstration in our study that people with diabetes are simply unable to meet these targets, these recommendations [7 8] may need to be further revised. Furthermore, randomized controlled trials assessing sodium intake and hard outcomes such as cardiovascular events and mortality are greatly needed [8].

Strengths and Limitations

The relatively large sample size of 3689 urine collections is considered a major strength. Furthermore, utilizing 24h uNa and uK excretion to estimate dietary intake is considered more accurate [25] over other methods such as dietary recall [24]. The study design being prospective and longitudinal enables the detection of changes in dietary sodium and potassium intake over time and helps in determining the intra-individual variability and the likelihood of change by participants over time. We acknowledge that medications known to affect sodium and or potassium excretion were not excluded. Excluding participants based on the use of these medications, would have significantly reduced the number of participants whose data is available to analyze given these medications are routinely prescribed to people with diabetes. Furthermore we have previously shown that treatment with thiazide diuretics for > 4 weeks does not affect urinary sodium excretion[54]. Additionally, the number of urine collections and the time interval between each collection were not consistent between participants. However, a major strength is having an average of four urine collections per participant which more accurately reflects habitual dietary patterns.

Our findings demonstrate that people with diabetes are not adhering to dietary sodium and potassium recommendations at an individual or at a population level. There is a low likelihood of adhering to such guidelines over time as dietary intake of sodium and potassium remains stable. Our findings, coupled with the previous demonstration in observational studies of an association between low dietary sodium intake and adverse cardiovascular health outcomes, questions the necessity for stringent sodium lowering. Long-term interventional studies are greatly needed to determine the ideal yet feasible dietary sodium intake targets in people with diabetes.

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Author Contributions:

SB, AM, and EIE were involved in the study design. SB and AM were involved in data interpretation. All authors were involved in the editing of the manuscript. EIE approved the study design and the final content of the manuscript. We thank Dr. Que Lam who assisted with data collection and Professor Leonid Churilov for statistical guidance.

Conflict of Interest:

EIE is an investigator in separate studies sponsored by Novo Nordisk, Bayer, Sanofi, and Mobius Medical and the research funds are paid to EIE's institution to contribute towards diabetes research. The remaining authors have no conflict of interest to declare.

List of abbreviations

uCr: 24-hour urinary creatinine excretion

uNa: 24-hour urinary sodium excretion

uK: 24-hour urinary potassium excretion

uVol : 24-hour urinary volume

uUrea: 24-hour urinary urea excretion

uGlu : 24-hour urinary glucose excretion

BMI: Body mass index

CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration

eGFR: estimated glomerular filtration rate

HbA1c: Glycosylated haemoglobin A1c

HDL: High-density lipoprotein

HRV: Heart rate variability

LDL: Low-density lipoprotein

NHF: National Heart Foundation

OR: Odds Ratio

SD: Standard deviation

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Tables

Table 2 - Baseline characteristics of study participants

Variable	Mean±SD	95% Confidence Interval
Total number of participants	904	
Male sex	551	
Age (years)	60±13	
Duration of diabetes (years)	13±9	-5 – 32
Body Mass Index (kg/m^2)	31.4±6.7	18.0 – 44.7
Systolic blood pressure (mmHg)	134±16	103 – 165
Diastolic blood pressure (mmHg)	73±10	54 – 93
24h uNa (mmol/24h)	181.2±72.7	35.9 – 326.6
24h uK (mmol/24h)	76.1±25.4	25.4 – 126.8
24h uCr (mmol/24h)	12.8±4.4	4.0 – 21.5
24h uGlu (mmol/24h)	52.0±104.6	-157.3 – 261.2
24h uUrea (mmol/24h)	441.2±156.8	128 – 755
HbA1c (%), [mmol/mol]	7.7±1.2, [61]	5.3 – 10.0
Fasting Glucose (mmol/l)	8.5±2.6	3.4 – 13.7
CKD-EPI eGFR (ml/min/1.73m^2)	77.9±23.9	30.0 – 125.6
Cholesterol (mmol/l)	4.1±0.9	2.3 – 5.9
HDL (mmol/l)	1.2±0.7	-0.1 – 2.6
LDL (mmol/l)	2.2±0.8	0.7 – 3.7
Triglycerides (mmol/l)	1.6±1.1	-0.5 – 3.8

Data are expressed as mean±standard deviation with 95% confidence interval. Abbreviations: IQR, interquartile range; 24h uNa, 24-hour urinary sodium excretion; 24h uK, 24-hour urinary potassium excretion;24h uCr, 24-hour urinary creatinine excretion; 24h uGlu, 24-hour urinary glucose excretion; 24h uUrea, 24-hour urinary urea excretion; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; eGFR, estimated glomerular filtration rate estimated using the CKD-EPI equation.

Table 2 – Results of Univariate and Multivariable analysis

Explanatory Variable	Urinary Sodium Guidelines						Urinary Potassium Guidelines					
	Univariate			Multivariable			Univariate			Multivariable		
	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value
Age (years)	1.01	(1.00 - 1.02)	0.06	1.00	(0.98 - 1.01)	0.54	0.97	(0.96 - 1.00)	**0.007**	0.99	(0.96 - 1.00)	0.21
Sex (males)	**0.40**	**(0.31 - 0.52)**	**<0.001**	**0.38**	**(0.28 - 0.53)**	**<0.001**	**6.13**	**(3.39 - 11.1)**	**<0.001**	**5.73**	**(2.91 - 11.3)**	**<0.001**
Duration of diabetes (years)	**1.04**	**(1.02 - 1.05)**	**<0.001**	**1.04**	**(1.02 - 1.05)**	**<0.001**	0.96	(0.93 – 0.99)	**0.006**	0.98	(0.95 - 1.01)	0.12
HbA1c (%)	0.93	(0.84 - 1.04)	0.19	**0.86**	**(0.76 – 0.97)**	**0.01**	0.77	(0.63 - 0.94)	0.009	**0.77**	**(0.62 – 0.96)**	**0.02**
Fasting glucose (mmol/l)	0.97	(0.94 - 1.01)	0.15				0.95	(0.89 - 1.01)	0.13			
CKD_EPI eGFR (ml/min/1.73m^2)	**0.99**	**(0.99 - 1.00)**	**0.003**				1.01	(1.00 - 1.02)	0.008			
Serum urea (mmol/l)	1.01	(0.98 - 1.05)	0.48				0.96	(0.88 - 1.04)	0.33			

Cholesterol (mmol/l)	0.90	(0.79 - 1.03)	0.13				1.08	(0.86 - 1.35)	0.53			
HDL (mmol/l)	1.00	(0.95 - 1.06)	0.9				0.88	(0.51 - 1.53)	0.65			
LDL (mmol/l)	0.86	(0.73 - 1.00)	0.05				1.03	(0.78 - 1.35)	0.85			
Triglycerides (mmol/l)	**0.81**	**(0.71 – 0.92)**	**0.002**				1.06	(0.90 - 1.25)	0.51			

Data are expressed as odds ratio, 95% confidence interval and p-value. The univariate logistic regression analyses was performed on 11 variables as potential predictors of adhering to dietary guidelines. The multivariable analysis was performed on four variables. Bold values indicate statistical significance. Abbreviations: OR, odds ratio; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; eGFR, estimated glomerular filtration rate estimated using the CKD-EPI equation

Figures

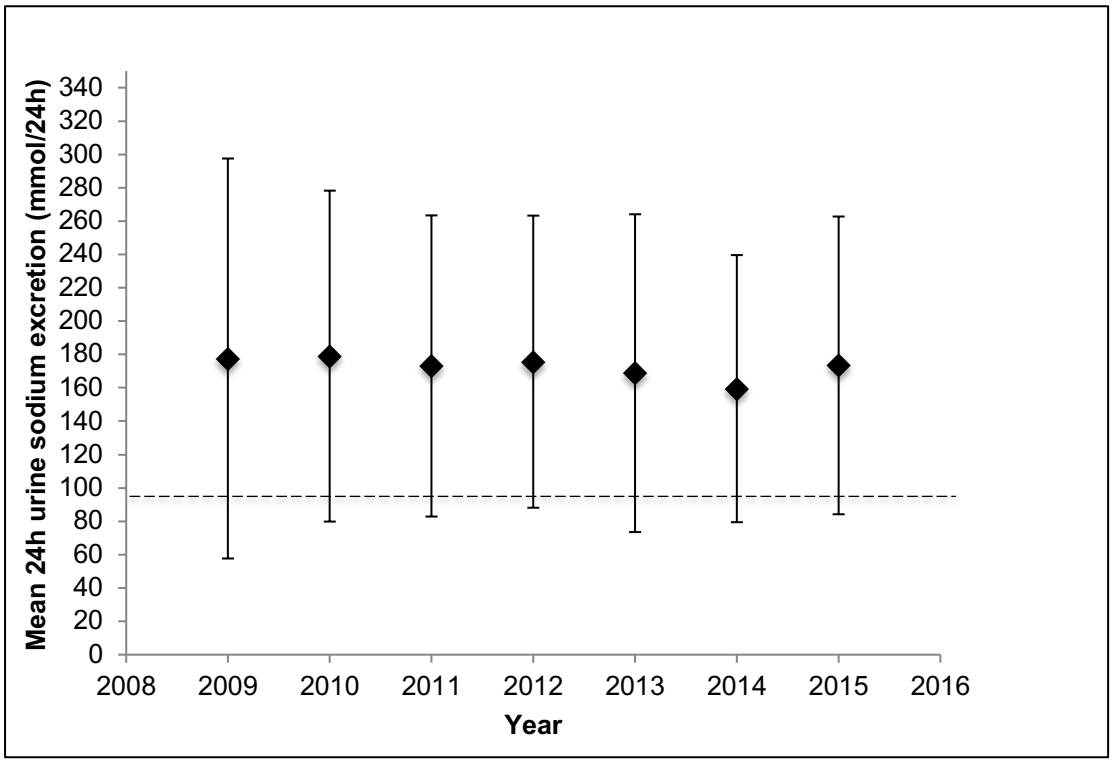

Figure 1 – Mean yearly urinary sodium excretion of total cohort from 2009 - 2015

Results are presented as mean +/- standard deviation. The horizontal dashed line represents the dietary sodium intake target as per the American Diabetes Association guidelines <100mmol/24h [7].

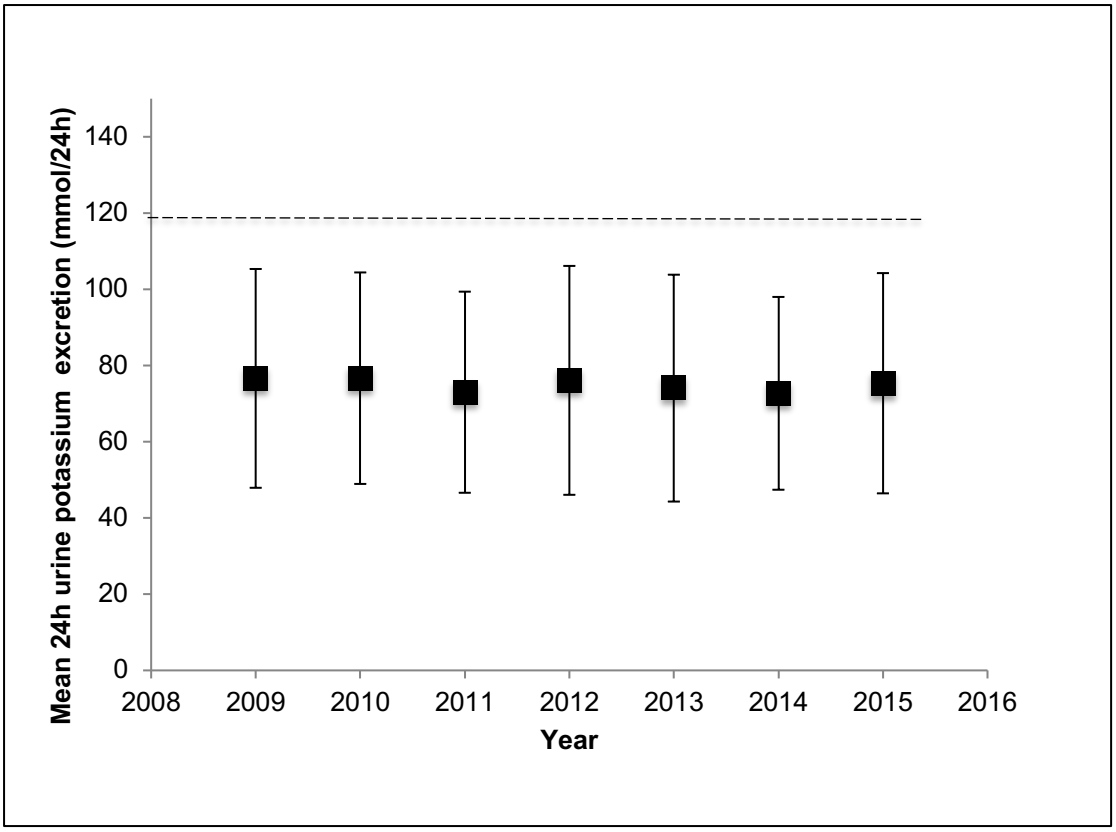

Figure 2 – Mean yearly urinary potassium excretion of total cohort from 2009 - 2015

Results are presented as mean +/- standard deviation. The horizontal dashed line represents the dietary potassium intake target as per the Institute of Medicine guidelines >120mmol/24h [8].

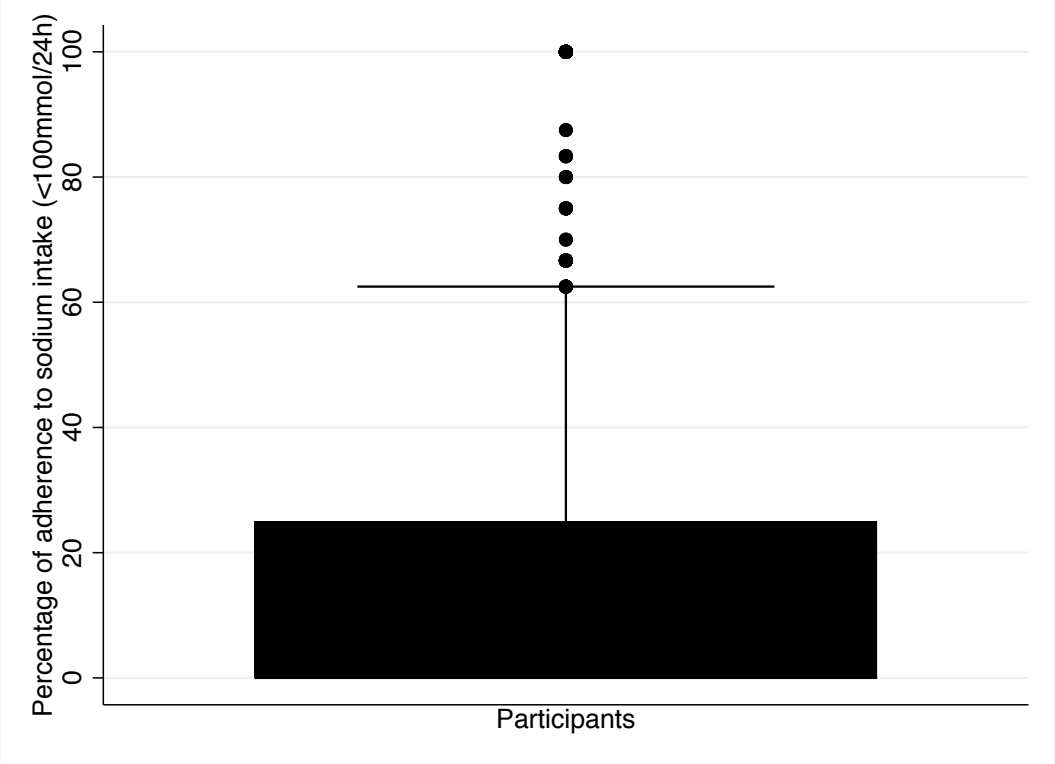

Figure 3 – Adherence to the dietary sodium intake guidelines at an individual level

Box plot representing a participant's likelihood to adhere to the dietary sodium guidelines over the seven-year period. The box-plot suggests that 50% of patients are not likely to adhere to the sodium intake guidelines and of the remaining 50%, 50% are only likely to adhere to the guidelines approximately 20% of the time and the other 50% adhere to the guidelines approximately 60% of the time.

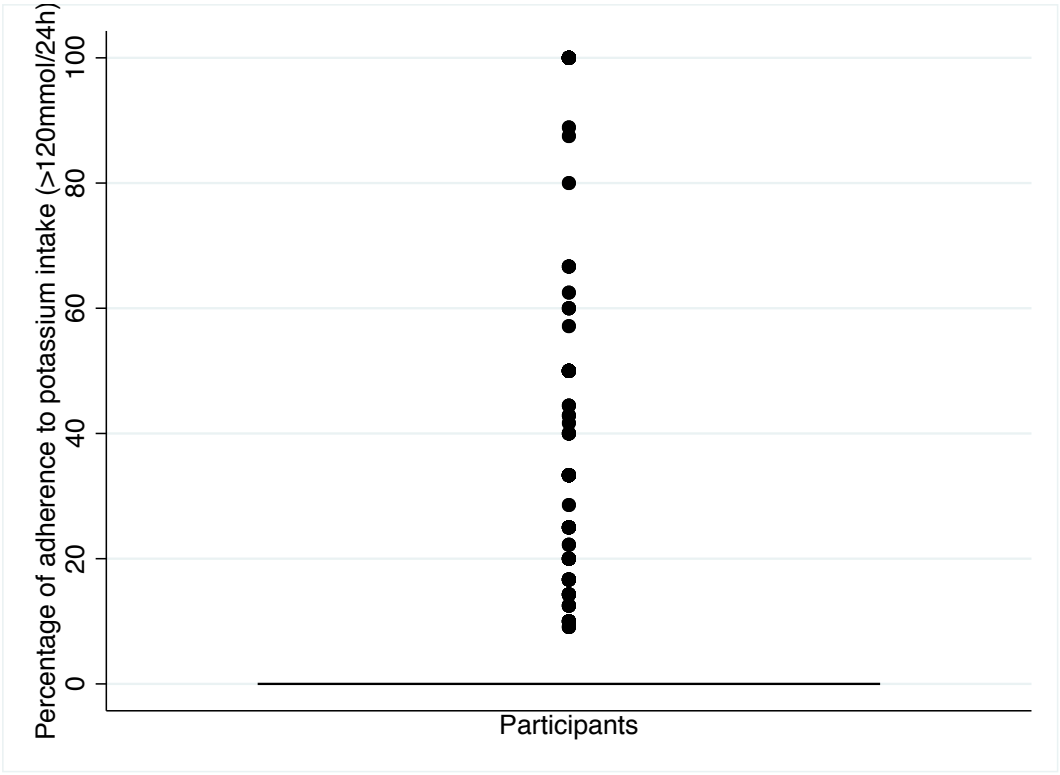

Figure 4 – Adherence to the dietary potassium intake guidelines at an individual level

Box plot representing a participant's likelihood to adhere to the potassium intake guidelines over the seven-year period. The box-plot suggests that 100% of participants are unlikely to adhere to the potassium intake guidelines.

Chapter 7: Comparison of 24hr urine sodium excretion measurements and 24hr dietary sodium recall in determining sodium intake in individuals with type 2 diabetes.

This chapter will be presented as the author-submitted manuscript.

Submitted to *Diabetologia 27*th June 2020.

Abstract

Aims: The relationship between sodium and cardiovascular outcomes remains unclear, possibly due to differences in methods estimating dietary sodium intake. We aimed to compare the main methods used in cardiovascular outcome studies, 24h dietary recall and 24hUNa, in estimating dietary sodium intake in people with type 2 diabetes.

Methods: This prospective study recruited individuals with type 2 diabetes, aged 50-75 years, with one to three, paired 24hUNa measurements and 24h dietary recalls following the multi-pass method. Pearson's correlation and Bland-Altman analysis were performed to determine the relationship and level of agreements between methods. Underreporting was determined by comparing energy intake (EI), reported from 24h dietary recall, with estimated basal metabolic rate (BMR_{est}). A cut-off value of <1.55 identified under-reporters.

Results Participants (n=22) had a mean (SD) age of 64(8) years, BMI of 29.9 (5.7) kg/m^2 and 56 total paired measurements. Mean (SD) sodium for 24h dietary recall was 1942.5 (1045.5) mg/24h and 3119.4 (1100.9) mg/24h for 24hUNa with a mean (SD) difference of 1176.9 (1245) mg/24 (p<.001). Whilst positive correlation between the methods (r=0.33, p=.01) was observed, Bland-Altman analysis showed little agreement, with dietary recall underestimating sodium intake at higher levels of sodium excretion. Mean (SD) $EI:BMR_{est}$ ratio was 1.24 (0.5) with underreporting in 73% (n=16) of participants.

Conclusions: Little agreement exists between 24hUNa and 24h dietary recall in type 2 diabetes. Dietary recall underestimates sodium intake, likely due to underreporting. In individuals with type 2 diabetes, 24hUNa is the preferred method to assess sodium intake.

Clinical Trial Registration Number:
Australian and New Zealand Clinical Trials Registry (ACTRN12613000127707).

Key Words:

Dietary recall, twenty-four-hour urinary sodium excretion, type two diabetes

Research in context:

What is already known about this subject:

- There is an ongoing debate regarding the role of sodium on cardiovascular health given observational studies suggest a paradoxical association between low sodium intake and higher cardiovascular morbidity and mortality.
- Public health policy, however, has based dietary sodium intake recommendations on observational data which has relied mainly on dietary recall, a method known to underestimate sodium intake
- Whilst sodium intake estimation through 24hour urine sodium excretion measurement is considered more accurate than recall , it is not widely used due to poor uptake by individuals in studies

What is the key question:

- Is 24hour urine sodium excretion more accurate and more feasible than 24hour dietary recall in estimating sodium intake in people with type 2 diabetes considered at high-risk of cardiovascular related disease?

What are the new findings:

- 24hour urine sodium excretions are not only more accurate, they are also performed more reliably and have a better uptake than 24hour dietary recall in individuals with type 2 diabetes.
- Studies assessing the correlation between two methods should use the Bland-Altman method of analysis as opposed to correlation analysis.

How might this impact on clinical practice:

- Future studies considering the evaluation of sodium intake on cardiovascular outcomes, especially in people with diabetes, should not use 24hour dietary recall and use 24hour urine sodium excretion measurements given improved accuracy.

Introduction

As lower sodium intake lowers blood pressure[1], public health bodies, such as the American Diabetes Association, recommend lowering sodium intake to 2300mmg per day (100mml/24h)[2]. However, our group [3] and others [4] have demonstrated an association between low dietary sodium intake and a higher all-cause and cardiovascular mortality in people with type 2 [3] and type 1 diabetes [4]. Others have [5] demonstrated a positive relationship[1] between sodium intake and cardiovascular disease in those with type 2 diabetes. This discrepancy is not surprising given methodologies estimating dietary sodium intake differ considerably, limiting the ability to compare results across studies adequately. Given cardiovascular related diseases are the leading cause of morbidity and mortality in those with diabetes, [6] it is important to have a standardized approach to determine optimal sodium intake in this population.

Methods including 24h dietary recall [5], whilst convenient, are limited by measurement error due to recall bias, variability in sodium content in foods [1] and underestimation of sodium intake [7]. Underestimation can occur by 30% to 50% [8] and may be due to factors such as underreporting, especially in those who are overweight [9], difficulty in quantifying discretionary sodium use and incomplete food composition databases [10, 11]. Furthermore, a single 24h dietary recall does not account for daily variability in dietary intake and is less likely to reflect habitual dietary patterns [10]. Estimation of sodium intake by 24h dietary recall in cardiovascular outcome studies have therefore been criticized [12-14]. Moreover, the National Health and Nutrition Examination Survey (NHANES) recently demonstrated that 24h dietary recall underestimates mean sodium intake[15] and attenuates any true associations between sodium intake and health outcomes.

Measurement of 24h urine sodium excretion (24hUNa), utilised by our group and others [3, 4], is widely considered to provide more accurate estimates of habitual sodium intake given approximately 90% of sodium intake is excreted by the kidneys[10]. A systematic review demonstrated that 24h dietary recalls are less accurate in estimating dietary sodium intake in comparison to 24hUNa[16]. We have previously demonstrated that a single 24hUNa is able to predict habitual dietary sodium intake in people with type 2 diabetes, with a day-to-day variation in sodium intake of approximately 20% [17].

However, the average of multiple, non-consecutive, 24h urine collections minimizes random error from day-to-day variability in sodium intake and provides the most accurate estimation of habitual sodium intake [10, 16].

To date, studies have usually excluded adults aged ≥ 70 years with co-morbidities due to poor response rates for 24hUNa collections in this age group [15]. We therefore aimed to assess paired, multiple, non-consecutive 24hUNa collections and 24h dietary recalls in people with type 2 diabetes aged 50-75 years as this cohort is more reflective of a high-risk population that have previously been excluded in studies of this nature [15]. We hypothesised there will be limited agreement between the two methods and there will be underreporting of sodium intake from the 24h dietary recalls.

Research Design and Methods

Study Design and Participants

In this prospective study, individuals with type 2 diabetes, who previously participated in an interventional study conducted at our university teaching hospital between June 2013 to June 2016 [18], were selected if they had a minimum of one paired 24h dietary recall and 24hUNa measurement. Importantly, individuals with a prior history of cardiovascular or cerebrovascular disease were not excluded given they represent the population at highest cardiovascular risk. In the original study[18], salt supplementation (NaCl 100mmol/24h) or matched placebo capsules were consumed daily for three weeks. Following a three-week washout period, the protocol was repeated in reverse. For the current analysis, the post salt supplementation 24hUNa and 24h dietary recall measurements were excluded. No participant was on sodium-glucose co-transporter 2 inhibitors. The study protocol was approved by the Human Research Ethics Committee at Austin Health. All participants provided written informed consent. The original trial [18] was registered with the Australian and New Zealand Clinical Trials Registry (ACTRN12613000127707).

Outcome measures

The primary endpoints of this study were to (i) provide an estimate of sodium intake utilising paired, multiple, non-consecutive 24hUNa measurements and 24h dietary recalls and to (ii) evaluate the relationship and the degree of agreement between these methods. The secondary endpoints were to investigate the reporting of sodium intake using the ratio between energy intake (EI) and estimated basal metabolic (BMR_{est}) ratio, $EI:BMR_{est}$.

Sodium intake assessed by 24h urine collections

At each of the four, non-consecutive, study visits; baseline, week 3, 6 and 9 of the original study, [18] participants collected 24hUNa collections. In addition to sodium excretion, urinary volume (24hUVol) and urinary creatinine (24hUCr) were analysed on Roche Cobas 8000 and were reported in mmol/24h. Urinary volume was recorded by weighing the specimen. No preservative was used for the 24h urine collection. To ensure accuracy of urine collections, participants were provided with written and verbal instructions by the primary investigator. Participants were instructed to empty their bladder upon waking in the morning, discard this urine and then collect any urine excreted during the following 24hours, inclusive of the next morning void. Urinary creatinine excretion measurements verified completeness of the collections.

Sodium intake assessed by 24h dietary recall

In the original study[18], participants received written and verbal dietary education from an Accredited Practicing Dietitian (APD), familiar with food science and nutrition research, on sources of sodium in commonly consumed foods. Patients were advised to avoid high salt foods during the original study[18], as part of their habitual low salt dietary intake. The APD provided written and verbal information on following a low-salt diet and understanding food labels. A multi-pass 24h dietary recall [19] was conducted to determine all foods and beverages consumed on the day the 24hUNa was being collected, from midnight to midnight. A telephone interview was conducted by the APD with the participant the day after the 24hUNa collection was completed at each of the four, non-consecutive, study visits; baseline, week 3, 6 and 9 of the original study [18] . For the baseline visit, however, two days were recalled, and an average of these days was used for dietary analysis[20]. In brief, the multi-pass method includes three stages: (i)

participants report a quick list of foods and beverages consumed in an uninterrupted manner, (ii) the interviewer then asks probe questions relevant to each quick list item to gather detailed information, including time of consumption, brand names of food items, portion sizes, combinations used such as milk in coffee and, (iii) a recall review to validate information and make necessary adjustments [19]. The APD was consistent throughout the trial and conducted all dietary recalls. Portion sizes were estimated using standard household measures and the APD's knowledge of the Australian food supply for standard packaged food with support from the manufacturer's website, where applicable. Additionally, participants were asked about discretionary salt use (adding salt at the table or during cooking).

Anthropometric measurements

Given there has been reports of underestimation of sodium intake by overweight people [9] we measured body weight (kg) and height (cm) at the baseline visit. The Body Mass Index (BMI) kg/m^2 was then calculated as both a continuous variable and a categorical variable as follows: normal BMI (18.5-24.9 kg/m^2), overweight BMI (25-29.9 kg/m^2) and obese BMI ($\geqslant$30 kg/m^2).

Estimation of energy requirements

To determine if there was any underreporting of sodium intake from 24h dietary recall, the BMR_{est}, which indicates the amount of resting energy used in a 24hour day, was calculated from the Schofield equation[21], adjusted for gender, age and weight as below.

$$Male\ BMR_{est}\ (kcal) = [48\ x\ weight(kg) + 3653]/\ 4.184\ (30 - 60\ years)$$
$$Male\ BMR_{est}\ (kcal) = [49\ x\ weight\ (kg)\ + 2459]\ /4.184(\geqslant 60\ years).$$
$$Female\ BMR_{est}\ (kcal) = [34\ x\ weight(kg) + 3538]/\ 4.184\ (30 - 60\ years)$$
$$Female\ BMR_{est}\ (kcal) = [38\ x\ weight\ (kg)\ + 2755]/\ 4.184\ (\geqslant 60\ years).$$

The reported EI for this study was the mean sodium intake of the 24h dietary recalls per participant. The ratio between the EI and the BMR_{est} was determined as follows:

$EI \div BMR_{est} = EI{:}BMR_{est}$.

An EI:BMR_{est} ratio cut-off value of 1.55 was used to define underreporting of energy intake as an EI:BMR_{est} ratio below this level is not compatible with long-term survival [22]. Participants were categorised as under-reporters (<1.55) and adequate reporters (≥1.55).

Biochemical serum analyses

Serum creatinine, estimated glomerular filtration rate (eGFR) as calculated by Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula, glycated haemoglobin (HbA1c), fasting glucose, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides and c-peptide were collected at each time point when the 24h urine collection was returned. The biochemical parameters were analysed on Roche Cobas 8000. The HbA1c was measured by immunoassay on Roche Integra and LDL was calculated using the Friedewald equation[23].

Statistics and data analysis

The primary investigator analysed the 24hUNa measurement data. A 10% random validity check was performed to assess for accuracy. The 24h dietary recall data was analysed by the APD using Xyris FoodWorks, a computerized database of Australian foods (FoodWorks Professional, Xyris Software, Highgate Hill, Australia, version 8). Sodium intake was calculated using the Australian Nutrient Composition Database AUSNUT 2013 with supplementary databases; AusBrands 2015 and AusFoods 2015[24]. (3). Sodium intake estimated via dietary recalls were reported in mg/24h. The molecular weight of sodium (23mg/mol) was used to convert 24hUNa in mmol/24h to mg/24h using the following formula: $24hUNa\left(\frac{mmol}{24h}\right) * 23 = 24hUNa\left(\frac{mg}{day}\right)$.

Continuous variables were reported as mean ± standard deviation (SD), median (interquartile range (IQR)) or as percent frequency as appropriate. Data is otherwise represented as mean ± standard deviation (SD) as indicated. A paired t-test was run to determine whether there was a statistically significant mean difference between the sodium intake estimated from 24hUNa compared to the total number of 24h dietary recalls. A Pearson's correlation coefficient (r) was run to evaluate the relationship

between 24h dietary recalls compared with 24hUNa measurements and was visualised on a scatter plot with a regression line. The Bland-Altman analysis was performed to demonstrate the level of agreement between 24h dietary recall and 24hUNa given it is the preferred method for validation studies as compared to correlation[16]. The data was displayed on the Bland-Altman plot with the difference between 24hUNa and 24h dietary recall (Y axis), plotted against the average of the two methods (X axis). Horizontal lines were drawn at the mean difference and at the limits of agreement. The limits of agreement were defined as the mean difference ±1.96 times the standard deviation of the differences. A mean difference of > 22 % was considered a significant difference between the two methods. [25] A multiple regression was calculated to predict EI:BMR_{est} based on BMI (as a continuous variable), weight, age and gender. Statistical analyses and creation of the scatter plot were performed using STATA version 15.1 (StataCorp. 2017. *Stata Statistical Software: Release 15*. College Station, TX: StataCorp LLC). The Bland-Altman plots were created using MedCalc Statistical Software version 19.2.0 (MedCalc Software bv, Ostend, Belgium). A P-value of <0.05 indicated statistical significance.

Results

Baseline cohort characteristics

Baseline characteristics are summarized in Table 1. The baseline mean (SD) sodium intake as assessed by 24hUNa was 134 (49) mmol/24h or 3082 (1127) mg/24h and by 24h dietary recall was 2010.9 (851) mg/24h. Participants had a mean (SD) age of 64(8) years with a median (IQR) diabetes duration of 13 (9) years. Glycaemic control was adequate with a mean (SD) HbA1c of 7.3 (1.5) (%) or 56 (3.6) mmol/ mol. The lipid profile was acceptable given the majority of the participants (77.3%) were on HMG-CoA reductase inhibitors. Participants had comorbidities such as hypertension (86.4%), ischemic heart disease (22.7%) or diabetes related kidney disease (40.9%).

Number of 24h urine collection and 24h dietary recalls available

As shown in Figure 1, participants (n=22) from the original study [18] were assessed for eligibility. Of the 88 possible 24hUNa excretions, based on four collections per

participant, 84 urine collections (95.5%) were available. Of the 88 possible 24h dietary recall records, 77 recalls (87.5%) were available. Therefore, 75 paired 24hUNa and 24h dietary recall results were available for analysis. There were 19 paired 24hUNa and 24h dietary recall samples in the post salt supplementation phase of the original study [18] and these were excluded in order to assess only the effects from dietary sodium intake and not from salt supplementation. The final analysis therefore included 22 participants with 56 paired 24hUNa and 24h dietary recalls. In 15 participants (68.2%), three paired urine and recall data were available (Figure 1).

Estimate of sodium intake utilising multiple measurements of 24hUNa and 24h dietary recall

From the 56 measurements available, the mean (SD) for 24hUNa was 3119.4 (1100.9) mg/24h and 1942.5 (1045.5) mg/24h for 24h dietary recall (Supplementary table 1). The mean (SD) difference in sodium intake between the two methods was 1176.9 (1245) mg/24 ($p<.001$) (see also Figure 3).

Correlation analysis with urine sodium excretion and dietary recall

Figure 2 demonstrates a moderate positive correlation between 24h dietary recall and 24hUNa, , r = 0.33 ($p= 0.01$).

Bland-Altman analysis

Figure 3 demonstrates the differences between the two methods per paired samples available (n=56). The upper limit of agreement was 3617 mg/24h and the lower limit of agreement was -1263.3 mg/24h . The mean difference as a percentage was 32.5%. The limits of agreement are wide and indicative of poor agreement between the two methods. The plot further demonstrates that 24h dietary recall underestimates sodium intake at higher levels of sodium excretion.

Figure 4 demonstrates the differences between the two methods for individual participants (n=22). The mean (SD) for 24hUNa was 2943 (1068) mg/24h and 1908.7(9771.3) mg/24h for 24h dietary recall. The upper limit of agreement was 3609.1 mg/24h and the lower limit of agreement was -1522.6 mg/24h. The mean (SD)

difference in sodium intake between the two methods was 1043.3 (1244.4) mg/24 (p=.0004). The mean difference as a percentage was 28.9%. Similar to Figure 3, 24h dietary recall was shown to underestimate sodium intake at higher levels of sodium excretion.

Anthropometric characteristics

The mean (SD) BMI was 29.9 (5.7) kg/m^2 (Table 1). Participants were categorized as normal (n=5), overweight (n=6) and obese (n=10). One participant had no corresponding height therefore BMI could not be calculated. There was no statistically significant relationship between the BMI category and gender (Fisher's exact test, p=.45). A one-way MANOVA did not demonstrate any significant difference between BMI categories on the combined dependent variables of 24 h dietary recall and 24hUNa (p=.24)

Estimation of reported sodium intake

The mean (SD) of EI:BMRest was 1.24 (0.5) for all participants (n=22), less than the expected requirement of 1.55. There was no difference in mean (SD) EI:BMR_{est} between females 1.24 (0.4) and males 1.23 (0.6) (p=.96). Sixteen participants (73%) were classified as under-reporters. The regression equation was not significant for BMI, weight, age and gender (p=.45) with an R^2 of 0.19.

Discussion

Principal Findings

The findings of the present study demonstrate that mean sodium intake in people with type 2 diabetes, as assessed by 24hUNa, is above the recommended dietary intake[2]. When comparing 24hUNa to 24h dietary recall, there was little agreement between these two measures. Dietary recall was shown to underestimate sodium intake, likely due to underreporting. Our study supports the use of 24hUNa rather than 24h dietary recall to improve accuracy in assessing sodium intake in individuals with type 2 diabetes.

The American Diabetes Association [2] has revised their recommendations of sodium intake from 1600mg/24h (< 70mmol/24h) to 2300mg / 24h (< 100mmol/24h) in people with diabetes, consistent with the general population. However, our study demonstrates people with diabetes are still not meeting these recommendations as the mean 24hUNa of 3523.7 mg/24h was well over this limit. We have recently demonstrated that people with diabetes are not adhering to dietary sodium recommendations at an individual or at a population level and there is a low likelihood of adhering to guidelines as sodium intake remains stable and is unlikely to change over time [26] Together with previous demonstrations of an association between low dietary sodium intake and adverse cardiovascular outcomes[3, 4], randomised interventional studies are greatly needed[27] to determine the most practical and safe dietary sodium intake target for people with diabetes.

However, the Standards for Salt Research Group has raised concern about the quality of previous research in estimating dietary sodium intake [28] and attempts to develop clear methodological criteria for future studies to ensure robust data are available for public health bodies to make more meaningful recommendations[28]. In response, a recent systematic review was commissioned, comparing 24h dietary recall with 24hUNa[16]. This demonstrated that 24h dietary recall inaccurately measures sodium intake compared with 24hUNa [16] and future studies should not rely on 24h dietary recall to assess associations between dietary sodium intake and cardiovascular health outcomes[16].

Our study likewise demonstrated poor agreement between 24h dietary recall and 24hUNa. Whilst the mean 24h dietary recall was 1942.5 mg/24h, lower than the mean 24hUNa and in target with recommended guidelines[2], these results are misleading as the Bland-Altman plots showed little agreement between the two methods, similar to previous studies [29, 30]. The mean difference of 32.5% in the total paired sample size (n=56) and of 28.9% at an individual level (n=22) was considerably more than the prespecified mean difference of > 22%. This was considered a generous criteria given previous studies have demonstrated dietary recall underestimates sodium intake by 9% [31] to 22% [25] compared with urine sodium excretion.

Furthermore, the Bland-Altman plots showed underestimation of sodium intake occurs at higher levels of sodium excretion, a similar finding to previous studies[30]. This observation may be explained by underreporting of higher sodium intake given we demonstrated the mean EI:BMR_{est} of 1.24 was less than the accepted mean EI:BMR_{est} of 1.55 and 73 % of our study participants were deemed to be under-reporters of sodium intake. The underreporting in our study was considerably greater than 5%-10% underreporting of sodium intake using 24h dietary recall described previously in a pooled analysis [32]. However, our results are not unexpected, given individuals with diabetes are known to be widespread under-reporters of energy intake[33]. Interestingly, a lower EI:BMR_{est} ratio in our study was not associated by higher body weight or BMI category unlike previous findings[32]. Given the majority of individuals with type 2 diabetes (up to 80%)[34] are likely to be overweight, this further highlights poor reliability in using 24h dietary recalls as a method of assessing sodium intake in individuals with diabetes, given the higher likelihood of underreporting in this population.

Furthermore, our study stresses the importance of interpreting results according to the method of analysis used to validate comparisons between two methods. In the present study, the correlation analysis demonstrated a positive, moderate correlation between 24h dietary recall and 24hUNa. However, whilst correlation is the most commonly used method for reporting relationships between 24h dietary recall and 24hUNa [16], correlations may be misleading as they are not able to give an indication of bias at different levels of intake[35] Therefore, the Bland-Altman analysis is preferred over correlation when validating two different methods of assessing a measurement of the same parameter[16, 35].

To the best of our knowledge, only two other validation studies have utilised the Bland-Altman method to compare 24hUNa with 24h dietary recall[29] [30]. However, the aforementioned studies were in either healthy volunteers with a mean (SD) age of 38 (8) years [30] or in volunteers with a wide age range of 18-64 years with only 12 % having a comorbidity (hypertension). In contrast, the present study participants had a mean (SD) age of 64 (8) years with comorbidities other than type 2 diabetes present such as hypertension (86%), ischemic heart disease (22.7%) and diabetic related kidney disease (40.9%). Therefore, the population we studied is considered to be at highest risk of

adverse cardiovascular health outcomes and, in turn, the most likely to benefit from understanding the complex interplay between sodium and cardiovascular health.

Additionally, the above-mentioned studies only used a single 24hUNa collection and compared this with either a single 24h dietary recall[15] or with two 24h dietary recalls[29]. This is in contrast to our study which utilised one to three paired urine collections and dietary recalls per participant, with 68.2% of participants having three paired urine and recalls available. Additionally, these paired measurements were collected in a non-consecutively at three-weekly intervals. Whilst 24hUNa over several days is the ideal method for assessing habitual sodium intake[10, 16], this method is often not utilised by large epidemiological studies as response rates by participants have been low in the past [36]. However, in the current study, we interestingly saw greater completion rates for 24hUNa (95.5 %) as compared with 24h dietary recall (87.5 %). This may be explained by a single investigator providing the instructions for urine collection to participants on the background of being routinely provided written and verbal instructions on accuracy of urine collections when previously attending outpatient appointments.

Strengths and limitations

The major strength is that, to the best of our knowledge, this is the first study to use the Bland-Altman analysis for validating the two common methods of sodium intake assessment (24hUNa and 24h dietary recall) in people with type 2 diabetes. Moreover, the population studied represents those at highest risk of cardiovascular disease, who have previously been excluded in such studies. Additionally, we used an Australian nutrient composition database which includes an extensive list of manufactured foods that are estimated to contribute to more than 75% of daily salt consumption [37, 38]. Furthermore, the APD quantified discretionary salt use, which accounts for 10-15% of daily salt intake [39]. Whilst this study is limited by small participant numbers, the majority of participants had four paired urine and recall data available leading to a sizeable total number of paired methods (n=56). We acknowledge that we did not assess potential confounders such as education level or socioeconomic status. Whilst we did not measure the physical activity level, a level of 1.55 for study participants was assumed as a conservative estimate for 'light activity' [40].

Individuals with type 2 diabetes are not currently meeting sodium intake recommendations. However, these recommendations have relied on previous studies with inconsistent methodologies. We demonstrated poor agreement between 24h dietary recall and 24hUNa. Underestimation and underreporting of sodium intake via 24h dietary recall is associated with a significant difference between 24h dietary recall and 24hUNa measurements. Adding to the growing concerns regarding the accuracy of 24h dietary recall in estimating dietary intake of sodium [16, 28], this study further highlights the importance for future studies to utilise 24hUNa in individuals with type 2 diabetes to validate dietary sodium intake, especially in cardiovascular outcome trials.

Acknowledgments and Contribution Statement

Author Contributions:

SB and EIE were involved in the study design. SB and MB were involved in analysing urine sodium and dietary recall results, respectively. SNW preformed the Bland-Altman analysis and produced the Bland-Altman plots. SB prepared the manuscript. All authors were involved in the editing of the manuscript. EIE approved the study design and the final content of the manuscript. SB is the guarantor of this manuscript.

Data availability

Data are available on request from primary study author SB.

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Conflict of Interest

EIE is an investigator in separate studies sponsored by Novo Nordisk, Bayer, Sanofi, and Mobius Medical and the research funds are paid to EIE's institution to contribute towards diabetes research. SB, MB and SNW have nothing to declare.

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Tables

Table 1: Baseline Demographic Characteristics

Variable	Total participants (n=22)	95 % Confidence Interval
Age, years (mean, SD)	64 (8)	60 – 67.5
Male sex	10 (50%)	
Diabetes duration, years (median, IQR)	13 (9)	9 – 17
Body mass index, kg/m^2 (mean, SD)	29.9 (5.7)	27 - 32.5
Ambulatory Blood pressure, mmHg (mean, SD)		
Systolic Blood Pressure	133 (9)	129.6 – 137.8
Diastolic Blood Pressure	70.5 (8)	67 – 74
Fasting serum lipid profile, mmol/L (mean, SD)		
Total cholesterol	4 (0.95)	3.7– 4.6
High-density lipoprotein cholesterol	1.4 (0.4)	1.2 – 1.5
Low-density lipoprotein cholesterol	2 (0.9)	1.7 – 2.5
Triglyceride	1.5 (0.9)	1.2 – 1.9
Fasting serum glucose level, mmol/L (mean, SD)	6.9 (2)	5.9 – 7.8
HbA1c % (mean, SD)	7.3 (1.5)	6.6 – 8
HbA1c mmol/mol (mean (SD)	56 (3.6)	49-64
C-peptide nmol/L (mean, SD)	0.98 (0.6)	0.7 – 1.2
eGFR, mL/min/1.73m^2 (CKD-EPI) (mean, SD)	74 (3)	67 – 81
Creatinine μmol/L (mean, SD)	84 (20)	75 – 93
CRP, mg/ L(mean, SD)	2.2 (1.4)	1.5 – 2.8
24hour urine biochemistry mmol/24h (mean, SD)		
Sodium excretion	134 (49)	112.5– 156
Potassium excretion	71 (26)	59.5 – 83
Creatinine excretion	11 (3.6)	9.6 – 12.8
24hour dietary recall (mg/24h)		
Sodium	2010.9 (851)	1633.5 – 2388.4
Pre-existing comorbidities (n (%))		
Hypertension	19 (86.4%)	
Dyslipidaemia	19 (86.4%)	
Ischaemic heart disease	5 (22.7%)	
Stroke	2 (9.1%)	
Transient ischaemic attack	1 (4.6%)	

Peripheral vascular disease	1 (4.6%)	
Diabetic microvascular complications (n (%))		
Diabetic retinopathy	6 (27.7%)	
Diabetic neuropathy	0 (0%)	
Diabetes related kidney disease	9 (40.9%)	
Gastroparesis	1 (4.6%)	
Types of dyslipidaemia medication (n (%))		
Statin	17 (77.3%)	
Fibrate	1 (4.6%)	
Ezetimibe	1 (4.6%)	
Types of diabetic medication (n (%))		
Metformin	20 (90.9%)	
Sulfonylurea	6 (27.3%)	
Dipeptidyl peptidase 4 inhibitor	1 (4.6%)	
Glucagon-like peptide 1 receptor agonist	2 (9.1%)	
Thiazolidinedione	0 (0%)	
Acarbose	1 (4.6%)	
Insulin Basal	4 (18.2%)	
Insulin Bolus	2 (9.1%)	
Insulin Premix	4 (18.2%)	
Sodium Glucose Transport 2 Inhibitors	0 (0%)	

Data are expressed as mean (Standard Deviation), or number (percentage), or median (interquartile range). Abbreviations: eGFR, estimated glomerular filtration rate estimated using the CKD-EPI equation; CRP, C-Reactive protein.

Figures

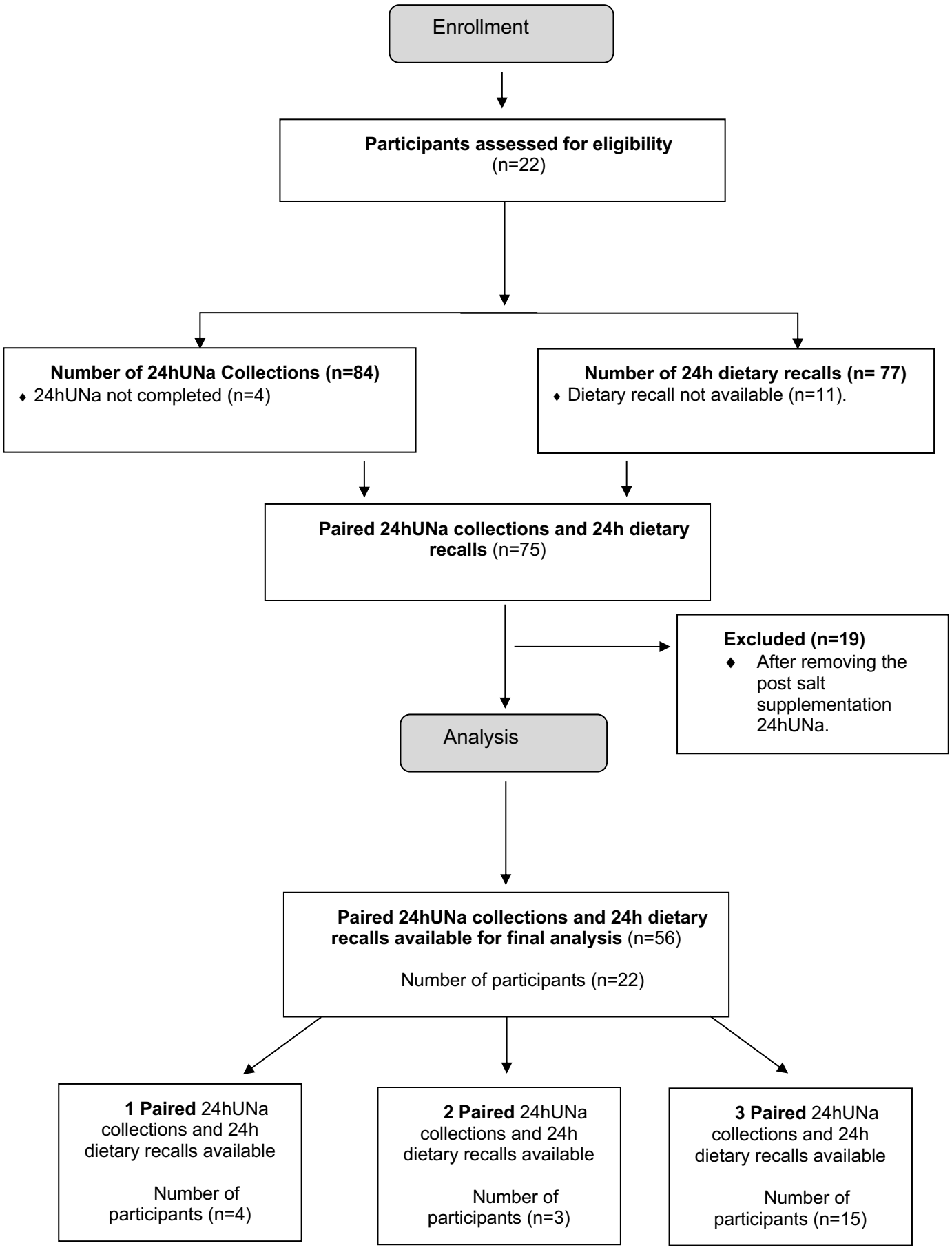
Enrollment
Participants assessed for eligibility (n=22)
Number of 24hUNa Collections (n=84)
♦ 24hUNa not completed (n=4)
Number of 24h dietary recalls (n= 77)
♦ Dietary recall not available (n=11).
Paired 24hUNa collections and 24h dietary recalls (n=75)
Excluded (n=19)
♦ After removing the post salt supplementation 24hUNa.
Analysis
Paired 24hUNa collections and 24h dietary recalls available for final analysis (n=56)
Number of participants (n=22)
1 Paired 24hUNa collections and 24h dietary recalls available
Number of participants (n=4)
2 Paired 24hUNa collections and 24h dietary recalls available
Number of participants (n=3)
3 Paired 24hUNa collections and 24h dietary recalls available
Number of participants (n=15)

Figure 1: Flow diagram of study selection process

Abbreviations: 24hUNa, twenty-four-hour urine sodium excretion

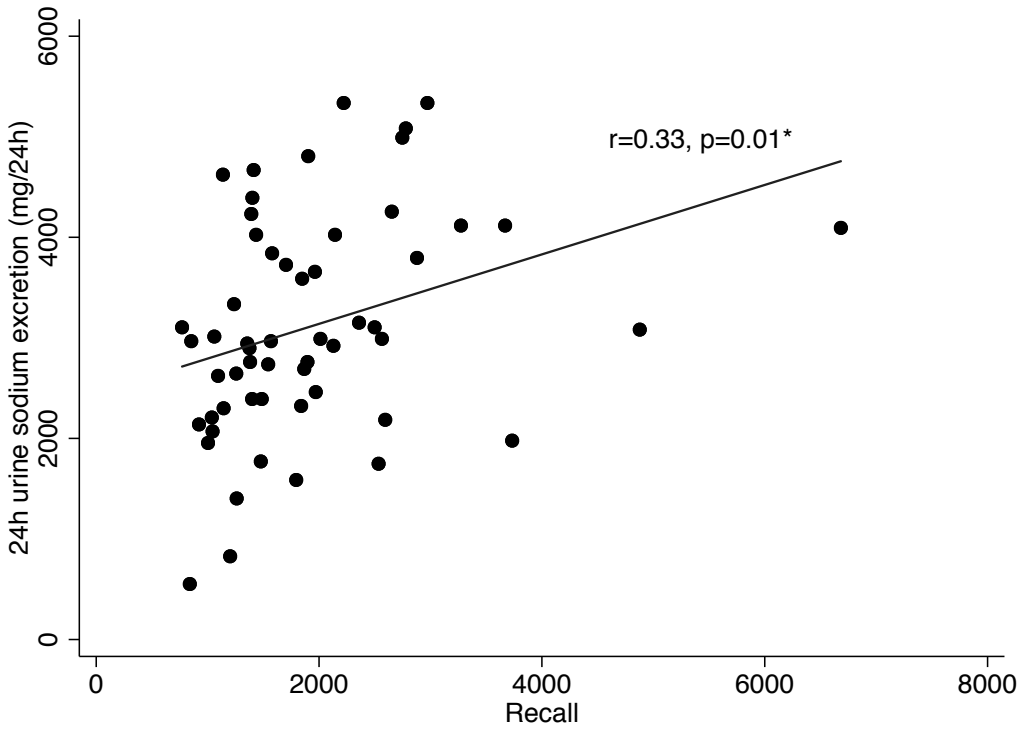

Figure 2: Correlation [a] between 24h urine sodium excretion and 24h dietary recall

Scatter plot demonstrating the relationship between 24h urine sodium excretion and 24h dietary recall. [a] Pearson's correlation coefficient (r) is shown alongside the regression line. The p-values are derived from Pearson's correlation. * indicates statistical significance.

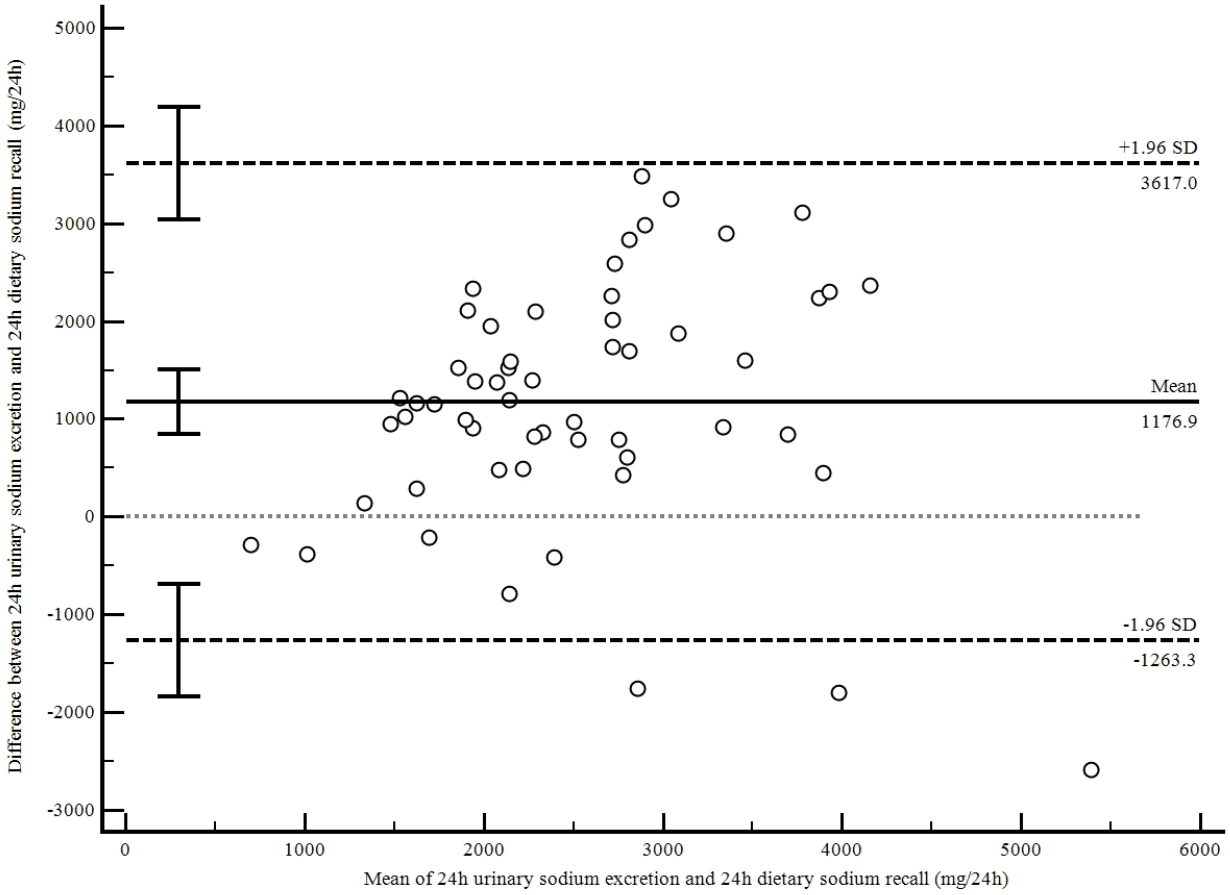

Figure 3: Bland-Altman plot of multiple measures of 24h dietary recall and 24h urine sodium excretion per paired sample

Bland-Altman plot of the difference between 24h dietary recall and 24h urine sodium excretion measurements (Y axis) against the mean of their measures (X axis). Each marker corresponds to a single pair of measurements(n=73). The bold horizontal line represents the mean difference between the 24h dietary recall and 24h urine sodium excretion. The dashed horizontal line represents the upper and the lower limits of agreement. The dotted horizontal line represents a mean difference of 0, if no difference between the two methods existed.

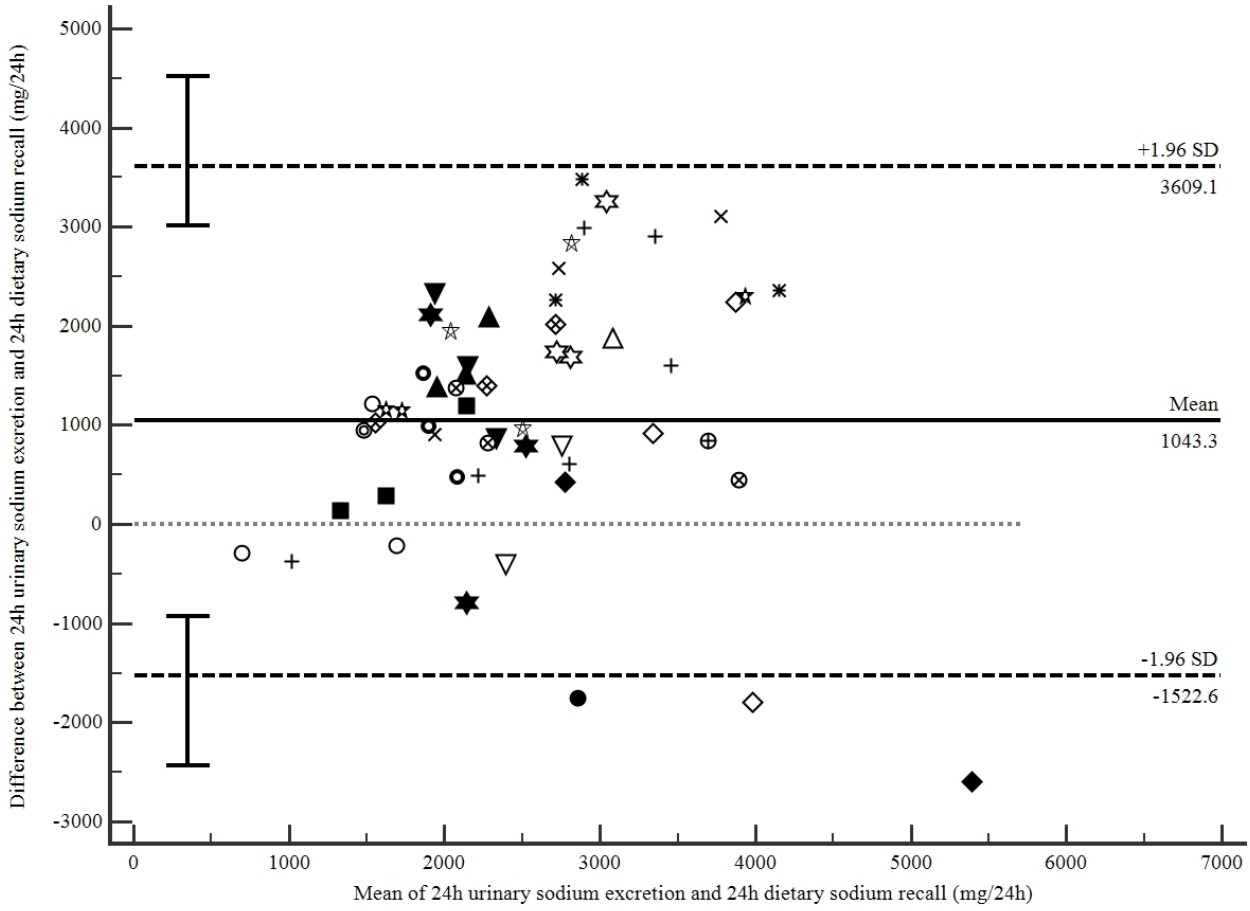

Figure 4: Bland-Altman Plot with multiple measures of 24h dietary recall and 24h urine sodium excretion per individual participant

Bland-Altman plot of the difference between 24h dietary recall and 24h urine sodium excretion measurements (Y axis) against the mean of their measures (X axis) in individual participants (n=20). Each distinct marker corresponds to an individual participant and represents the pair of their measurements. The bold horizontal line represents the mean difference between the 24h dietary recall and 24h urine sodium excretion. The dashed horizontal line represents the upper and the lower limits of agreement. The dotted horizontal line represents a mean difference of 0, if no difference between the two methods existed.

Supplementary Information

Supplementary Table1: Raw data of 24h dietary recall and 24hUNa measurements

Paired sample number	24h Dietary Recall (mg/24h)	24hUNa (mg/24h)	Mean paired 24UNa and 24h Dietary recall (mg/24h)	Difference in paired 24UNa and 24h Dietary recall (mg/24h)
1	1794.959	1587	1690.9795	-207.959
2	922.4052	2139	1530.7026	1216.5948
3	840.0108	552	696.0054	-288.0108
4	1477.479	1771	1624.2395	293.521
5	1260.76	1403	1331.88	142.24
6	1543.4055	2737	2140.20275	1193.5945
7	3733.3065	1978	2855.65325	-1755.3065
8	2143.3665	4025	3084.18325	1881.6335
9	2653.015	4255	3454.0075	1601.985
10	1902.882	4807	3354.941	2904.118
11	1970.8	2461	2215.9	490.2
12	1237.202	3335	2286.101	2097.798
13	1256.944	2645	1950.972	1388.056
14	1375.554	2898	2136.777	1522.446
15	2564.129	2990	2777.0645	425.871
16	6683.375	4094	5388.6875	-2589.375
17	2879.127	3795	3337.0635	915.873
18	4878.598	3082	3980.299	-1796.598
19	2747.117	4991	3869.0585	2243.883
20	1435.186	4025	2730.093	2589.814
21	2221.311	5336	3778.6555	3114.689
22	1485.712	2392	1938.856	906.288
23	1202.281	828	1015.1405	-374.281
24	1401.507	4393	2897.2535	2991.493
25	2498.906	3105	2801.953	606.094
26	2593.9095	2185	2389.45475	-408.9095
27	2358.931	3151	2754.9655	792.069
28	1578.696	3841	2709.848	2262.304
29	2972.403	5336	4154.2015	2363.597

30	1137.1706	4623	2880.0853	3485.8294
31	1896.095	2760	2328.0475	863.905
32	1355.549	2944	2149.7745	1588.451
33	770.5284	3105	1937.7642	2334.4716
34	1413.0385	4669	3041.01925	3255.9615
35	1962.855	3657	2809.9275	1694.145
36	1848.342	3588	2718.171	1739.658
37	3273.7735	4117	3695.38675	843.2265
38	2534.579	1748	2141.2895	-786.579
39	853.1335	2967	1910.06675	2113.8665
40	2128.9735	2921	2524.98675	792.0265
41	1391.68	4232	2811.84	2840.32
42	1060.018	3013	2036.509	1952.982
43	2012.397	2990	2501.1985	977.603
44	1381.0975	2760	2070.54875	1378.9025
45	3669.722	4117	3893.361	447.278
46	1866.866	2691	2278.933	824.134
47	1143.474	2300	1721.737	1156.526
48	2778.843	5083	3930.9215	2304.157
49	1037.848	2208	1622.924	1170.152
50	1003.765	1955	1479.3825	951.235
51	1568.044	2967	2267.522	1398.956
52	1703.704	3726	2714.852	2022.296
53	1044.279	2070	1557.1395	1025.721
54	1093.761	2622	1857.8805	1528.239
55	1839.182	2323	2081.091	483.818
56	1399.275	2392	1895.6375	992.725
Mean [a]	1942.5	3119.4		1176.9
Standard Deviation [b]	1045.5	1100.9		1245

Data expressed as raw data of single pair of measurements available (n=73)

[a] mean difference in 24h dietary recall and 24hUNa (mg/24h). [b] standard deviation of [a]

Abbreviations: 24hUNa, 24h urine sodium excretion

Chapter 8 : General Discussion and Conclusions

Overview

Based on statistical models, it is estimated that lowering sodium will lead to a lowering of blood pressure and, in turn, a reduction in the incidence of cardiovascular related disease. However, no large randomized trial has been conducted to document reductions in the risk of cardiovascular disease occurs with lowering sodium intake [40]. Furthermore, contrary to the prevailing view, despite a low sodium intake having beneficial effects on blood pressure, we [60] and others [335] have previously reported that low sodium intake was unexpectedly associated with higher overall mortality in people with diabetes. Data from larger studies examining the association between sodium intake and cardiovascular disease in the general population, such as the PURE study[40], similarly reported that sodium excretion below 3000mg/day (130mmol/24h) was associated with higher risk of death and major cardiovascular events[40]. There has been great inconsistency in the literature regarding the association between low dietary sodium intake and all-cause and cardiovascular mortality, with findings demonstrating an association of either higher mortality [53 60 336,337], non-linear relationships [40,55], or lower mortality [41,42,45,47-49,64,338].

This prompted The Institute of Medicine to examine the evidence on dietary sodium intake and health outcomes[339] concluding that the evidence on health outcomes found no benefit and possible harm in lowering sodium intake to 1500 to 2300mg/day (65-100mmol/24h) within subgroups with chronic illness such as those with diabetes [339]. Public health authorities, such as the American Diabetes Association, accordingly updated their recommendations advocating that individuals with diabetes should aim to lower their sodium intake to 2300mg/day (100mmol/24h) [70]. However, recognizing that the existing literature consists largely of observational data, the IOM also concluded that more randomized controlled trials are needed to fully understand the mechanistic links behind the observations of low sodium intake and adverse cardiovascular related outcomes among higher-risk subgroups such as those with diabetes[339].

The studies presented in this thesis, therefore sought to examine biologically plausible mechanisms behind previous observations of higher mortality [53 60 61,337] associated with low sodium intake. Previous studies, mainly experimental in design, have demonstrated that low sodium intake has pleiotropic effects which may counterbalance its blood

pressure lowering benefits. For example, sodium is known to play a critical role in normal human physiology, and activation of the renin–angiotensin–aldosterone system occurs when sodium intake falls below approximately 3000mg/day (130mmol/24h) [40] as well as activation of the sympathetic nervous system as evidenced by increased circulating levels of adrenaline and noradrenaline [340] which in turn can lead to endothelial dysfunction and ultimately progression to cardiovascular disease [242]. However, mechanistic studies exploring these findings in people with type 2 diabetes are generally lacking. This thesis therefore sought to examine the effects of sodium intake primarily on the sympathetic nervous system and endothelial function, considered to be precursors to the development of cardiovascular disease. Given that many earlier studies have been criticized [91-93] for their use of estimating sodium intake either through dietary recall, which can underestimate intake by up to 50% [88], or from spot urine samples with application of various formulas [59, 94, 95], the studies presented in this thesis used 24h urine sodium excretion as the method of sodium intake assessment given this has greater accuracy [77, 78].

Differences in metabolic risk on sympathetic and endothelial system outcomes

To get a general overview, the study presented in chapter three aimed to compare sympathetic nervous system activity and endothelial function in the setting of low sodium intake along the glucose continuum in individuals who had either: normal glucose tolerance, impaired glucose tolerance, treatment naïve, diabetes and treated type 2 diabetes with established cardio-metabolic risk factors. The findings demonstrated that despite the lower sympathetic activity in individuals with treated type 2 diabetes, endothelial function, baroreflex function and heart rate were impaired as compared to those with normal glucose tolerance, impaired glucose tolerance and treatment naïve type 2 diabetes. This occurred even though individuals with treated type 2 diabetes were appropriately managed for their cardio-metabolic risk factors and importantly, adhered to recommended sodium intake guidelines.

Whilst autonomic dysfunction can be estimated from indirect measurements such as heart rate variability[341, 342], only a few studies [135, 258, 343], have used microneurography, the gold standard for assessing sympathetic activity [260], in people with diabetes and was

the method of choice for this study as well as that in chapter four. It has previously been demonstrated that individuals in the early phase of type 2 diabetes have an augmented sympathetic drive characterized by higher MSNA [258] [343], regardless of blood pressure status [135] compared to the non-diabetes population. However, in contrast, we observed that the individuals with established type 2 diabetes had lower MSNA levels as compared to the other groups. These differences may be explained in part, due to the duration and control of diabetes, sodium intake and the use of lipid-lowering and anti-diabetic agents.

Diabetes duration in the established type 2 diabetes group was 11 years, which was longer compared to a previous study where individuals had newly diagnosed diabetes [258], yet shorter compared another study [135] where participants had a mean duration of diabetes of 22 years. Thus, factors other than diabetes duration are likely to account for the differences in MSNA we observed. Whilst not the focus of this thesis chapter, other factors such as differences in insulin levels or other unmeasured factors such as fitness levels would be ideal to review in any future studies of this design and nature.

Individuals with established type 2 diabetes had lower levels of total cholesterol and LDL, attributed to the high use of statin therapy. In the previous studies [258] [135] participants were not on statin therapy. Given statin therapy is known to have pleiotropic effects[344] and interactions with neuro-humoral systems[345], in particular being able to reduce sympathetic nervous activity[346], it may be plausible that in the present study, individuals in the established type 2 diabetes group had lower sympathetic activity than the other groups in previous studies [135, 258] due to the use of statin therapy. The information regarding the type of statin administered and the dose of statin used in individuals from the treated type 2 diabetes group was not recorded. This information would have been advantageous. However, in a meta-analysis comparing previous studies utilizing microneurography in assessing the effects of statins on sympathetic outflow[346], the meta-analysis concluded that the sympatho-inhibitory effect of statins was not dose dependent and furthermore, the effects of statin on sympathetic outflow may be independent of changes in plasma cholesterol [346]. Therefore, although the information regarding the dose of statin in the chapter three study is not available, based on the results from the meta-analysis [346], the observations reported may not be

dependent on the dose of statin used. Furthermore, at this stage, the findings reported may present an opportunity for future investigations to determine if the type of statin used is important to clarify further the presented observations, as well as from those of the meta-analysis[346]. This may well have important practical implications for the management of cardiovascular disease in those with established type 2 diabetes whereby the use of statins, even in those with normal total cholesterol levels, may help to reduce sympathetic hyperactivity.

Despite the more optimal lipid profile in the established type 2 diabetes group, endothelial function measures, as derived from PAT ratio and RHI, were still lower. The effect of a disturbed lipid profile on EndoPAT remains unclear. Whilst other studies [347] concur with the findings presented in chapter three of no significant associations between LDL, HDL and triglycerides with EndoPAT measurements, this is contrary to previous studies where high levels of LDL[348], triglycerides[300] and HDL [349] were predictors of reduced PAT ratio. Together these findings raise the necessity for further clarification regarding the impact of lipids on EndoPAT measurements and endothelial function. Nevertheless, as statin therapy use was not associated with improved endothelial function, as expected[350], it becomes difficult to comment on the overall contribution of statin therapy on the sympathetic activity observations in the study presented in chapter three. Therefore, to elucidate these observations further, dedicated research is warranted into assessing the effects of dose and type of statin therapy on sympathetic activity simultaneously with endothelial function.

The use of anti-diabetic therapy was an unavoidable difference, with over 90% in the established type 2 diabetes group receiving Metformin. The impact of metformin on MSNA in people with diabetes is largely unknown. Participants in a previous study [135] were also mainly treated with metformin however no differences in MSNA were seen in those with or without metformin therapy. Additionally, metformin did not alter MSNA in a small group of non-diabetic insulin resistant obese individuals [351]. However, treatment with other insulin-sensitizing agents such as Pioglitazone, has been inconsistent with studies demonstrating a decrease MSNA in people with type 2 diabetes[352, 353] or, conversely, no effect on MSNA in obese individuals with either impaired glucose tolerance or treatment naïve type 2 diabetes [354]. None of the

participants in the present study were on thiazolidinedione agents. Overall the effect of anti-diabetic agents on sympathetic nervous system activity remains unclear. Exploring the possibility of whether insulin-sensitizing agents are able to extend their therapeutic benefit, beyond improvements in insulin resistance and glycemic control, by reducing sympathetic hyperactivity would be advantageous given the increase in cardiovascular related disease seen people with diabetes[355].

An intact baroreflex is essential for cardiovascular homeostasis[356] with impairment leading to higher mortality rates [152, 357]. Consistent with previous reports of reduced baroreflex function in individuals with type 2 diabetes[258, 358] the present study found that baroreflex effectiveness index was lower in the established type 2 diabetes group. However, the baroreflex effectiveness index did not differ significantly between the other subgroups. This finding is consistent with a previous study where the baroreflex function in patients with established coronary artery disease did not differ between patients with normal glucose tolerance, impaired glucose tolerance or newly diagnosed diabetes[359]. Together, these findings are in accord with previous research[360] suggesting that the duration of diabetes possibly contributes to the extent of baroreflex impairment.

Furthermore, the baroreflex impairment occurred in the presence of low sympathetic activity which is surprising given baroreflex function typically decreases in the presence of sympathetic hyperactivity[361]. No previous studies have demonstrated this particular finding. A possible explanation for these unexpected outcomes could be that the baroreflex effectiveness index was reduced due to underlying differences in renal function between subgroups. Whilst participants in subgroups other than established type 2 diabetes had no documented kidney disease, 41 % of the established type 2 diabetes participants had evidence of early chronic kidney disease. Despite all participants being chosen with an eGFR of > 45mL/min/1.73m2, complete data for urinary albumin excretion for the entire cohort was not available. This is an important area given baroreflex function is known to be reduced in patients with chronic kidney disease [362]. Thus, to explore the role of renal function further, correlation between eGFR and urinary albumin excretion will be important in future studies. Previous findings have shown that reduced baroreflex sensitivity is seen in those with a high sodium intake based on dietary recall [363]. In contrast, Couruzi et al [364] demonstrated that

spontaneous arterial baroreflex sensitivity was improved after a high-sodium diet of one week. The inconsistent findings require further studies to elucidate the role of sodium on baroreflex function.

Likewise, despite expecting a lower heart rate given the reduced sympathetic activity, heart rate remained higher in the established type 2 diabetes group. Individuals with type 2 diabetes [365] with a higher fasting glucose [366, 367] are known to have a higher resting heart rate. There are previous reports of a low sodium intake increasing heart rate [368] and a high sodium intake decreasing heart rate in both hypertensive [364]and normotensive[369] individuals. However, these studies represented the non-diabetes population. As impaired baroreflex function[152] and an increased heart rate [370, 371] are known to be independent risk factors for increased cardiovascular and all-cause mortality, the observation of an elevated baroreflex effectiveness index and high resting heart rate despite lower sympathetic activity is important to further characterize in future studies. The additional measurement of catecholamines may be advantageous given hyperglycemia [372] and low urinary sodium excretion [373] elevate plasma norepinephrine levels.

Ultimately, despite the observation of a lower sympathetic profile, it is of concern that individuals with established type 2 diabetes, who followed guidelines, had a low sodium intake with adequate blood pressure, lipid and glycemic control, nonetheless had impaired baroreflex and endothelial function. These findings add to the concern that despite preventative strategies, as aforementioned, the risk of cardiovascular disease is still elevated [36]in people with type 2 diabetes in the setting of low sodium intake.

The effect of salt supplementation on sympathetic and endothelial systems

To further explore the observations demonstrated in chapter three, it was deemed important to conduct a randomized controlled trial specific to individuals with type 2 diabetes, using salt supplementation as an intervention to review the outcomes on the SNS and endothelial function. As low sodium intake has been shown to increase sympathetic activity[27], we aimed to investigate whether salt loading reduces

sympathetic nervous activity and improves endothelial function. This design, using salt supplements (compared to placebo) has the advantage that changes in sodium intake are not dependent on dietary change and diets can remain constant for the duration of the study.

Given the need for such trials[339], the study presented in chapter four is considered timely as there are surprisingly no previous studies that have assessed the effect of sodium intake on cardiovascular parameters in people with type 2 diabetes despite, over the last decade, observational studies demonstrating that a low sodium intake is paradoxically associated with a higher mortality rate [53 60 61, 337]. The study therefore addresses an issue of considerable importance to human health considering the increased risk of cardiovascular disease in patients with type 2 diabetes [138].

Overall, the study findings presented in chapter four demonstrated that salt supplementation was associated with a small increase in sympathetic activity , that not reach levels associated with pathological disease states. Importantly, there were no detrimental effects on endothelial function or alterations in blood pressure. Interestingly improvements in baroreflex function and RAAS activity were seen. Salt-resistant individuals demonstrated a trend towards improved endothelial function after salt supplementation. Overall, these findings suggest the role of stringent sodium lowering in people with type 2 diabetes, especially those who are salt-resistant, may be at present, unnecessary.

The study in chapter four is the first study to utilize MSNA to examine the direct effects of sodium intake on sympathetic nervous system activity in people with type 2 diabetes. We demonstrated that salt supplementation of 100mmol/24h resulting in an average urinary sodium excretion of approximately 200mmol/24h, led to an increase in MSNA, rather than a decrease as we had hypothesized. Conversely, Grassi et al who also used MSNA but in those without diabetes with untreated essential hypertension, found a lower salt intake of 80mmol/24h was associated with an increase in MSNA [144]. The discrepancy in findings may be due to mechanisms underpinning diabetes itself, which usually renders a higher sympathetic nervous system activity as compared to those without diabetes. It has been previously reported that MSNA is increased in people with

type 2 diabetes regardless of blood pressure status compared to the non-diabetic population [135] as is arterial noradrenaline concentration, cardiac enlargement and diastolic dysfunction [136].

However, whilst higher sympathetic nervous system activity was observed with salt supplementation in the present study, it is important to note that these levels were comparable to MSNA levels seen in healthy individuals. When we categorized the participants based on their salt-sensitivity status, the salt-sensitive individuals had higher MSNA levels post salt supplementation as compared with the salt-resistant individuals. Despite the higher MSNA levels observed, for the overall cohort, the measures of endothelial function were not altered significantly. Likewise, in the salt-sensitive individuals, the endothelial function markers were not altered significantly. Moreover, the salt-resistant individuals demonstrated a trend towards improved endothelial function post salt supplementation as compared to placebo as measured by the PAT ratio (0.07) and reactive hyperemic index (0.09) . There may be a rationale at this stage, until more robust evidence becomes available, that in individuals belonging to high-risk sub-groups, such as those with type 2 diabetes, sodium intake lowering recommendations may be of benefit to only those individuals who have a habitual sodium intake> 3450 mg/day (150mmol/24h) with concomitant salt-sensitive hypertension.

The study in chapter four is not able determine whether the improved cardiac baroreflex response can negate the effects from the increased sympathetic nervous system response from salt supplementation. Certainly, a balance between both systems would be ideal for optimal functioning of the cardiovascular system. The observation of an improvement in cardiac baroreflex function in participants post salt supplementation, questions the possibility of a brief period of salt supplementation being associated with increased baroreceptor sensitivity. The basis for these findings may be explained by the increase in plasma volume seen with salt loading, which in turn activates cardiopulmonary receptors and results in a reduction in sympathetic efferent activity. Therefore, overall, the increase in MSNA we observed in our study was unexpected.

Whilst acknowledging that the current study may be considered of short duration, based on previous studies that have assessed the pleiotropic effects of salt loading, three weeks was considered appropriate to detect an effect on the primary endpoints such as endothelial function [374] and SNS [142]. Moreover, previous studies by our research group have demonstrated that two weeks of salt supplementation was tolerated and lead to changes in RAAS activity [375]. Furthermore, data from short-term interventional studies are required to determine the usefulness of embarking on longer term studies. Whilst a dedicated outcome-based study is ideal, the study presented in chapter four should provide the proof of concept for a longer-term interventional study. The observations in the chapter four are therefore intended to stimulate further research into this area. Longer study duration and an evaluation in those with and without diabetes are approaches that can be incorporated in future studies.

The sample size being small is a limitation of the study. As this study was an exploratory study, and the first study to have directly assessed the mechanistic effects of sodium intake (via salt supplementation) on cardiovascular parameters in people with type 2 diabetes, there was limited data from previous studies in which to derive a sample size calculation for the present study. Additionally, the factors that may have led to participant recruitment challenges primarily were that there was an overall poor acceptance by potential participants willing to take salt supplementation in a capsule form. This may be due to the public health messages surrounding the potential benefits of salt restriction. The invasiveness of procedures (MSNA) further added to recruitment limitations. However, despite the above limitations, 22 participants completed the study. This is in comparison to the previous studies which formed the basis for the MSNA [144] and EndoPAT [376] power calculations whereby 11 and 19 participants were assessed respectively.

The practice of individualized medicine and the need to understand the complex cardiovascular physiology in people with type 2 diabetes is becoming increasingly important. The findings in chapter four have attempted to underpin the mechanistic interactions between sodium and cardiovascular health in people with type 2 diabetes. The study attempts to fill the gap in knowledge required to understand the role of sodium on cardiovascular outcomes in this patient population given the inconsistencies

in the current literature[69]. As highlighted by the recent American Diabetes Association 2019 Nutrition Consensus Report on Nutrition Therapy for Adults with Diabetes or Prediabetes, lowering dietary sodium intake in people with diabetes to targets less than 2300mg/day must be considered on an individualized basis[377]. Therefore, the study in chapter four raises the importance of establishing optimal sodium intake guidelines specific to high risk sub-groups, such as those with type 2 diabetes, to help improve public health outcomes. In particular, the study addresses the need to further classify people with diabetes according to their salt-sensitivity status given salt-sensitivity itself is capable of increasing the risk of renovascular and cerebrovascular disease, independent of its hypertensive effects[378]. Furthermore, a recent review pinpointed 21 genetic variants involved in hypertension[379]. Of these 21 genes studies, 18 were associated with salt-sensitivity[379]. This highlights the need to understand a person's unique genetic profile in order to fine-tune and practically implement individualized medicine in the future.

The findings observed in chapter three, whereby a low sodium intake in the established type 2 diabetes group was associated with lower MSNA which in turn was associated with poorer baroreflex and endothelial function combined with the study findings in chapter four, that demonstrated MSNA was higher post salt supplementation yet was associated with a more improved baroreflex and RAAS activity without alteration to endothelial function, suggest that perhaps, the effect of sodium may be have a more prominent role on the endothelial and RAAS system.

The effect of sodium intake on microparticles as a measure of endothelial function

As above, given the study in chapter four demonstrated that salt supplementation had no detrimental effects on endothelial function, there became a need to explore this observation further using an alternative method of endothelial function assessment through the use of microparticles. Therefore, the study in chapter five aimed to investigate the associations between habitual dietary sodium intake on circulating microparticle levels in individuals with type 2 diabetes in the setting of RAAS blockade use given a majority of individuals with diabetes are on RAAS blockade. There have been no previous studies, of any study design, that have examined these associations.

Despite expecting higher endothelial microparticles being associated with lower sodium intake, we did not demonstrate any significant association between sodium intake and endothelial microparticles. However, a trend towards higher CD36+/CD235a+ erythrocyte-derived microparticle levels being associated with lower sodium excretion and higher platelet-derived microparticles being associated with the lowest tertile of 24hour urine sodium excretion was observed. Overall, given the pro-atherogenic roles of CD36+/CD235a+ microparticles and the procoagulant effects of platelet-derived microparticles, these findings warrant investigation to further explore the role of sodium on microparticle levels. Additionally, as RAAS blockade was not associated with lower microparticles counts in the setting of a low sodium intake, these findings question whether the therapeutic benefits of RAAS blockade may be attenuated during lower sodium intake.

As the data represents the diabetes population with hypertension and other cardiovascular risk factors, the use of antihypertensive agents in this prospective study was high and unavoidable, as expected in this population. As the combination of RAAS blockade with thiazide diuretics improves anti-hypertensive efficacy, reduces pill-burden and improves compliance [380] this may in turn explain the higher usage of thiazide diuretics in the RAAS blockade group (32%) as compared to the non-RAAS blockade group (11%). One of the proposed blood pressure lowering mechanisms of thiazide diuretics relates to its natriuresis effects via inhibition of the sodium chloride cotransporter in the renal distal convoluted tubule[381] which accounts for approximately 7% of total sodium reabsorption[382]. However, previous studies have demonstrated that the maximal sodium loss occurs within the first 48 hours after the initiation of thiazide therapy and by the third day, sodium output reaches equilibrium with sodium intake [383]. In support of this finding, our group has previously demonstrated that after a period of four weeks, the addition of hydrochlorothiazide to telmisartan did not lead to a significant increase in urinary sodium excretion[375]. Therefore, in the chapter five study, the 24h urine sodium excretion is deemed to be reflective of habitual sodium intake.

Participants who had RAAS blockade use had a longer duration of diabetes, with, in general, higher rates of co-existing macrovascular and microvascular comorbidities. However, these are the very patients who need to be studied as they represent the group

that has the highest cardiovascular risk. The group on RAAS blockade, despite their longer duration of diabetes and comorbidities, had a more favorable outcome with overall lower microparticle counts as compared to those who were not on RAAS blockade. Therefore, these findings are promising, and further studies could be conducted to evaluate this further. For example, no studies are available that have looked at whether this is a dose-dependent effect.

Interestingly however, it was surprising that there was no association between RAAS blockade and circulating microparticles in the setting of low sodium intake. This is undesirable as the therapeutic, beneficial effects of RAAS blockade seemed to be counteracted by the low sodium intake, possibly due to ongoing RAAS activation. This is important to evaluate further given the significant proportion of people with diabetes who are on RAAS inhibitors. If the intended therapeutic benefit on endothelial health from RAAS blockade is potentially being counteracted by a low sodium intake, this may therefore have significant public health implications.

Size variation in the microparticles counted may play a role in the findings. It is well established that depending on the methods used to prepare and analyze microparticle levels, microparticle level counts may vary significantly between studies. Minimizing the occurrence of pre-analytical and analytical variables, as was done for the study in chapter five, can reduce discrepancies in results[384]. However, it is well known that flow cytometry is biased towards detection of larger particles (0.5 - 1um) and it may well be the smaller endothelial derived vesicles that are important determinants of endothelial dysfunction may be less accounted for. Whilst new technologies are emerging, flow cytometry is currently the most practical and widely employed method for the analysis of individual subsets of microparticles [331]. The study in chapter five was designed to follow on from others who have shown that endothelial microparticles detected by flow cytometry are elevated in type 2 diabetes [189,201,321,322] and correlate with arterial stiffness and impaired endothelium-mediated vasodilation [201]. Therefore, the study is still relevant and able to be compared to others employing similar techniques.

Unfortunately, angiotensin II levels were not measured for any participants. Measurement of angiotensin II and plasma renin levels would have been advantageous

to further assess the effect of RAAS blockade. However, as stated in the limitations section, assessment of angiotensin II and plasma renin should be incorporated into future studies of this nature. It was not possible to measure angiotensin II and plasma renin activity during the study period because their measurement can be affected by the duration of storage [385,386]. As the blood samples were collected between June 2013- June 2016, many of the stored plasma samples had extended refrigeration and were deemed inappropriate, especially as the potential for cryoactivation of prorenin to renin, may lead to falsely higher renin results[385]. In addition to the duration of storage, specific EDTA plasma tubes, which are usually recommended for angiotensin II and plasma renin analysis[386], were not used in the chapter five study for the preparation of platelet-free plasma. Instead, sodium citrate tubes were used for microparticle assessment according to previously published protocols [305]. Therefore, immunoassays that would be used for any potential angiotensin II and plasma renin analysis may be affected by our collection methods. For 15 participants, it was fortunate as their plasma renin was measured at the time of their testing. Therefore, the methodology used for those samples were appropriate. As described in chapter five, a multiple regression was run to predict the levels of microparticles from 24h urine sodium excretion and plasma renin activity. These variables did not significantly predict any of the microparticle subsets.

Regarding the statistical power of, the sample size estimation for the study in chapter five was based on the statistical power from a previous study, which required 41 participants to detect a 20% difference in the levels of platelet derived microparticles (80% power, two-sided alpha 0.05) [230]. Despite aiming for 41 participants however, the aforementioned study had managed to only have 21 participants who were included in the final analysis [230]. Therefore, whilst the absolute numbers of participants are low within each tertile group in the chapter five study, its sample size is still comparable to other studies of this nature [230]. Importantly, given the recent findings from the community-level study, whereby inverse associations were demonstrated between low sodium intake and myocardial infarction and mortality [387], the findings from chapter five are particularly relevant now. This exploratory study serves as a platform for larger studies aimed to investigate whether the observations in chapter five provide

mechanistic insights surrounding the paradoxical associations between low sodium intake and adverse cardiovascular health outcomes for people with diabetes [60, 61].

Are the sodium intake guidelines being met?

One of the main difficulties in recruiting participants for the study in chapter four, as outlined in Supplementary figure 1 of chapter four, was that during the screening process, only a small proportion of people presenting to the diabetes clinics were found to have habitual low dietary sodium intake. Therefore, chapter six was dedicated to providing a recent update on whether people with diabetes are adhering to sodium intake guidelines. In line with previous reports that have demonstrated only 10% of the general population are meeting targets[72], the findings in chapter six go on to confirm that people with diabetes are not adhering to dietary sodium and potassium recommendations at an individual or at a population level. Furthermore, there is a low likelihood of adhering to such guidelines over time as dietary intake of sodium and potassium remains stable. In short, despite health bodies advocating for lowering dietary sodium and increasing potassium, these guidelines are simply not being met. Therefore, together with the previous demonstration in observational studies of an association between low dietary sodium intake and adverse cardiovascular health outcomes[53 60 61, 337], coupled with the current observations from this thesis as presented in chapters three to five, an important question arises regarding the feasibility and practicality of such guidelines.

The reasons for the findings in chapter six are likely multifactorial in nature as variations in sodium intake are complex and may depend upon salt appetite, taste perception and an interplay between individual genetics and population dietary habits. These parameters were not formally assessed in chapter six, as they were not the primary or secondary outcome measure, however it would be prudent to explore these concepts to gain further insight into an overall lack of ability to meet guidelines in people with diabetes.

Encouraging a population-wide optimal level of sodium intake may not be the best approach. As abovementioned, a focus may now need to shift towards formulating guidelines to help individuals and healthcare workers manage dietary sodium intake on

a more individualized basis[377]. For example, people with type 2 diabetes who live in Japan will have higher "healthier" sodium intake, by virtue of their diet consisting mainly of soy-based products, salted fish and vegetables, however despite this, they nonetheless have a lower risk of cardiovascular disease [388]. This is in comparison to westernized populations where sodium intake is derived from "unhealthy" processed foods, most of which are considered staple foods such as breads, cereals and cheese [389]. Therefore, reducing sodium at a population level may be more meaningful if sodium content in foods were reduced, especially in westernized locations, especially given the majority of people with diabetes in Australia were found to consume high sodium content foods despite appropriate delivery of health education[389]. Given the ongoing debate over the optimal level of sodium intake on cardiovascular health outcomes, this chapter further reinforces the need for long-term interventional studies to determine not only the most optimal target but also the most achievable dietary sodium targets in high-risk individuals.

Improving measurement of sodium intake is important for ongoing future studies

Overall, at present, it remains to be shown whether or not sodium lowering has the intended impact on cardiovascular outcome in those with diabetes, independent of its blood pressure lowering effects. Perhaps a significant reason, other than studies not assessing hard cardiovascular end points, of why studies have not been able to reach a consensus in the great salt debate, is that the most crucial step, that of sodium intake estimation, is methodologically varied. Therefore, the final chapter in this thesis, chapter seven, was designed to compare the most common method of sodium estimation used in previous studies which provided basis for the sodium dietary recommendations [25 41], that of dietary recall, against the accepted gold standard, 24hour urine sodium excretion[84]. This was made possible as for each participant in study four, both measures of 24hour urine sodium excretion as well as 24hour dietary recall information was collected simultaneously. For the present chapter study, it was additionally important to perform the Bland-Altman analysis given correlation analysis between the two measures is not as meaningful as compared to the Bland-Altman analysis when assessing validity between two methods of measuring an outcome[84].

Comparing recall with urine excretion with the Bland-Altman analysis has surprisingly not been assessed before in those with type 2 diabetes.

The study presented in chapter seven demonstrated poor agreement between 24h dietary recall and 24hUNa. This is likely due to underestimation and underreporting of sodium intake via 24h dietary recall. The use of multiple paired urine and recall measures is considered a major strength of this study as multiple, non-consecutive analyses are considered more advantageous than a single collection in estimating habitual sodium intake[84].

It is acknowledged that the reporting of sodium intake was assessed using an estimation of the basal metabolic rate. Ideally, the doubly labelled water technique would have provided a more accurate estimate of the basal metabolic rate [390] however, at the time of 24hour dietary recall collection for study four, the technique required to do the doubly labelled water experiment was not implemented in the protocol. Furthermore, the equations used to estimate the basal metabolic rate have mainly relied on datasets from doubly labelled water studies, which did include individuals from Australia[391]. Therefore, given the common use of the estimated basal metabolic rates in studies assessing nutrient intake[392], the use of this technique is considered acceptable for the study in chapter seven.

Overall, this study remains important as it highlights to future investigators and to public health policy bodies that only studies which use 24hour urine sodium excretions, which convincingly provide a more accurate estimation of dietary sodium intake[84], should be used for any future cardiovascular outcome trials.

Conclusions

In individuals with type 2 diabetes with low sodium intake, salt supplementation increased sympathetic activity without altering endothelial function. Despite public health bodies advocating for lowering dietary sodium, these guidelines are not being met and intake is unlikely to change over time. The findings presented in this thesis collectively question the necessity for stringent sodium lowering and call for further interventional studies to determine the ideal yet feasible dietary sodium intake targets

in people with diabetes. Future studies should explore the associations between sodium and the sympathetic and endothelial systems in the context of SGLT-2 inhibitor use. Although the feasibility of conducting large randomized trials with sodium as an intervention is considered difficult, these trials are greatly needed and called upon by the IOM [68, 69]. This is essential if we are able to resolve the ongoing controversy that surrounds optimal sodium intake and make meaningful recommendations to guide overall public health policy.

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