EFFECTS OF DIETARY SODIUM INTAKE ON VASCULAR FUNCTION

A thesis submitted by

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DECLARATION OF ORIGINALITY

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Kacie Dickinson

DESCRIPTION OF THESIS

All of the studies have either been published (Chapter 3 and Chapter 4) or have been submitted for publication (Chapter 2) prior to completion of this thesis. Therefore the thesis was prepared in a Thesis by Publication style. Each chapter is formatted to conform to the style of the journal to which it was submitted. The methodologies are included within the relevant chapters. Author contribution statements are at the beginning of each chapter.

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ABSTRACT

Background

Increased dietary salt (sodium chloride) intake may increase the risk of cardiovascular disease independently of the effects on blood pressure by altering vascular endothelial function. It has previously been shown that reducing dietary salt intake can improve endothelial function after a short period of time however the effects of chronic moderate salt reduction and acute effects of a high salt meal on vascular function are not well studied in controlled trials. The thesis presents studies exploring the effects of manipulating dietary salt intake on endothelial function in normotensive overweight and obese and healthy adults.

Aims

To assess the effects of 1) longer term moderate salt reduction on vascular function in overweight and obese adults 2) a high salt meal on post-prandial vascular function in healthy adults and 3) explore potential mechanisms underlying effects of acute and chronic modification of salt intake on vascular function.

Results

In the first study overweight and obese adults (n=25) with normal blood pressure followed a moderately reduced salt diet (100mmol Na/day) and a usual salt diet (150mmol Na/day) for six weeks each in a randomised cross-over design. Following the reduced salt diet flow-mediated dilatation (FMD) was improved and endothelin-1 (a biomarker of endothelial function) improved significantly compared with the usual salt diet. The change in FMD occurred after two days, was sustained at 6 weeks and was significantly related to the change in 24hr urinary sodium to creatinine ratio. There were no changes in other markers of vascular stiffness (pulse wave velocity, augmentation index), plasma

nitrate/nitrite, asymmetric dimethylarginine, renin, aldosterone or blood pressure between treatments.

Population salt intakes are in excess of recommendations and published data suggest it may be common to consume in excess of 6g salt in a single meal. In the second study we tested the hypothesis that a high salt meal has adverse effects of vascular function in the postprandial period. The results showed that compared with a low salt meal (5mmol Na), the high salt meal (65mmol Na) impaired postprandial FMD and that the FMD response was not related to changes in blood pressure.

In the third study, the mechanisms underlying the effects on endothelial function observed following the high salt meal in Study 2 were investigated. The results showed that augmentation index (a measure of arterial stiffness), serum sodium and osmolality increase significantly in response to the high salt meal (65mmol Na) compared with the low salt meal (5mmol Na). No differences in plasma nitrate/nitrite, vasopressin, atrial natriuretic peptide or blood pressure were observed between treatments.

The main findings in of this thesis are that a modest reduction in dietary salt intake (3g/day) improves FMD rapidly after 2 days, which persists after 6 weeks, which may be explained by a fall in endothelin-1. Second, a single high salt meal has acute adverse effects on post-prandial arterial stiffness that is not accounted for by changes in plasma nitrate/nitrite or other vasoactive hormones. These results suggest mealtime sodium intakes as well as total daily salt intake may have implications for cardiovascular disease risk through altering endothelial function. Further work should be done to define the underlying short and long-term mechanisms by which salt affects endothelial function and long-term cardiovascular disease risk.

PUBLICATIONS ARISING FROM THIS THESIS

Peer Review articles

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ABBREVIATIONS

AI	Adequate intake
ANOVA	Analysis of variance
ADH	Anti-diuretic hormone
AUC	Area under the curve
ADMA	Asymmetric dimethyl arginine
ANP	Atrial natriuretic peptide
AVP	Arginine vasopressin
AIx	Augmentation index
BP	Blood pressure
BMI	Body mass index
CVD	Cardiovascular disease
CRP	C-reactive protein
CV	Coefficient of variation
DBP	Diastolic blood pressure
ET-1	Endothelin-1
FMD	Flow-mediated dilatation
HSM	High salt meal
HR	Heart rate
ICAM-1	Intracellular adhesion molecule - 1
kJ	Kilojoule
LSM	Low salt meal
K	Potassium
MAP	Mean arterial pressure
NO	Nitric oxide

NRV	Nutrient reference value
PAT	Peripheral arterial tonometry
PWV	Pulse wave velocity
RHI	Reactive hyperaemia index
RAAS	Renin angiotensin aldosterone system
Na	Sodium
NaCl	Sodium chloride
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of the mean
UL	Upper limit
UNa	Urinary sodium
UNa:C	Urinary sodium creatinine ratio
UNa:K	Urinary sodium: potassium ratio
VAS	Visual analogue scale
VCAM-1	Vascular cellular adhesion molecule – 1

Chapter 1: Research Background

Cardiovascular disease (CVD) is the leading non-communicable cause of morbidity and mortality of worldwide. The most important pathophysiological process underlying CVD is coronary and peripheral artery atherosclerosis. Endothelial function is impaired early in this disease process while clinical sequelae occur late in the disease process [1]. Therefore interventions to improve or reverse endothelial dysfunction are considered an important therapeutic target for preventing atherosclerosis progression [1].

Dietary salt reduction reduces blood pressure BP and is associated with a reduced risk for CVD. Independent of its BP lowering effects, it has been demonstrated in small number of studies to have benefits on vascular function, which is a potential mechanism by which salt reduction can reduce CVD risk. However the underlying mechanisms are not well understood. It is not known if small reductions in salt intake from current levels can improve endothelial function and whether this occurs independently of any changes that might occur with BP. Clarification of the role of sodium in vascular function is the focus of the research in this thesis.

In the thesis *salt* refers to sodium chloride

1 mmol sodium = 23 mg sodium

1 gram of sodium chloride (salt) = 390mg (17 mmol) of sodium

1 teaspoon (6 grams) of sodium chloride (salt) = 2300mg (100mmol) of sodium

1.1 Cardiovascular Disease

1.1.1 Prevalence

CVD refers to all the diseases of the coronary, cerebral and peripheral vasculature. Thirty-four per cent (48,456) of all deaths in 2008 in Australia were attributable to CVD with the main causes of death being coronary heart disease (49%) and stroke (18%) [2]. It is estimated from self-reports that 3.4 million Australians (17%) of the population had one or more long term CVD condition [2]. CVD is also a leading cause of death globally. In 2008, 17million deaths (30% of global population deaths) were attributable to CVD [3] This is expected to rise to 2 5million deaths annually by 2030 [3].

CVD affects men and women with the prevalence increasing dramatically with age with 62% of those aged 75 years and over estimated to have the condition (Australian Institute of Health and Welfare 2011). With an ageing population in Australia it can be expected the disease burden will increase significantly. There is also a higher prevalence of CVD among Australians in the low SES segment of the population [2].

CVD also inflicts a substantial economic burden accounting for the largest share of health expenditure in Australia generating costs of $5.9 billion (2004-2005) as well as a substantial personal burden and cost resulting from years of life lost and disability [2]. The high prevalence of CVD in Australia, its impact on morbidity and mortality, and the potential for health improvements through prevention and treatment programs emphasises the need for effective prevention and treatment strategies.

1.1.2 Brief Pathophysiology - Atherosclerosis

Atherosclerosis of coronary and peripheral arteries is the most important pathophysiological process underlying CVD [4]. The atherosclerotic lesion is an inflammatory-fibroproliferative response to injury usually caused by cholesterol accumulation [4]. Atherogenesis involves the trans-endothelial passage of lipoproteins, particularly low-density lipoprotein (LDL) and inflammatory cells and in the later stages aggregation of platelets on the damaged surface and formation of a fibrous plaque. The atherosclerotic plaques result from inflammatory cells attaching to the endothelium, migrating into the underlying intima along with smooth muscle cells from the media all of which accumulate cholesterol and other lipid debris, which eventually result in narrowing of the blood vessels. Ongoing inflammation weakens the shoulders of the fibrous caps of the plaques. These unstable plaques can rupture and are exposed to the circulating blood, where aggregation of platelets and ultimately a clot (thrombus) forms which can occlude blood flow to vital organs resulting in acute myocardial infarction or stroke.

1.2 Vascular endothelial function overview

1.2.1 Endothelium

The endothelium is a monolayer of cells lining the basement membrane of the vascular system, which makes numerous contributions to normal cardiovascular function. Under homeostatic conditions the endothelium is responsible for the regulation of vascular tone, balance of pro- and anti-atherogenic factors and control of coagulation [5, 6]. This is achieved by the balanced secretion of a variety of vasoactive substances. Of these vasodilator substances, nitric oxide (NO) is the most potent secreted by the endothelium. Other vasodilators produced by the endothelium are prostacyclin and endothelium derived hyperpolarising factor (EDHF) prostaglandins PG E1 and E2 (which can also oppose the vasodilating effects of bradykinin). Opposing the effects of these substances are vasoconstrictors produced by the endothelium including endothelin-1 and thromboxane.

1.2.2 Nitric Oxide

NO is the most potent vasodilator produced by the endothelium and its availability is a key biomarker of endothelial function. NO is produced by endothelial cells via the enzymatic conversion of the amino acid L-arginine to NO and L-citrulline by endothelial nitric oxide synthase (eNOS). A number of physiological stimuli can increase NO synthesis. Shear

stress is the most important physiologic stimuli for endothelial NO production [7] and it plays a key role in flow-mediated dilatation. Importantly NO exerts additional protective effects on the endothelium by inhibiting cytokine activation and platelet aggregation and smooth muscle cell proliferation and migration at early and later stages of atherosclerosis [8-10].

1.2.3 Endothelial dysfunction

Endothelial dysfunction can be defined as a by a loss of endothelial control of vascular homeostasis. It is characterised by impaired vasodilator function and is thought to precede the initial formation of the atherosclerotic plaque [1, 11]. This loss of vasodilator capacity is also accompanied by a disruption of the balance between pro and anti-thrombotic factors at the endothelial level which ultimately contributes to atherosclerotic plaque progression. One of the primary mechanisms of endothelial dysfunction is thought to be the diminished bioavailability of NO resulting from either decreased production or increased degradation of NO or both [12][13][14]

Asymmetric-dimethylarginine (ADMA) is an endogenous inhibitor of endothelial nitric oxide synthase (eNOS) which reduces NO via competitive inhibition of L-arginine, the substrate for eNOS production. Increased plasma ADMA levels contribute to vascular resistance and stiffness and correlate with the severity of endothelial dysfunction, which can be reversed with l-arginine administration. ADMA is derived from the catabolism of proteins that contain methylated arginine residues and is constantly produced as a part of normal protein turnover The plasma level of ADMA is normally in the range of ~0.5-1μmol/L with concentrations shown to be increased two-fold in subjects with risk factors for vascular disease and up to 10-fold in subjects with atherosclerosis and established cardiovascular disease [15]. A number of large studies have also shown plasma ADMA to be an independent biomarker for CVD morbidity and mortality [16].

Endothelin-1 (ET-1) is a potent vasoconstrictor produced by the endothelium, which contributes to the maintenance of vascular tone [17]. It is released continuously by endothelial cells and under normal physiological conditions the plasma concentration of ET-1 is around 1pM. NO inhibits the release of ET-1 from the vascular endothelium, and ET-1 has a strong inhibitory effect on endothelium-dependant dilatation in the coronary and cerebral arteries, making ET-1 and NO closely interdependent [18]. In addition, ET-1

is also produced by vascular smooth muscle cells and at elevated concentrations ET-1 exerts pro-inflammatory effects and promotes vascular smooth muscle cell proliferation [19]. Increased circulating levels of ET-1 have been reported in patients with coronary disease and atherosclerosis and in patients with hypertension associated with renal failure [20] [21] [19].

1.2.4 Clinical assessment of endothelial dysfunction

Endothelial function can be non-invasively assessed in arteries using high-frequency B-mode ultrasound as first described by Celermajer & Deanfield in 1992 [22]. Endothelial dysfunction has historically been studied using a variety of invasive techniques namely coronary angiography with acetylcholine (Ach) infusion [23] and more recently magnetic-resonance imaging (MRI) [24] as well as venous occlusion plethysmography of the forearm with arterial Ach infusion. Because of their invasive nature or cost, routine use or application in assessment outside the research laboratory is limited. The development of a non-invasive ultrasound technique to measure endothelial function in peripheral arteries, known as flow-mediated dilatation (FMD), was first described by Celermajer & Deanfield in 1992 [22]. FMD is measured in the peripheral brachial artery and is thought to mirror the response seen in the coronary arteries [7, 11]. During the test, increased shear stress is caused by a markedly enhanced blood flow following release of forearm ischemia (induced by compressing the forearm with a conventional cuff inflated to suprasystolic pressure for 5 minutes) [25]. This increased flow stimulates the production of endothelium derived nitric oxide (NO) causing vascular smooth muscle relaxation and arterial dilatation [7, 25]. The increase in blood flow and vascular diameter is measured by high-resolution ultrasound and is expressed as a percentage of the increase of the baseline values [26, 27]. In healthy people, reported FMD values range from around 5-15% of the baseline diameter [28]. This response is impaired or absent when the vascular endothelium is not functioning normally [29]. 2003). It has been established that FMD measured in the peripheral (brachial) artery is positively correlated with acetyl choline-induced endothelial dependant vasodilatation of coronary arteries [11, 26, 30, 31]. FMD has also been shown to be reduced in subjects with risk factors for CVD such as high BP [32] and type 1 and type II diabetes [33].

Impairment of endothelial vasodilator responses as assessed by ultrasound determination of the brachial artery vasoreactivity, precedes the appearance of atherosclerotic intimal

lesions and ultimately leads to clinical events [1]. There are other methods available for the assessment of vascular endothelial function *in vivo* [34, 35] however the non-invasive nature and reproducibility of brachial artery FMD for assessment of endothelial function has been a useful tool for atherosclerosis research.

1.2.5 Endothelium-independent vasodilation

This FMD response (endothelium-dependant production of NO) is compared with the vasodilatation produced by NO donors, such as nitroglycerin, which is referred to as endothelium-independent vasodilation. In experimental approaches that use flow- or endothelium-dependant measures such as FMD, endothelium independent responses to exogenous NO provide a measure of sensitivity of vascular smooth muscles cells to NO [22]. When no differences in endothelium-independent are observed in response to interventions, we can be confident that the differences in FMD observed can be attributed to changes endothelium dependant changes in NO.

1.2.6 Endothelial dysfunction and cardiovascular disease risk

Endothelial dysfunction, characterised by impaired vasodilatation is evident in patients with established CVD as well as those with CVD risk factors such as diabetes (Lucas, Pitari et al.), high BP [22, 36] [23] hyperlipidemia, obesity and metabolic syndrome [37, 38]. FMD has also been demonstrated as a predictor of CVD risk and events. In a longitudinal study of 2264 post-menopausal women after a mean 45 months follow-up, it was found women with FMD in the lowest tertile (FMD<4.5%) compared with the middle FMD tertile (4.6-8.0%) was associated with a ~3 fold increase in CVD event [39].

A meta-analysis of published cohort studies involving 5,547 participants demonstrated a significant relationship between FMD and the risk of CVD events [40]. For every 1% increase in brachial FMD, adjusted for other risk factors there was a 30% reduction in events Most of the studies contained older men with pre-existing coronary heart disease. It has also been shown that patients with coronary artery disease who had impaired brachial artery responses to intra-arterial acetylcholine measured using venous occlusion plethysmography were more likely to have coronary events over the next 4.5 years [13]. If the forearm blood flow was below the median level patients were twice as likely to have an event compared with it being above the median. Therefore improving endothelial function

is of clinical importance because it can potentially shift individuals from a high risk to lower risk category for cardiovascular events.

1.2.7 Other markers of vascular function

1.2.7.1 Augmentation index

Augmentation index (AIX) is a measure of arterial stiffness and measures the timing and strength of the arterial reflected wave. It is assessed by 1 artery tonometry of the radial (or femoral) artery which is transformed to represent the central aortic pressure profile [41]. In 5960 people in the Multiethnic study of Atherosclerosis a 10% increase in AIX at baseline was associated with an 8% increase in hard CVD endpoints over 7.6 years [42]. A meta-analysis of 11 studies showed a 10% increase in Aix was associated with a 38% increase in all-cause mortality [43]. Low fat diets have been shown to lower AIX while weight loss on a low carbohydrate, high saturated fat diet does not [44]. Wycherley et al found no effect of a weight loss diet, either very low or high in carbohydrate, on AIX [45]. Low salt diets in salt resistant subjects do not lower blood pressure nor do they lower AIX while both occur in salt sensitive subjects [46]. Salt restriction is more effective than exercise at lowering SBP and AIX [47].

1.2.7.2 Pulse wave velocity

Pulse wave velocity is another measure of vascular stiffness and is assessed by measuring impulse transit time between central and peripheral arteries (usually carotid and femoral arteries. A recent meta-analysis showed that a 1SD increase in PWV was associated with a 45% increase in CVD events and after adjustment for conventional risk factors, including blood pressure, was still associated with a 30% increase in CVD events [48]. In an earlier meta-analysis of 17 studies and 15,877 subjects followed for 7.7 years an increase in aortic PWV by 1 m/s corresponded to an age-, sex-, and risk factor-adjusted risk increase of 14%, 15%, and 15% in total CV events, CV mortality, and all-cause mortality, respectively [43]. Avolio et al (1986) showed that a low salt diet prevented an age related rise in PWV and that this was independent of blood pressure [49]. Short term dietary salt loading increases blood pressure and PWV and the two were correlated but changes in PWV were not completely accounted for by changes in BP.

1.2.7.3 Peripheral artery tonometry

This is an alternative technique of assessing vascular function and looks at the flow response in digital arteries after 5 minutes of forearm ischemia. It is not operator dependent like FMD. Values tend to be lower in women with IHD particularly non obstructive disease [50] and also reflect coronary vascular dysfunction [51].

All three methods of assessing vascular function have been used in this thesis

1.2.8 Inflammatory molecules derived from the endothelium

Cellular adhesion molecules play an important role in the immune response to inflammation as they are involved with recruitment and attachment of leukocytes and subsequent trans-endothelial passage of leukocytes to the subintima, the earliest stage in the development of the atherosclerotic lesion [52]. Increased plasma levels of intracellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), E-selection and P-selectin) shed from the surface of the endothelium are thought to also reflect endothelial dysfunction and have been associated with both cardiovascular risk factors and events[53]. Hajilooi [54] found ICAM-1 and P-selectin values were significantly higher in patients with coronary artery disease (CAD) than in controls after adjusting for traditional CVD risk factors. Brevetti [55] also found an association between raised levels of soluble adhesion molecules and impaired FMD. It was demonstrated that patients with lower FMD (<5.5%) had higher plasma sICAM-1 compared with those with an FMD>5.5%.

1.3 Dietary salt overview

1.3.1 Overview of sodium physiology and regulation

Sodium (salt) is an essential nutrient required to maintain vascular volume and osmolality blood pressure and tissue perfusion. It is also required for active transport of molecules across cell membranes and maintaining membrane potential. Approximately 95% of total body sodium content is contained within the extracellular compartment (135-145mmol/L).

Total body sodium balance is normally very tightly controlled through the inter-play of a number of homeostatic mechanisms which ensure that in healthy normotensive people changing salt intake has little effect on BP. The major renal mechanism responsible for control of sodium excretion and reabsorption and long-term blood pressure control is pressure-natriuresis mechanism, first proposed by Guyton [56]. Briefly, increases in dietary salt intake produce a transient rise in BP and the kidneys alter sodium excretion rates so that excess sodium is excreted in the urine. Absorption of dietary sodium (primarily occurring in the large intestine) is efficient with an estimated 98% of ingested sodium being absorbed, regardless of the magnitude of sodium consumption [57]. As intakes far exceed physiological requirements, the majority of sodium chloride ingested is excreted in the urine (provided sweating is not excessive) via this mechanism. The result is that large changes in salt and water intake can be accommodated with only slight changes in blood volume, extracellular fluid volume and arterial pressure.

1.3.2 Renin-Angiotensin Aldosterone System

Other mechanisms are required to maintain blood pressure. Neurohormonal mechanisms are involved in maintenance of sodium balance and BP including the renin-angiotensin aldosterone system (RAAS). Low blood pressure and low sodium levels stimulate renin production which leads to the increased production of angiotensin 1 to angiotensin II which is a potent smooth muscle contractile agent. Angiotensin II also stimulates aldosterone production from the adrenal gland which increases sodium reabsorption from the distal tubule in the kidney. In this way sodium and water balance and blood pressure are maintained. Low sodium diets stimulate rennin and angiotensin production and this may have negative effects. This will be discussed later in the chapter.

1.3.3 Atrial Natriuretic Peptide

An important counter-regulatory system is atrial natriuretic peptide (ANP) which has vasodilator effects and renal effects that lead to natriuresis and diuresis by increasing GFR and decreasing renin release. ANP inhibits arginine-vasopressin (AVP) secretion, in part by inhibiting Angiotensin II-induced stimulation of AVP secretion [58, 59].

1.3.4 Arginine Vasopressin

AVP plays an important role in regulating water balance in humans [60]. Its secretion is under control of several mechanisms, some of which are not completely understood. Its two primary functions are to retain water in the body and to constrict blood vessels [61]. Vasopressin regulates the body's retention of water by acting to increase water absorption in the collecting ducts of the kidney nephron. The main stimulus for secretion of vasopressin is increased osmolality of plasma and thus can be influenced by sodium intake acutely [62]. Angiotensin 11 also stimulates AVP secretion [63].

1.3.5 Food sources of dietary sodium

The majority of sodium in Australia is consumed via processed foods. A comprehensive dietary survey of adults estimated 75% of sodium intake consumed from processed foods alone [64]. It is estimated only 5-10% of sodium intake in the diet is from discretionary salt – that is added to foods or cooking [65]. It has also been shown that se of salt in cooking was associated with a higher daily sodium excretion of 15mmol Na/day [66]. Fresh foods are naturally very low in sodium.

A recent systematic survey of the food supply in Australia of 7221 foods determined the foods groups containing the greatest amounts of sodium were sauces (1283mg/100g) and processed meats (846mg/100g) [67]. This is the first comprehensive evaluation of the sodium content of processed foods which indicates the possibility that some meals and food combinations can result in consumption in excess of 2300mg (or 6g NaCl - upper limit of daily consumption) in a single eating occasion. However frequency of consumption of highest sodium foods was not addressed in this study. The relatively high sodium content of processed foods is supported by results of comprehensive evaluation of takeaway foods in Australia [68]. Most items described the quantities of single takeaway meals and products in excess of 4g per serve (i.e. 66% daily NHMRC Upper Limit (UL))

Other studies examining contributions of food groups to sodium intakes have demonstrated in women with Bread alone contributed 19% of Na intake, with an overall contribution from the breads and cereals group of 32.5%. Meat products contributed 14.4% of intake, the dairy and eggs group (excluding cheese) 9.6% and combination dishes (e.g. pizza, quiche, sandwiches and stir fry dishes) 8.4% [69]. In patients with Type 2 diabetes the biggest contributors to salt intake included core foods such as breads and cereals (23% of daily sodium intake) [70]. Foods such as breads and cereals contribute substantially to sodium intake because of the *frequency* of consumption rather than absolute sodium content. This has implications when considering as much as 5-6 serves of breads and cereals recommended as part of a healthy diet [71] Other countries have demonstrated similar findings [72]. In the United States, the foods contributing the most sodium amongst the 2005-2006 National Health and Nutrition Examination Survey (NHANES) cohort were breads (7.3%), chicken and mixed chicken dishes (6.8%), pizza (6.3%), pasta and pasta dishes (5.1%) and condiments (4.4%) [73]. In Canada, an examination of relative contribution of food groups to sodium intake revealed similar results, with the greatest

sources of sodium being bread (14%, 430mg/day), processed meats (9%, 276mg/day), pasta dishes (6%, 176mg/day), cheese (5%, 159mg/day) [74].
Cultural differences in foods intake also explain differences in sources of sodium in the diet In the People's Republic of China sample, from the INTERMAP study most (76%) dietary sodium was from salt added in home cooking, about 50% less in southern than northern samples [75]. In Japan, most (63%) dietary sodium came from soy sauce (20%), commercially processed fish/seafood (15%), salted soups (15%), and preserved vegetables (13%) [76].

Salt plays an important role processed food industry due to its role in preservation (Crocco 1982), food safety (by inhibiting microbial activity) [77] and enhancing flavours [78]. Because sodium is ubiquitous in the food supply reducing salt levels could have implications of safety, taste and cost of foods [79]. If salt levels are decreased, it will be necessary to use other preservatives to ensure safe foods with a reasonable shelf life, which may impact on taste and flavor [77].

In summary, sodium present in processed foods contributes to the majority of a person's total daily salt intake. In Chapter 3 and 4 will explore the effects of a high salt meal on various aspects of vascular function in the post-prandial phase.

1.3.6 Recommended intakes of dietary sodium

The Nutrient References Values (NRVs) for Australia and New Zealand [80] endorsed by the National Health and Medical Research Council (NHMRC), include information on specific nutrient requirements on a daily basis for sustenance and prevention of deficiency states. An Adequate Intake (AI) and upper limit (UL) of intake has been established for sodium intakes to ensure basic requirements for sodium are met (accounting for insensible sodium losses in sweat and faeces) and to allow adequate intakes of other nutrients [80]. The continuous BP – sodium relationship makes it difficult to precisely determine the UL for sodium (Table 1) which has been established to reduce the adverse effects of higher levels of sodium consumption on blood pressure and cardiovascular disease in the population and prevent chronic disease.

TABLE 1: Nutrient ReferenceVales for Sodium [80]

Age	Adequate Intake (AI)		Upper Level of Intake (UL)	
	mg/day	mmol/day	mg/day	mmol/day
1-3 years	200-400	9-17	1000	43
4-8 years	300-600	13-26	1400	60
9-13 years	400-800	17-34	2000	86
14+ years	460-920	20-40	2300	100

1.3.7 Current dietary sodium intakes in Australian adults

Current reported sodium intakes in Australia exceed recommendations. However, the existing data on sodium intake in Australia is limited. The most recent estimate of urinary sodium excretion (to estimate dietary sodium intake) in Australian adults was 155± 63mmol/day (9±4 g NaCl/day) [66]. This was estimated from a sub-sample (n=783; Mean age 64±6.3years) of the Melbourne Collaborative Cohort study, which used a single 24-hr urine sample to estimate sodium intake. Women had lower urinary sodium excretion than men. There were also a very low proportion of men (1.6%) and women (7.9%) (4.9% total) who met the Australian recommendation for chronic disease prevention of sodium intake of <70 mmol Na/day (< 4 g salt/day) [66].

These data on sodium intake assessed using 24-hr urine collection are similar to the last comprehensive population based evaluation of sodium intake by Beard et al. published in 1997 [81]. Mean urinary sodium excretion was 170mmol/day (9.8 g salt/day) for men (n=87) and 118mmol/day (6.8 g salt/day) for women (n=107). A more recent study evaluating sodium intake in community sample of women reported Na excretion of 126±42) mmol Na/day (7.6±2.5g NaCl/day) [69].

Other studies have reported 24hr sodium excretion values in Australian adults ranging from 105–210mmol/day (6–12 g NaCl/day) although many of these are from smaller convenience samples [66, 82] [83]. In these groups overweight and obese adults (BMI>25kg/m2) had highest levels of sodium excretion, likely related to their higher calorie intakes compared with leaner controls.

1.4 Evidence for the effects of salt on cardiovascular health in humans

1.4.1 Benefits of salt reduction on blood pressure

Data from several epidemiological studies confirm the association between dietary salt intake and BP [84, 85]. Several early studies investigating the relationship between salt and BP have demonstrated that people living in nonindustrial, unacculturated communities urinary with a sodium excretion of less than 50mmol per day maintain low average blood pressures that do not increase with age [86]. Migration studies also provided evidence that blood pressure increases when such populations adopt modern lifestyles, including increases in dietary salt intake [87]. However the exposure to an urban lifestyle and inevitably to other risk factors known to increase BP (smoking, stress, obesity, poor dietary intake etc.) may confound this type of study design.

The INTERSALT study examined the relationship between dietary salt intake (as measured by 24hr urinary sodium excretion) and blood pressure in 10,079 men and women from 52 centers around the world [75]. A significant association between sodium excretion and BP was observed within the all the centres. However, the 4 centres with low dietary salt intakes (<3g NaCl/24h or 1.1g of Na/24h) had low blood pressure, and a very slight upward slope of blood pressure with age. When these centres were excluded from the analysis, the association between salt and BP was no longer significant across populations. A major limitation in this type of study, in addition to single samples of urinary sodium excretion to estimate intake, is that so few populations had low sodium intakes, making it difficult to see potential dose-response associations between sodium and BP.

Several meta-analyses of interventions have shown convincing evidence of a direct cause-and-effect relationship between salt consumption and blood pressure. The findings of meta-analyses prior to 2013, demonstrate reductions in sodium of 70-100mmol (4.1-5.9 g of NaCl/day) significantly reduces SBP by 3-5mmHg and DBP by 1-2mmHg in hypertensive adults. In normotensive people this same level of salt reduction results in significant, but smaller reductions in SBP and DBP are observed (1-2/0-1 mmHg). Many of these RCTs included were of short duration of a few weeks and very varied protocols, which included quite severe salt loading and depletion.

The most recent meta-analysis published of 37 randomised controlled trials of greater than 4 weeks duration in adults found a reduction in sodium intake significantly reduced SBP

pressure by 3.39 mmHg (95% CI 2.46 to 4.31) and DBP by 1.54 mm Hg (0.98 to 2.11). When sodium intake was <2g/day versus ≥2 g/day, SBP was reduced by 3.47 mmHg (0.76 to 6.18) and DBP by 1.81 mm Hg (0.54 to 3.08) [88].

Another recent meta-analysis of 34 RCTs (>4 weeks duration) reached similar conclusions [89]. A mean reduction in sodium intake from usual intakes by 75 mmol/24-h (4.4 g NaCl/day) reduced SBP by 4.18 mmHg (95% CI: -5.18 to -3.18) and DBP by 2.06 mmHg (95% CI: -2.67 to -1.45). BP status (hypertensive or normotensive) and the change in 24-h urinary sodium were all significantly associated with the fall in SBP. A 100mmol reduction in 24hr UNa (6g NaCl/day salt) was associated with a fall in systolic BP of 5.8 mmHg (95%CI: 2.5 to 9.2) after adjusting for age, ethnic group and BP status. Collectively, the evidence suggests salt reduction reduces BP. The challenge however is getting individuals to reduce their salt intakes long term.

1.4.2 Salt and cardiovascular disease risk and mortality

Prospective cohort studies evaluating the association between sodium intake and cardiovascular outcomes however have been inconsistent. A meta-analysis of 19 independent cohort samples from 13 studies involving a total of 177,025 participants (follow-up, 3.5 to 19 years) and 11,000 cardiovascular events reported that a high salt intake is associated with increased risks of stroke and total cardiovascular disease [90] although an inverse trend with respect to the association between salt intake and the risk of cardiovascular disease was observed in three cohorts.

In contrast to these findings a number of recent observational studies have suggested no association of salt intake with CVD or an increased prevalence of CVD with low salt intake.

In the largest of these studies, a J-shaped association between sodium intake and CV death and heart failure was found [91]. This was a post-hoc analysis of patients with CVD risk factors from the ONTARGET and TRANSCEND trials (n= 28 880). After a 56 month follow up, it was found that 24hr UNa excretion of > 7g per day was associated with higher CVD events while increased CVD mortality was associated with intake of less than 3g per day. As compared with participants who had a baseline sodium excretion of 4 to 6 g per day (10 to 15 g per day of sodium chloride), participants who excreted more than 6 g of sodium (15 g of sodium chloride) per day and those who excreted less than 4 g of sodium

(10 g of sodium chloride) per day in that study showed an increase in cardiovascular deaths, strokes, or heart attacks.

In the EPOGH study [92] lower sodium excretion was associated with higher CVD mortality. Among 3681 participants followed up for a median 7.9 years, CVD deaths decreased across increasing tertiles of 24-hour sodium excretion, from 50 deaths in the low (mean, 107 mmol), 24 in the medium (mean, 168 mmol), and 10 in the high excretion group (mean, 260 mmol; P<.001), resulting in respective death rates of4.1% (95%confidence interval [CI], 3.5%-4.7%), 1.9% (95% CI, 1.5%-2.3%), and 0.8% (95% CI, 0.5%-1.1%). In multivariable-adjusted analyses, this inverse association retained significance (P=.02): the HR in the low tertile was 1.56 (95% CI, 1.02-2.36; P=.04). Baseline sodium excretion predicted neither total mortality (P=.10) nor fatal combined with non-fatal CVD events (P=.55).

Thomas et al (2011) reported that sodium excretion was associated with all-cause mortality in people with type 1 diabetes [93]. Those with the highest and lowest urinary sodium excretion had reduced survival. Ekinci et al (2011) found in patients with type 2 diabetes that lower sodium excretion was associated with increased all-cause and cardiovascular mortality [94]. The reasons for these associations are not known but need clarification.

However, observational approaches of this kind have inherent limitations such as confounding variables (such as concomitant disease e.g. heart failure and diuretic therapy) and short follow up duration. All the studies reviewed so far also suffer from the fact that none were designed to address the relation between daily sodium intake and the risk of cardiovascular disease so may be underpowered to detect meaningful associations.

1.4.3 Low Salt Interventions and CVD

The relationship between salt intake and CVD events has not been conclusively demonstrated. In a long-term follow-up of the trials of hypertension prevention (TOHP) [95] reported from 200 CVD events that there was a 25% lower risk of a CVD event in subjects in the low salt intervention group. In contrast in a systematic review of the long term effects of advice to reduce dietary salt in 11 trials including 3572 subjects [96] reported only 17 deaths equally divided equally between intervention and control groups. Overall there were small reductions in SBP and DBP (1.1 mm Hg, and 0.6 mm Hg respectively) and reduction in 24 hour urinary sodium excretion of 35.5 mmol/24 hours. In

a systematic review and meta-analyses of intervention and cohort studies [88] reported reductions of 3.47 mmHg SBP and 1.81mmHg DBP when sodium intake was <90mmol/day but found insufficient data to report on mortality or morbidity. In cohort studies an increased risk of stroke and coronary heart disease death was found. In a meta-analysis of randomized controlled trials from which there were 665 deaths from 6,250 participants [97] report reductions in SBP of 1 - 4 mm Hg, with little evidence of any effect of salt reduction on CVD morbidity and mortality. The report that salt restriction increased the risk of all-cause mortality in those with heart failure. Some of the adverse effects to salt restriction may occur through RAAS activation.

1.4.4 Salt and neurohormonal activation

Such responses to salt reduction may have adverse cardiovascular consequences [98]. Recent meta-analyses confirmed long-term (>6 months) modest reductions of salt intake have shown only small increases in renin activity. Salt restriction to 30 mmol sodium/day is associated with increases in plasma renin (from 10mU/L to 35mU/L) and aldosterone (from 250pmol/L to 850pmol/L) [99] but it is unclear whether a moderate salt reduction to 100mmol/day has the same effect. Stimulation of renin produces a rise in Angiotensin II, which mediates vasoconstriction to restore blood pressure. However Angiotensin II, acting through the angiotensin II receptor (AT1) can also adversely affect NO bioavailability by increasing superoxide production which results in increased NO destruction [100].

In a recent meta-analysis He and Macgregor found that sodium reduction of approximately 75 mmol/24 h (equivalent to 4.4 g/day salt) was associated with modest increases in plasma renin activity and aldosterone in the presence of blood pressure reductions of 4 mm Hg for SBP and 2 mm Hg for DBP [89]. In a study by Meland and Aamland there was no change in aldosterone after modest reduction in sodium of 38 mmol Na/day in hypertensive patients in which DBP improved after low sodium diet [101]. Benetos et al found no change in aldosterone & renin after 9 weeks on a low salt diet with 4 weeks of either salt capsules (3.5g/58mmol Na) or placebo in 20 people with essential hypertension [102]. In contrast in a study by Grassi et al in which sodium was reduced by 140mmol in a small study of 11 untreated mild - moderate essential hypertensive patients that there was an increase in plasma renin activity and a doubling in aldosterone from 75 to 150 pg/ml in the presence of a BP reduction of 7 mmHg SBP and 4 mmHg DBP [103]. Similarly Fotherby et al found an increase in plasma renin activity and an increase in aldosterone by 20% following an decrease in sodium of 80 mmol Na from a baseline of 160mmol Na in

17 subjects with untreated hypertension in a 10 week cross-over study It appears that modest sodium reductions of 60mmol or less have little effect on the renin-angiotensin-aldosterone-system [104].

1.4.5 Salt and obesity

Obese people may be more susceptible to the adverse cardiovascular effects of salt. Analyses from the NHANES I (National Health and Nutrition Examination Survey) study reported salt intake in overweight people was associated with increased mortality from CVD but not in people of normal weight [105]. In a study of persons with the metabolic syndrome it has been found urinary sodium is associated with obesity and higher blood pressure [106]. Dietary salt reduction may be a more effective treatment for reducing cardiovascular disease and improving blood vessel health in those with the metabolic syndrome than in normal healthy people, as they may be a group more sensitive to the BP lowering effects of salt reduction [107].

Despite the current recommendations of leading health authorities considerable scientific debate still exists regarding optimum level of dietary salt consumption for the general population as well as those with high blood pressure for prevention of cardiovascular disease Because population intakes are high, there is a general consensus that population reduction in salt intake from current levels (9-12g/day) to 5-6g/day would be beneficial. The lower limit of sodium intake for the greatest benefit on CVD risk reduction remains to be defined.

1.5 Impact of Dietary Salt on Vascular Endothelial Function

1.5.1 Dietary salt and endothelial function –Evidence from intervention studies

A small number of studies have examined the effects of salt on endothelial function in normotensive and pre-hypertensive adults. Tzemos et al demonstrated a blunted endothelium dependant response to acetylcholine in response to salt loading (200mmol/day) after 5 days in young healthy normotensives compared with low salt diet/placebo (70mmol Na/day) [108]. Similarly Dishy showed in a short term study a large salt load (400mmol Na/day) reduced endothelium dependant responses and that this was independent of changes in BP [109]. Similarly Bragulat reported significantly lower forearm endothelium-dependant dilation responses to oral salt loading in 26 salt-sensitive hypertensive adults compared with 16 salt-resistant hypertensives, suggesting salt-sensitive hypertensives are more susceptible to the NO lowering effects of salt loading [110].

We have previously demonstrated endothelial function, as measured by brachial artery FMD, was improved following a 2 week low salt intake (50mmol Na per day) compared with a usual salt intake (150mmol Na per day) for 2 weeks in overweight and obese normotensive subjects (FMD low salt 4.89±2.42% vs. Normal Salt 3.37±2.10%; p=0.001) [111]. The magnitude of salt reduction and loading was similar to that described by Tzemos and the finding was independent of changes in BP [108]. In another investigation of salt and endothelial function before and after weight loss, it was reported that FMD was negatively correlated with 24hour sodium excretion after weight loss (r=-0.28; p=<0.05) [112].

A study in haemodialysis patients demonstrated that FMD was improved by 1.5% when dialysate sodium concentration was reduced from 140mmol/L to 137mmol/L over a 6-month period [113] suggesting FMD may be directly related to serum sodium levels. Carotid intima media thickness (CIMT), a marker of atherosclerotic disease was significantly decreased with decreasing dialysate sodium concentration. Systolic BP was decreased by 8.5mmHg (not significant) by the intervention.

Since the work in this thesis was completed Jablosnki et al [114] has demonstrated endothelial function is improved with sodium reduction from 150mmol to 70mmol in pre-hypertensive older adults (n=17). In this randomised cross over study FMD was improved by 68% with a reduction in salt intake of 83mmol Na/day (4.9g NaCl/day) after 4 week. It

was also found this level of salt restriction enhanced nitric oxide bioavailability as measured by change in forearm vasodilation response to eNOS inhibition. The authors propose that reduced oxidative stress is the mechanism underlying the mechanism the changes in NO. Some limitations warrant discussion. The sample was very small and the group was pre-hypertensive, with medicated participants still included. This may have meant the participants were more sensitive to changes in BP that occurred with salt reduction, which could potentially confound the results. Indeed a large significant reduction in SBP after the low sodium intervention compared with baseline and the normal sodium intervention was observed (10-12mmHg), but this was not significant for the decrease in DBP (3-4mmHg). It is somewhat surprising there was no significant correlation between FMD and BP. Interestingly, despite increases in plasma renin activity aldosterone and angiontensin-II (all non-significant) with the low salt intervention, vascular function still improved.

Also since this work was completed DuPont et al studied 14 healthy, salt resistant adults and FMD responses to a 7-day high sodium diet (300-350mmol Na/day) compared with a 7-day low sodium diet (20mmol Na/day) [115]. FMD was significantly lower on the high sodium diet compared with the low sodium diet (7.3±0.7% vs. 10.3±0.9% respectively). The high sodium diet also significantly suppressed plasma renin activity, plasma angiotensin II, and aldosterone. Interestingly the FMD improved while BP did not change with either intervention.

1.5.2 Salt and Endothelial Dysfunction - Potential Mechanisms

Dishy et al showed salt loading (400mmol Na/day) decreased NO production independently of changes in BP [109]. Campese et al similarly found high salt intake reduced serum NO in both normotensive and hypertensive individuals independently of their blood pressure response [116]. Fujiwara also demonstrated reduced plasma nitrates/nitrites with sodium loading with a greater magnitude of change in nitrates/nitrites among salt-sensitive (SS) hypertensives compared with salt-resistant hypertensives & normotensives [117]. Bragulat et al showed that a high salt intake (250 mmol Na/day) decreased the plasma concentration and urinary excretion of nitrates , suggesting this level of salt intake may adversely affect endothelial cell function [118].

Increased vascular endothelial cell stiffness and reduced nitric oxide release from endothelial cells in higher media sodium concentration has also recently been demonstrated in vitro [119]. Notably stiffness was increased with acute increases in sodium concentrations within a physiological range (135-145mmol).

A suppressant effect of high salt intake on NO production could occur via changes in a nitric oxide synthase inhibitor - plasma asymmetric dimethylarginine (ADMA) [117, 120]. Fujiwara showed that plasma ADMA concentration increased after salt loading and decreased after salt reduction, and these changes were inversely correlated with the change in plasma NO ($r = -0.64$, $P=0.003$) [117]. Osanai et al similarly demonstrated an adverse effect of salt loading in patients with essential hypertension [121]. Plasma ADMA increased significantly with salt loading and correlated significantly with percentage change in plasma nitrate/nitrites. A strength of this study was that dietary nitrates were controlled for in both salt loading and salt-restriction diets which reduces potential dietary confounding of plasma nitrate/nitrite levels. More recently, Fang et al reported plasma ADMA concentrations increased significantly and plasma nitrate/nitrites reduced considerably after salt loading in salt-sensitive normotensive subjects [120]. Significant correlations were demonstrated between plasma ADMA, mean BP, and level of plasma nitrate/nitrites after salt loading.

ADMA may also quench NO production and impair endothelium dependent dilatation via promotion of superoxide production which increases the destruction of NO [122]. Ketonen et al also linked high salt intake to the development of atherosclerosis via increased superoxide production [123]. Li et al also demonstrated high salt inactivates NOS in endothelial cells of bovine origin [124]. A shift in the Na concentration from 137 to 142mmol (5mmol) resulted in a 25% decreased in NOS activity. A 20mmol Na increase was associated with 70% reduction in NOS activity.

Another line of evidence suggests that impaired endothelial response to salt loading could be a product of reduced responsiveness of vascular smooth muscle to NO. Normally, endothelial derived nitric oxide acts on surrounding smooth muscle cells and activates soluble guanylyl cyclase (sGC) to form cyclic guanosine monophosphate (cGMP) [125]. Vascular smooth muscle relaxation is caused by the increase in cGMP level. The endothelium-independent response can be assessed using high-resolution B-mode ultrasound to assess arterial vasodilation relative to baseline diameter after sub-lingual

administration of nitroglycerin, which acts as a nitric oxide donor. Matrougui demonstrated reduced responsiveness to NO donors in smooth muscle cells in rat models with a high salt intake [126]. In contrast, the endothelium independent response to nitroprusside in humans was shown to be increased by salt [127].

Angiotensin II also plays an important role in the regulation of vascular inflammatory responses by modulating the expression of adhesion molecules, chemokines and cytokines, including tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) [128]. Angiotensin converting enzyme (ACE) inhibition has been demonstrated to improve FMD and lower C-reactive protein (CRP) [129]. Theoretically, a large reduction in salt could lead to a rise in angiotensin II and a fall in NO bioavailability. These interactions between salt, RAAS and the endothelium appear to be complex and an important aspect to clarify is whether the acute rises in renin and aldosterone in response to salt reduction revert to baseline once the intravascular volume is re-equilibrated so that any potentially damaging effects of a rise in angiotensin II are transient.

Several studies in humans have shown that NO production increases after salt reduction [109, 114, 116, 117, 119] in vivo and in vitro. It is not clear from these studies whether a smaller reduction in dietary salt intake beyond 4 weeks would produce the same effects. Effects of a small salt reduction in normotensive adults on ADMA and ET-1 and the role of RAAS activation in vascular function response to salt reduction are less clear.

1.5.3 Effects of dietary salt intake on post-prandial endothelial function

To our knowledge no studies have directly evaluated the effects of a physiological salt load on post-prandial vascular function. Two studies have reported responses to oral salt loading which demonstrated a rise in plasma sodium in response to 100mmol Na (5.8g salt) loading in healthy people of 3mmol/L which occurred within 2-3 hours of consuming the test meal [130, 131]. It has been postulated a high salt intake may acutely impair vascular function by raising plasma sodium by this amount. Oberleithner et al has demonstrated, in culture, that increasing plasma sodium concentration within a narrow physiological range (135mmol/L to 145mmol/L) stiffens human endothelial cells assessed using atomic force microscope as a nanosensor and reduces NO production [132]. Li et al produced similar results in cultured bovine endothelial cells by increasing sodium concentration from 137 to 142mmol/L and demonstrating a subsequent 25% reduction in

endothelial nitric oxide synthase activity [124]. So it may be plausible that a high salt meal can induce changes in endothelial function via alterations in plasma sodium.

Conclusions and knowledge gaps

The effects of acute and chronic salt modification on vascular function and the mechanisms are still not clear. There is evidence from studies in humans to support a role for NO as the underlying mechanism for enhanced vascular function with salt reduction in chronic studies. However as much of the literature reports these effects in adults with elevated blood pressure the BP-independence of the vascular effects from these studies is not clear. In order to clarify these effects, normotensive adults were studied in the current research program. In addition, the novelty of the approach of the investigations in the current thesis is to study vascular function in response to dietary salt intake corresponding with current recommended upper limits (i.e. 2300mg Na/day) and to compare the effects with levels of current dietary salt consumption.

1.6 Thesis Scope, Aims and Hypothesis

1.6.1 Scope of thesis:

The efficacy of salt reduction for improving or reversing the endothelial dysfunction that occurs early in atherosclerosis and the associated mechanisms are not well understood. The studies in this thesis aim to address this gap in knowledge and to contribute to evidence regarding how salt impacts endothelial function.

The effects of a chronic moderate salt reduction on vascular function and mechanisms will be studied. Two studies will be conducted to test the impact of a high salt meal on post-prandial endothelial function and to explore the associated mechanisms.

1.6.2 Aims:

1. To determine the effects of a reduced salt diet (100mmol/Na day, 6g salt) compared to a usual salt diet (150mmol/Na day, 9g salt) on endothelial function as assessed by flow-mediated dilatation in overweight and obese adults in a 6-week randomised cross-over study.
2. To assess the effects of a high salt meal on post-prandial endothelial function.
3. To determine the short and longer term mechanisms underlying effects of salt on vascular function.

1.6.3 Specific Hypotheses:

1. Endothelial function as measured by flow-mediated dilatation will improve with a chronic modest reduction in dietary sodium and this will be due to increased nitric oxide bioavailability.
2. A high salt meal will impair endothelial function, as measured by flow-mediated dilatation compared with a low salt meal.
3. A high salt meal will increase post-prandial arterial stiffness and serum sodium and this will be due to reduced nitric oxide bioavailability.

Chapter 2: A reduction of 3g/day from a usual 9g/day salt diet improves endothelial function and decreases endothelin-1 in a randomised cross-over study in normotensive overweight and obese subjects

STATEMENT OF AUTHOR CONTRIBUTIONS AND RESPONSIBILITIES

Kacie Dickinson (Candidate)

Developed protocol, prepared ethics application, delivered dietary interventions, performed vascular measurements, assisted with laboratory analyses, performed statistical analyses, interpreted data, wrote the manuscript.

Signed Date 22.11.2013

Peter Clifton

My contribution to the paper involved: Developed hypothesis and study design, obtained funding, assistance with statistical analyses, data interpretation and manuscript evaluation. I give consent to Kacie Dickinson to present this paper for examination towards the degree of Doctor of Philosophy.

Signed Date 22.11.2013

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My contribution to the paper involved: Developed hypothesis and study design, obtained funding, data interpretation and manuscript evaluation. I give consent to Kacie Dickinson to present this paper for examination towards the degree of Doctor of Philosophy.

Signed. Date 22.11.2013

A reduction of 3g/day from a usual 9g/day salt diet improves endothelial function and decreases endothelin-1 in a randomised cross-over study in normotensive overweight and obese subjects

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Running Title: Modest salt reduction improves FMD in obesity

Figures: 2

Tables: 2

This trial was registered with the Australian and New Zealand Clinical Trials Registry Unique Identifier: ACTRN12609000321246

http://www.anzctr.org.au/ACTRN12609000321246.aspx

2.1 ABSTRACT

Background

It is unclear if a modest reduction in dietary salt intake has beneficial effects on vascular function. The aim was to compare the effects of 9g salt/day with 6g salt/day intake on measures of vascular function and explore mechanisms of effect in overweight and obese adults.

Methods

Twenty-five overweight/obese subjects (BMI 27 - 40kg/m^2) completed a randomised cross-over study of 6 weeks each on a reduced salt (RS) (6 g/day) and usual salt diet (US) (9g/day). Flow-mediated-dilatation (FMD), 24hr blood pressure (BP), augmentation index (AIx), pulse wave velocity (PWV), plasma and urinary nitrate/nitrite, asymmetric dimethylarginine (ADMA), renin, aldosterone and endothelin-1 and vascular adhesion molecules were measured after 2 days and 6 weeks. Adherence to the diets was determined from two 24 hour urine collections.

Results

Urinary sodium excretion was155±58 mmol/24h US vs 113±45 mmol/24h RS (p=0.002). Following the RS diet there was a significant improvement in FMD from 3.5±2.8% to 5.6±2.8% (P<0.001) and decrease in serum endothelin-1 from 1.45±0.38pg/ml to 1.25±0.39pg/ml (P<0.05). Endothelium-independent vasodilatation was also significantly different between treatments (P<0.05). AIx, PWV, serum ADMA and plasma and urinary nitrate/nitrite concentrations were not different between treatments. Change in FMD was related to the urinary sodium: creatinine ratio (r = -0.47, P<0.05) and was independent of blood pressure. Aldosterone and renin were unchanged.

Conclusioni

A small reduction in dietary salt intake of 3g/day improves endothelial function in normotensive overweight and obese subjects. This response may be mediated by serum endothelin-1. This small reduction in salt had no effect on aldosterone and renin concentrations.

2.2 INTRODUCTION

Endothelial function, as measured by flow-mediated dilatation (FMD), is improved when sodium intake decreases by 65 - 100 mmol Na (4-6g salt) per day in overweight and obese and pre-hypertensive adults [114, 133]. The mechanism by which sodium affects vascular function is unclear but is thought to involve nitric oxide (NO) bioavailability. It has recently been demonstrated that after 4 weeks FMD and NO bioavailability were increased in hypertensive individuals when salt decreased by 4g (65 mmol) per day [114]. In these studies the sodium intake was 50 mmol Na/day (1) and 80 mmol Na/day (2) which were substantial and possibly unsustainable decreases from the habitual sodium intake of the study participants of 150mmol/day. The studies had duration of 2 and 5 weeks respectively and provide no information on how quickly the improvement in FMD occurs. Earlier studies in humans evaluating the effects of extreme salt loading and depletion, (400 mmol Na/day or 10 mmol Na/day) on measures of NO production observed significant decreases in plasma nitrite and nitrate during salt loading [109]. There is evidence that the effect of sodium on vascular resistance is mediated by asymmetric dimethylarginine (ADMA) [134]. Schmidlin et al observed that salt loading in salt sensitive subjects increased ADMA after 2 days and it remained increased after 7 days. There is also evidence that reducing sodium intake reduces endothelin-1 (ET-1) and E-selectin concentrations in salt-sensitive individuals and decreases intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) in salt-resistant individuals [135].

The effects of a modest sodium reduction of e.g. 50 mmol/day (3g salt/day) on endothelial function as assessed by FMD, markers of NO production such as nitrate/nitrite and ADMA or biomarkers of endothelial function such as ET-1 and cellular adhesion molecules, to our knowledge, has not been investigated nor has the time course of change in FMD following sodium reduction been investigated.

Our aim was to assess the effects of a modest reduction in sodium intake of 50 mmol Na (3g salt) /day on measures of vascular function, indices of nitric oxide production, ADMA, ET-1 and cellular adhesion molecules in normotensive overweight and obese adults in a randomised cross-over design study.

We hypothesised that a diet containing 100 mmol Na (6g salt) /day would improve FMD compared to a diet containing 150 mmol Na (9g salt) /day after 2 days, that this change would be maintained after 6 weeks, and that this would be related to measures of nitrate/nitrite production. We also postulated that ET-1, ICAM-1 and VCAM-1 would increase on a diet containing 150 mmol Na/day and that these changes would not be related to blood pressure.

2.3 METHODS

Fifty overweight or obese (body mass index (BMI) 27 - 40kg/m^2) men and women were recruited in Adelaide, South Australia from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) volunteer database, by public advertisement at a local hospital and by local Adelaide newspaper advertisement and screened at the Clinical Research Unit, CSIRO Animal, Food and Health Science in Adelaide. Subjects were excluded if they had cardiovascular disease, blood pressure (BP) greater than 139/89 mmHg, were taking anti-hypertensive medication, had significant weight loss in the preceding 6 months (>2kg) or BMI <27 or >40kg/m^2. None of the participants were smokers and none had diabetes, dyslipidaemia, inflammatory bowel disease, pulmonary disease or vasculitis from a self-reported health questionnaire. Twenty-five subjects (8 men and 17 women) completed the study. The protocol was approved by the CSIRO Human Nutrition Human Research Ethics Committee (HREC09/09) and all subjects gave written informed consent. The trial was registered with the Australian and New Zealand Clinical

Trials Registry (ACTRN12609000321246). A CONSORT diagram is presented in **Figure 1**. http://www.anzctr.org.au/ACTRN12609000321246.aspx

This study was a 12 week single-blind randomised controlled cross-over study. All baseline measurements were conducted whilst participants were following their usual diets. Instructions were given to the participants not to alter physical activity for the duration of the study. They were asked at clinic visits to confirm there were no changes to activity levels. We did not objectively measure physical activity before or during the intervention.

Participants were advised by a dietitian to follow a diet containing 6g of salt per day for 12 weeks and provided with written instructions. They were asked to avoid rich sources of nitrates and to consistently consume 2.5 cups of vegetables daily. The diet was planned to ensure weight stability for the 12 week period with approximately 7000kJ/day for women and 8000kJ/day for men. Wholemeal bread (Tip Top, George Weston Foods 400mg Na/100g) and salt reduced margarine (360mg Na/100g Flora, Unilever Australia) were provided to participants to aid compliance. Participants were randomly allocated to take 6 slow sodium tablets (NOVARTIS Pharmaceuticals Australia Pty Limited, North Ryde NSW) each containing 10mmol sodium per tablet every day for 6 weeks or no supplementation whilst following the reduced salt diet. A salt tablet compliance checklist was completed daily by participants and submitted at the end of the intervention to the trial manager. Randomisation was carried out by a person independent of the study using Clinstat software (CLINSTAT software; Martin Bland, York, United Kingdom). After 6 weeks participants crossed over to the opposite intervention for 6 weeks. Personnel conducting the study were blinded to treatment allocation. Participants were not blinded to the intervention as there were no placebo tablets. Participants were instructed on how to collect a 3–day weighed food record and asked to do this for 2 weekdays and 1 weekend day every fortnight for the duration of the study and scales were provided to volunteers

where required. Dietary data was analysed using a computerised database of Australian foods (Foodworks Professional 2012 Version 7.0, Xyris Software Pty Ltd, Highgate Hill, Australia).

All outcome measures were assessed at baseline, day 2 and week 6 of each intervention, except for 24h BP which was recorded at baseline and Week 6 of each intervention.

Resting clinic BP was measured as previously described [111]. 24-hour ambulatory blood pressure was measured at baseline and during the last week (i.e. Week 6) of each intervention every 30 minutes from 0600-2200 and every hour between 2200-0600 as previously described [99].

Vascular measurements were taken in the fasting state. Endothelium-dependent FMD of the right brachial artery was measured as previously described [111]. Endothelium-independent vasodilation was also measured after a 10-minute rest phase as previously described [136]. All the vascular measurements were carried out by the same operator. The intraobserver CV for FMD was 16% (n = 12), which was calculated in healthy subjects who were scanned on 2 separate occasions after an overnight fast. AIx was measured by using the SphygmoCor BP analysis system (AtCor Medical, Sydney, Australia). The intraobserver CV for AIx was 12.8% on the basis of data for healthy individuals (n = 12) who were tested on 2 separate occasions [111]. Aortic PWV was measured via Doppler recordings at the carotid and femoral arteries. Approximately 10 consecutive beats were recorded to cover a complete respiratory cycle. A simultaneous ECG recording was used to calculate the interval between the R-wave and the upstroke of each sound wave. The difference between the average intervals for each artery was calculated. PWV was then determined by dividing the measured surface anatomical distance by this difference. The intraobserver CV for PWV was 20% for obese individuals (n = 25) who were tested on 2 separate occasions.

Fasting blood samples were collected in vacutainer tubes with no additives, for ET-1, ADMA, adhesion molecules and aldosterone or tubes containing EDTA for nitrate/nitrite and renin and stored on ice and centrifuged within 15 minutes of collection at 3000rpm for 10 minutes at 4 ^{o}C. The serum or plasma was then stored at -80 ^{o}C. All biochemical variables were measured at the completion of the study.

Two 24 hour urine samples were collected for measurement of sodium, potassium, creatinine and nitrate/nitrite approximately 3-7 days apart at baseline, day 2 and weeks 5 and 6 of the intervention. Urine was collected without preservatives, protected from UV light, kept refrigerated during collection and stored at -20^{o}C until required for analysis. Urinary sodium, potassium and creatinine were analysed by a certified commercial laboratory (Institute of Medical and Veterinary Science, Adelaide, South Australia).

Urinary and plasma nitrate/nitrite levels were measured in duplicate using a commercially available enzyme immunoassay kit (Nitrate/nitrite Colorimetric Assay Kit, Cayman Chemical Company Ann Arbor, MI). Urine was diluted 1:30 with assay buffer before measurement. For measurement in plasma, after filtration using 30-kD microfuge ultrafilters (Nanosep 30k Omega Centrifugal Device, PALL Life sciences Ann Arbor, MI, USA), 40 µL of plasma was diluted with 200 µL assay buffer and mixed with 10µL enzyme cofactor and 10µL nitrate reductase. After the plasma had been kept at room temperature for 3 hours to convert nitrate to nitrite, total nitrite was measured at 540 nm absorbance following reaction with Griess reagent (sulfanilamide and naphthalene–ethylene diamine dihydrochloride). The intra-assay CV was 2.7% and the inter-assay CV 3.4% and the limit of detection was approximately 2.5µmol/L**.** Plasma renin and serum aldosterone were analysed by a certified commercial laboratory (IMVS, Adelaide, South Australia). Serum endothelin-1 was measured using a commercially available ELISA kit (Quantikine Human Endothelin-1 ELISA, R&D Systems) according to the manufacturers

instruction. The intra-assay CV was 4.0% and the inter-assay CV was 7.6% and the limit of detection was 0.2pg/ml. Serum ADMA was measured using a commercially available ELISA kit (ADMA ELISA Kit, Immunodiagnostik) according to the manufacturer's instructions. The intra-assay CV was 10.5% and the inter-assay CV was 6.2% and the limit of detection was 0.05umol/l. Adhesion molecules (ICAM-1, VCAM-1, E-selectin and P-selectin) were analysed using a Human Adhesion Molecule MultiAnalyte Profiling Base Kit according to the manufacturer's instructions (Flurokine MAP LAD000 R&D Systems, Inc. Minneapolis, MN USA).

Statistical analysis

All participants who completed the 12 week study (n=25) were analysed regardless of their compliance to the usual or reduced salt interventions. Normality was assessed using Kologomorov-Smirnov test and inspection of histograms and normality plots. Non-normal data was log-transformed prior to analysis with normal scale values presented. Repeated measures Analysis of variance (ANOVA) (with diet (i.e. Usual Salt or Reduced Salt) and time (Day 2, Week 6) as within-subject factors) was used to assess the effects of diet intervention on dependent variables over time. Gender and treatment order was used as a between subject factors in separate analyses. Where there was a significant main effect, post-hoc tests with Bonferroni adjustment for multiple comparisons were performed. Covariates including, weight baseline 24hr urinary sodium excretion and baseline BP and changes in BP were used in specific analyses. Pearson correlation analyses were used to assess the association between variables. Analyses were performed with IBM SPSS 20.0 for Windows (IBM SPSS Inc, Chicago, IL). Significance was set at $P < 0.05$. Continuous data are presented as mean±SD unless otherwise stated. Sample size was determined based on the results of a previous randomised cross-over study investigating the effect of salt reduction on flow-mediated dilatation [111].

2.4 RESULTS

Subject recruitment and withdrawal are presented in Figure 1. Twenty-five participants completed the 12-week intervention and baseline data is presented in Table 1. Baseline sodium excretion was 154±58mmol/24h which is similar to recent population estimates of sodium intake [66] and at baseline FMD was inversely correlated with age (r= -0.42 P<0.04). Participants' weight remained stable during the intervention period as planned (Table 1).

Two participants did not tolerate the sodium tablets so were offered sachets of sodium chloride containing the equivalent of 60.6mmol Na as an alternative to be added daily to food. Dietary compliance was confirmed by the mean of two 24-hour urinary sodium collections at the end of day 2 and week 5 and 6 of each intervention and returned sodium tablet check sheets. 24-hr urinary sodium decreased (P=0.002) following the reduced salt diet after 2 days compared to the usual salt diet (US 142±55mmol/24hr; RS 94±36mmol/24hr) and after 6 weeks (US 155±58mmol/24hr; RS 113±45mmol/24hr). Urinary sodium to creatinine ratio was significantly different (P<0.001) between treatments after 2 days (US 12.3±3.9; RS 8.6±3.6) and at the end of Week 6 (US 13.4±4.9; RS 9.1±3.4); 24 hour urinary potassium excretion did not change (P=0.6) between treatments as planned (Table 1). Background dietary sodium intake was not significantly different between interventions (Table 2). Other dietary intake data revealed no significant differences between any nutrients except saturated fat intake was significantly greater during the Reduced Salt Diet (US 23±11g/day vs. RS 26±11g/day P=0.03) (Table 2).

FMD

Baseline brachial artery diameter was not significantly different between treatments (US 3.6±0.7mm vs. RS 3.5±0.6mm; P = 0.3). There was no correlation between weight and FMD at baseline. There was a significant effect of diet on FMD (P<0.0001) such that FMD

was significantly increased with the reduced salt diet at Day 2 (Table 1) and after 6 weeks compared with the usual salt diet (Week 6 US 3.5±2.8%; RS 5.6±2.8% P=0.001). There was a significant effect for diet (P=0.034) on endothelium independent vasodilatation (Table 1). The effect of diet on FMD remained significant after adjusting for gender, weight and the following covariates; change in daytime SBP (P=0.001), change in daytime DBP (P=0.002); change in saturated fat intake (P=0.001), and treatment order (P<0.0001) and lost significance after adjusting for change in urinary sodium to creatinine ratio (P=0.063). Change in FMD was significantly correlated with change in urinary sodium to creatinine ratio (r = -0.47 P<0.02) at the end of the intervention such that the greater the increase in 24h UNa:C, the greater the decrease in FMD (**Figure 2**).

AIx & PWV

There were no significant differences in AIx and PWV between treatments (Table 1).

Blood pressure

There was no significant difference in daytime, night-time, total 24h or fasting seated clinic blood pressure variables (SBP, DBP or MAP) between treatments (Table 1).

Nitrate/Nitrite

Urinary nitrate/nitrite concentration was calculated as the mean concentration from two 24-hr urine collections at the end of the last two weeks of each intervention. There were no significant differences between the Usual and Reduced Salt diets (US 1.12±0.57mmol/24h; RS 0.99±0.45mmol /24h *P*=0.3). Plasma nitrate/nitrite concentration was not significantly different between the treatments (US 19.7±11.2umol/L; RS 20.9±9.3umol/L P=0.7)

Renin and Aldosterone

Renin and aldosterone were not significantly different between treatments; aldosterone (US 299±184 vs RS 280±149 pmol/L P=0.7); renin (US 14±7 vs RS 15±10 ng/L P=0.7)

ADMA and Endothelin-1

ADMA was not significantly different between treatments (Table 1). Endothelin-1 was decreased at Week 6 and not day 2 on the Reduced Salt diet (diet overall P=0.07, time = 0.004) compared with the Usual salt diet (Week 6 US 1.45±0.38 pg/ml; RS 1.25±0.39 pg/ml P<0.02). There was no significant correlation between the change in FMD and change in ET-1 at Week 6 (r=0.15 P=0.9)

Adhesion molecules

There were transient effects on E and P selectin on day 2 with falls from baseline on the usual salt diet only which had disappeared by week 6. There were no changes in ICAM-1 or VCAM-1 (Table 1).

2.5 DISCUSSION

We have demonstrated that usual salt diet impairs FMD and increases endothelin-1 and that this is reversed by salt reduction of approximately 3 g/day. Endothelin-1 decreased by 14% after 6 weeks which and this was associated with a 45% increase in FMD. To our knowledge this change in endothelin-1 has not previously been observed in normotensive adults in response to modest dietary salt change. Previous studies in normotensive and hypertensive subjects in which diets were 50, 150 or 250mmol sodium/day (3, 9 and 15g salt respectively) did not change plasma endothelin-1 concentrations [137] [138]. In contrast Bragulat et al (2001) observed an increase in endothelin-1 after 7 days on a low salt diet (3g/day) and a decrease on a high salt diet (15g/day) in a group of patients with essential hypertension [118]. The reasons for these conflicting results require clarification but duration of the diet may be important as we saw no effects of a reduced salt diet after 2 days.

Inhibition of the action of ET-1 with its specific inhibitor bosentan increased FMD by more than an absolute 5% after 4 weeks and inhibition of endothelin-1 in heart failure has also been shown to increase FMD [139] [140]. Tsai et. al. [141] reported increased expression of circulating and aortic endothelin-1 protein levels in rats following a high salt diet providing further confirmation that a high salt intake may increase endothelin-1.

Casey et al found an inverse relationship between FMD and endothelin-1 levels in patients with coronary artery disease in a cross-sectional study [142]. However we did not observe any significant correlation between endothelin-1 and FMD.

These findings support our previous work in which a larger reduction of 6g/day improved FMD by 45% after 2 weeks [111]. The novelty of the present study is we have shown that the improvement in FMD happens quickly, after 2 days and is sustained at 6 weeks. Another study also demonstrated FMD was improved by 68% after reducing salt intake for

4 weeks [114]. FMD is a valid predictor of cardiovascular events [143] and the magnitude of change in FMD in the current study has the potential to shift an individual from a high to a low risk category for cardiovascular events [39]. We also found a significant relationship between change in FMD and change in urinary sodium to creatinine ratio that was not related to changes in blood pressure supporting recent findings that sodium intake has direct effects on endothelial function [114].

We also observed a significant change in the endothelium-independent vasodilation and speculate that this is mediated by the change in endothelin-1 as it is known that endothelin-1 exerts paracrine vasoconstrictive actions on vascular smooth muscle cells [144]. Endothelin-1 secretion is mostly into the subendothelial space. However endothelin-1 is also present in plasma and has autocrine actions on endothelial cells to release NO. It has been found that rats exposed to hypoxia had increased plasma endothelin -1 that inversely correlated with arterial PO2 [145] and that was due to increased endothelin -1 gene expression [146] so we believe a fall in plasma endothelin-1 reflects lower endothelin -1 mRNA expression and has physiological significance. In overweight and obese subjects infusion of an ET-A receptor antagonist restores ACh-mediated endothelium-dependent vasodilation so luminal events are also important [147].

We did not observe any changes in plasma or urinary total nitrate and nitrite concentrations between the reduced and usual salt interventions. Previous short term studies of salt loading have demonstrated a reduction in plasma nitrate/nitrite concentrations [109, 116] which was reversed with salt restriction. However when vascular responses were also measured, these were not related to the changes in nitrate/nitrite [109]. It may be our methods are not sensitive enough to detect small changes in NO production or the mechanism may not be via changes in NO production.

ADMA, an endogenous inhibitor of NO has been shown to be increased with salt loading and shown to be significantly related to the change in total plasma nitrate/nitrite concentration and BP changes [117] [120]. In contrast we did not did not observe any significant changes in ADMA or BP in the current study with modest salt intake changes.

Adhesion molecules (VCAM-1, ICAM-1, E-selectin and P-selectin) were used as measures of endothelial function and are involved in the pathogenesis of atherosclerosis [148] [149]. In the current study no changes were observed for ICAM-1 or VCAM-1 or E and P selectin. A previous study in salt-sensitive and salt-resistant hypertensive adults demonstrated VCAM-1, ICAM-1 and E-selectin increased in a dose dependent manner when sodium was increased from 20mmol Na/day to 220mmol Na/day [150]. In contrast a recent study has shown in apolipoprotein-E knockout mice, vascular expression of adhesion molecules increased along with increased atherosclerosis following a low salt diet [151]. This was thought to be mediated by activation of the renin-angiotensin system with very low salt diets, and hypothesised as a mechanism to explain some epidemiological evidence that very low salt diets promote atherosclerosis progression and cardiovascular deaths [152] [153]. It is of note we saw no change in aldosterone and renin with the modest salt reduction in the present study.

No changes in BP occurred between the diets in the current study, which is consistent with previous studies [114]. The change in FMD was not related to changes in BP which supports our hypothesis that salt reduction has beneficial effects on endothelial function independently of blood pressure.

We did not observe any significant changes in PWV or AIx, predictors of future cardiovascular events [154] and mortality [43], which could be explained in part by lack of change in blood pressure. Previous studies of dietary salt modification on arterial stiffness have not shown consistent results. Salt reduction over a 4 -6 week period was

shown to decrease PWV in hypertensive volunteers [155]. A 22% reduction in PWV following salt reduction in normotensives has also been shown [49].

Limitations

A limitation of the protocol is the absence of a placebo for the reduced salt intervention, so participants knew which intervention they were receiving. We also experienced a very high drop-out rate (50%) highlighting the difficulties of recruiting and keeping volunteers in a study involving burdensome urine collections.

The final sample size was small for some of the biochemical measures. We had 80% power $P<0.05$ to see differences of the order of 5-6% in blood pressure measures, <8% for ICAM-1, VCAM-1 and E-selectin, <15% for PWV and P-selectin, 20% for Aix and approximately 30-35 % for the other measures. The 30-35% changes are clearly much larger than would be expected to occur with this mild intervention. A further limitation of the study was the absence of a control group of non-obese people so we were not able to determine if non-obese subjects respond in a similar way.

We conclude that a diet containing 9g salt impairs FMD and that this is reversed by a modest reduction in salt of 3g/day. This small sodium reduction also decreased endothelin-1. The renin-angiotensin-aldosterone system was not altered by this change in salt intake.

Author contributions

JBK and PMC developed the hypotheses tested in the study. KMD contributed to study design, planned and conducted the study, performed the vascular measurements, developed diet materials, performed the statistical analyses, interpreted the results and wrote the manuscript. JBK and PMC contributed to statistical analyses, interpretation of the data and critically reviewed the manuscript.

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Disclosures

None of the authors had any conflict of interest in relation to this manuscript.

Table 2.1 : Changes from Usual Salt (US) to Reduced Salt (RS) diet after 2 days and 6 weeks

		BL	US Day 2	US Week 6	RS Day 2	RS Week 6	Diet p value
Weight (kg)		92±16	91±16	91±16	91±16	91±16	NS
Clinic BP	SBP	120±13	115±9	118±16	117±12	115±10	NS
mm Hg	DBP	77±7	73±8	74±8	74±7	73±6	NS
	MAP	91±8	87±9	90±9	88±8	87±7	NS
24ABPM	SBP	123±9		123±12		121±11	NS
mm Hg	DBP	72±5		73±7		71±6	0.07
	MAP	89±6		90±8		88±7	0.08
	HR	69±8		68±8		67±7	NS
	PP	52±8		51±8		50±8	NS
Vascular	FMD (mm)	0.13±0.07	0.15±0.09	0.12±0.09	0.20±0.08*	0.19±0.09†	<0.001
	FMD %	3.7±2.2	4.4±2.5	3.5 ±2.8	5.9±2.2*	5.6±2.8†	<0.001
	GTN %	20.5±9.2	22.7±8.9	21.8±7.1	25.3±8.7	23.2±7.9	0.04
	PWV (m/s)	11.0±4.6	10.9±4.7	9.6±2.9	10.7±2.6	10.7±3.5	NS
	AIx (%)	27.1±7.8	27.9±7.3	29.7±6.5	28.1±7.3	28.8±6.3	NS
Urine	Na	154±58	142±55	155±58	94±36*	113±45†	<0.001
mmol/24h	K	76±23	79±30	78±32	75±25	80±24	NS
	Cr	12.2±3.9	12.1±4.2	12.2±4.5	11.5±3.7	13.0±4.1	NS
	Na/Cr	12.2±3.9	12.3±3.9	13.4±4.9	8.6±3.6*	9.1±3.4†	<0.001
	nitrate/ nitrite	1.2±0.6	1.1±0.6	1.1±0.6	1.1±.7	1.0±0.5	NS
Plasma	Nitrate/ nitrite (umol/L)	21.6± 14.6	21.3± 12.8	20.0± 11.17	24.3±20.7	20.9± 9.3	NS
	ET-1 (pg/ml)	1.41±0.50	1.58±0.57	1.45±0.38	1.53±0.51	1.25±0.39†	0.07
	Renin (pg/ml)	14.3±8.9	15.4±8.6	14.7±7.4	14.9±10.1	14.7±9.7	NS

Aldosterone (pmol/L)	299±182	290±110	304±179	294±152	278±145	NS
ICAM-1 (ng/ml)	25.2±53.8	25.3±60.0	25.5±55.6	25.2±55.2	25.4±55.6	NS
VCAM-1 (ng/ml)	73.3±27.5	75.7±23.0	76.8±25.0	71.7±24.3	70.2±24.0	NS
E-selectin (ng/ml)	3.9±1.3	3.6±1.3	3.9±1.3	4.0±1.3	3.9±1.4	NS
P-selectin (ng/ml)	7.0±1.5	6.5±1.7	7.0±2.3	7.1±2.3	7.3±2.1	NS
ADMA (umol/L)	3.8±1.5	3.8±1.3	3.5±2.0	4.1±1.4	3.8±1.3	NS

* Significantly different from Usual Salt Day 2 (with Bonferroni adjustment for multiple comparisons)
† Significantly different from Usual Salt Day 6 (with Bonferroni adjustment for multiple comparisons)

Abbreviations

ADMA, asymmetric dimethylarginine
AIx, augmentation index
DBP, diastolic blood pressure
FMD, flow-mediated dilatation
GTN-MD, endothelium-independent dilatation
ICAM-1, intracellular adhesion molecule-1
MAP; mean arterial pressure
PP, pulse pressure
PWV, pulse wave velocity
SBP, systolic blood pressure
VCAM-1, vascular cell adhesion molecule-1

Table 2.2 : Mean dietary intake estimated from 3, 3-day weighed food records during the usual salt and reduced salt interventions (excluding sodium supplementation)

	Baseline	Usual Salt Diet	Reduced Salt Diet
Weight (g)	3073±840	3075±1041	3085±912
Energy kJ	9154±2491	8149±2224	8494±1875
Protein (g)	104±31	92±23	97±19
% kJ from Protein	19±3	20±3	20±3
Total fat (g)	84±28	69±24	74±22
kJ from Fat	33±4	31±4	32±4
Saturated fat (g)	31±11	23±11	26±11*
% kJ as saturated	14±1	11±2	12±2
Polyunsaturated Fat (g)	13±4	13±4	14±4
% kJ as polyunsaturated	6±1	7±1	7±1
Monounsaturated fat (g)	32±12	27±10	28±8
% kJ as monounsaturated	14±1	13±1	13±1
Cholesterol mg	342±148	299±95	312±108
Carbohydrate (g)	227±56	215±64	221±53
% kJ from Carbohydrate	42±6	43±6	43±5
Alcohol (g)	10±20	7±9	7±10
% kJ from Alcohol	3±5	3±4	2±4
Dietary fibre (g)	25±8	28±10	28±8
Total folate (ug)	311±94	334±135	310±85
Sodium mg	2761±1031	1729±627	1799±497
Sodium (mmol)	120±45	75±27	78±22
Potassium mg	3521±981	3417±1050	3539±793
Magnesium mg	370±99	378±102	395±87
Calcium mg	896±299	747±192	840±357

* Significantly different compared to Usual Salt Diet ($p<0.05$)

Figure 2.1: Flow diagram of a randomised control trial assessing the effects of modest salt reduction on endothelial function. Describes subject screening, enrolment, randomisation and completion of the 12 week protocol involving following a reduced salt diet, with and without sodium supplementation for 6 weeks each.

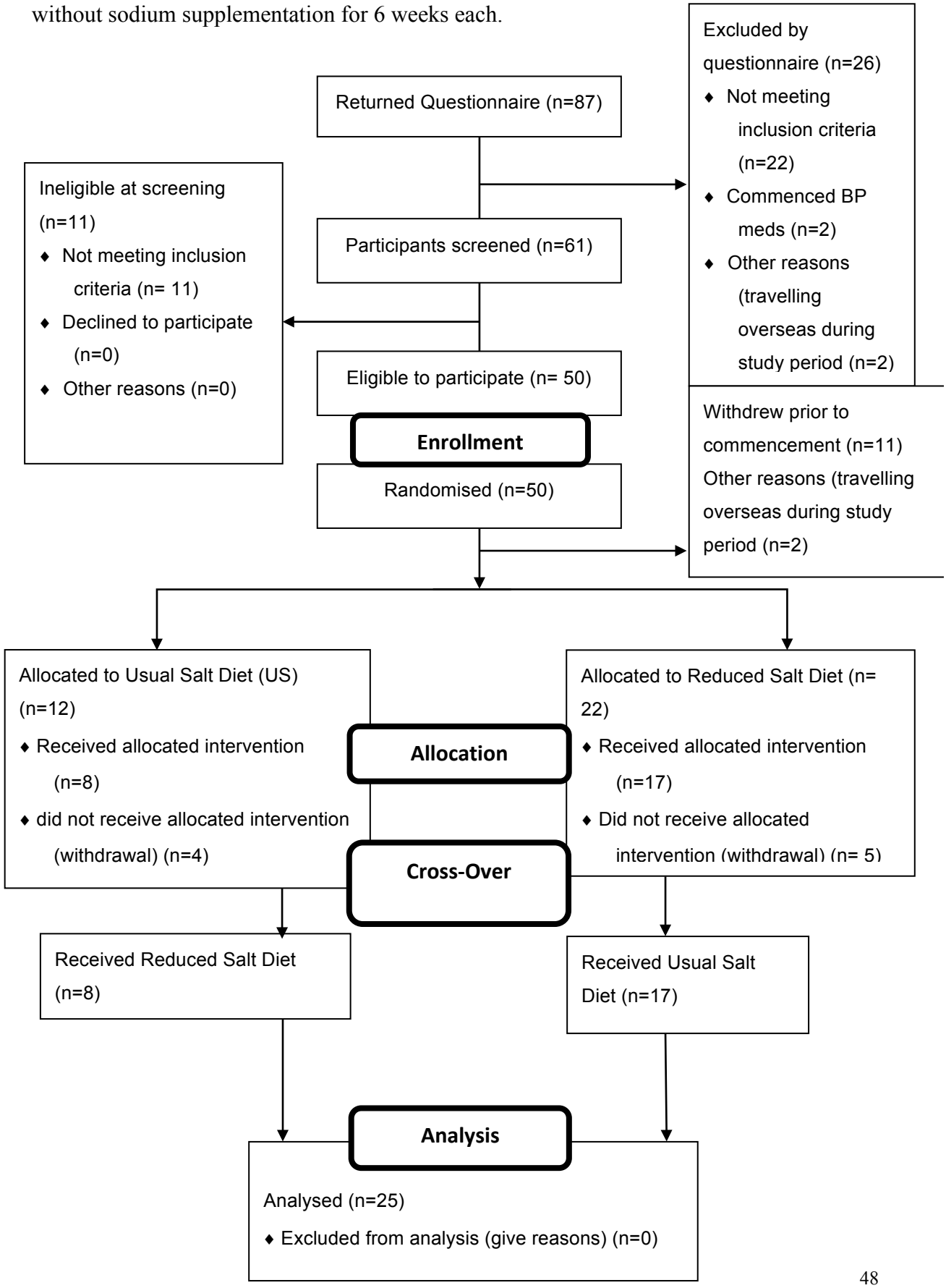
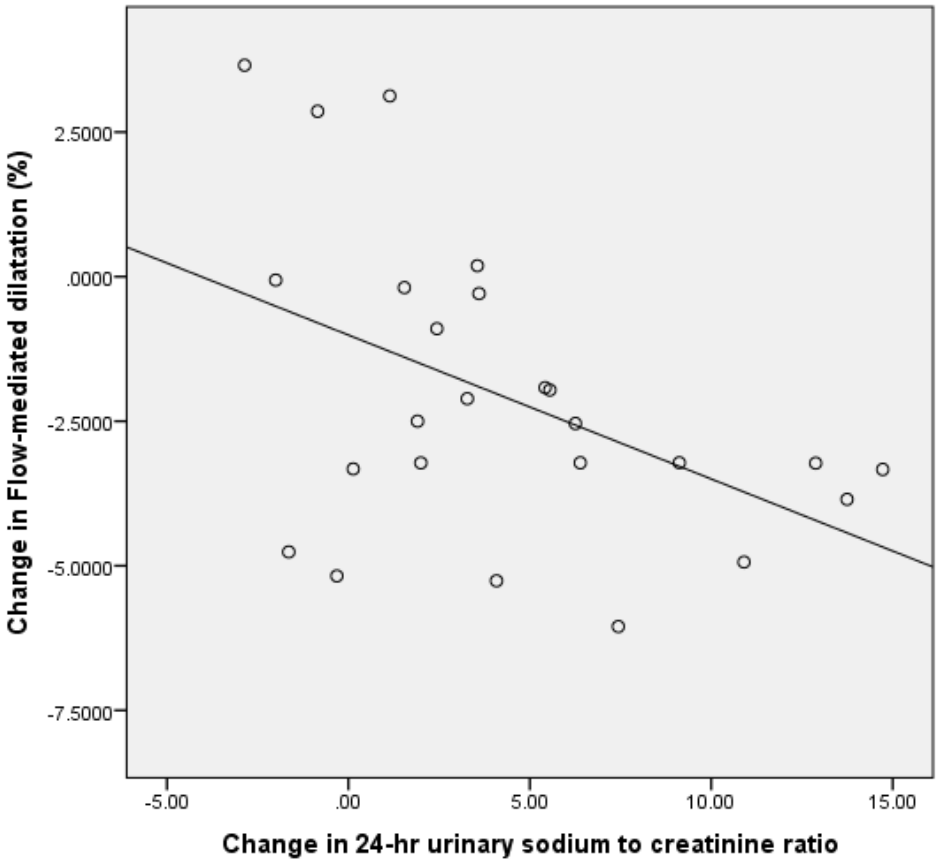


Figure 2.2: Correlation between change in 24h urinary sodium to creatinine ratio and change in flow-mediated dilatation (FMD) in 25 overweight and obese men and women following consumption of a usual salt diet and a reduced salt diet for 6 weeks each in a crossover study. Pearson correlation analyses were conducted to assess the association of change between variables. There was a significant correlation between variables ($r = -0.470$, $P<0.05$).



Chapter 3: Endothelial function is impaired after a high salt meal in healthy subjects

A version of this manuscript is published in Am J Clin Nutr 2011; 93; 500-505

STATEMENT OF AUTHORSHIP

Kacie Dickinson (Candidate)

Developed protocol, prepared ethics application, conducted the study, performed vascular measurements, performed statistical analyses, interpreted data, wrote the manuscript, acted as corresponding author.

Signed Date 22.11.2013

Peter Clifton

My contribution to the paper involved:

Developed hypothesis and study design, assistance with statistical analyses, data interpretation and manuscript evaluation. I give consent to Kacie Dickinson to present this paper for examination towards the degree of Doctor of Philosophy.

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Chapter 4: Postprandial effects of a high salt meal on serum sodium, arterial stiffness, markers of nitric oxide production and markers of endothelial function.

STATEMENT OF AUTHOR CONTRIBUTIONS AND RESPONSIBILITIES

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Signed Date 22.1.2013

Postprandial effects of a high salt meal on serum sodium, arterial stiffness, markers of nitric oxide production and markers of endothelial function.

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This trial was registered with the Australian and New Zealand Clinical Trials Registry Unique Identifier: ACTRN12611000583943

http://www.anzctr.org.au/trial_view.aspx?ID=343019

4.1 ABSTRACT

Aim: The aim of the study was to determine if a high salt meal containing 65mmol Na causes a rise in sodium concentrations and a reduction in plasma nitrate/nitrite concentrations (an index of nitric oxide production). Secondary aims were to determine the effects of a high salt meal on augmentation index (AIx) a measure of arterial stiffness and markers of endothelial function.

Methods and Results: In a randomised cross-over study 16 healthy normotensive adults consumed a low sodium soup containing 5mmol Na and a high sodium soup containing 65mmol Na. Sodium, plasma nitrate/nitrite, endothelin-1 (ET-1), C-reactive protein (CRP), vasopressin (AVP) and atrial natriuretic peptide (ANP) concentrations before and every 30 minutes after the soup for 2 hours. Blood pressure (BP) and AI were also measured at these time points.

There were significant increases in serum sodium, osmolality and chloride in response to the high sodium meal. However plasma nitrate/nitrite concentrations were not different between meals (meal p =0.812; time p=0.45; meal x time interaction p=0.50). Plasma ANP, AVP and ET-1 were not different between meals. AI was significantly increased following the high sodium meal (P=0.02) but there was no effect on BP.

Conclusions: A meal containing 65mmol Na increases serum sodium and arterial stiffness but does not alter postprandial nitrate/nitrite concentration in healthy normotensive individuals. Further research is needed to explore the mechanism by which salt affects vascular function in the postprandial period.

4.2 INTRODUCTION

There is substantial evidence of the adverse effects of high sodium intakes on blood pressure and cardiovascular health [85, 175]. Accumulating evidence suggests that there are adverse effects of a high sodium intake on endothelial function that are independent of blood pressure [111]. Endothelial dysfunction is regarded as an important initial event in atherogenesis and impaired nitric oxide (NO) production is thought to be a common pathway of endothelial injury and progression to clinical cardiovascular disease (CVD) [4, 176].

Endothelium dependent dilatation and endothelial NO production have been shown to be impaired by short term high salt intakes [108, 109, 116]. We previously demonstrated that flow-mediated dilatation (FMD), a measure of endothelium dependent vasodilatation, is significantly impaired after a meal containing 65mmol Na compared with a meal containing 5mmol Na/day but whether NO concentrations are altered following a high salt meal had not been demonstrated [133].

Arterial stiffness, a predictor of cardiovascular risk and mortality has been shown to improve with salt reduction [49, 177, 178]. However the postprandial effects of a high salt meal on measures of vascular stiffness as measured by augmentation index (AIx) it is unknown.

Elevated circulating levels of endothelin-1 (ET-1) are a hallmark of endothelial dysfunction. Chronic excess dietary sodium intake has been shown to increase ET-1 expression but it is not known if ET-1 is altered acutely by a high sodium meal [141]. Studies also suggest that inflammatory markers such as C-reactive protein (CRP) are

associated with higher dietary sodium intakes in hypertensive individuals but it is not known if CRP is altered in response to a high salt meal [179].

Both AVP and atrial natriuretic peptide (ANP) have vasoactive properties and may be altered acutely following a salt load, which may in part explain the effects observed on postprandial vascular function in response to salt loading [180, 181].

Our aim was to determine if a meal containing 65mmol Na, a sodium load which we have previously shown impairs flow-mediated dilatation [133] causes a reduction in plasma nitrate/nitrite concentrations (an index of nitric oxide production). We hypothesised sodium concentrations would increase and that nitrate/nitrite concentrations would decrease following a high salt meal. Secondary aims were to investigate the effects of the high salt meal on vascular function as measured by AIx and on plasma AVP, ANP, endothelin-1 and CRP.

4.3 METHODS

Subjects

Sixteen men and women aged between 18-70 years were recruited by advertisement at the local university and hospital and from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Food and Nutritional Sciences Adelaide. Inclusion criteria were body mass index (BMI) $\geq$18kg/m^2 and $\leq$27 kg/m^2, systolic blood pressure (SBP) < 130mmHg, diastolic blood pressure (DBP) <90mmHg, weight stable in the preceding 6 months, no use of anti-hypertensive medication, systemic steroids, folate supplementation or non-steroidal anti-inflammatory drugs. Participants were not excluded if they were taking other vitamin or mineral supplements provided their dosage and frequency remained

unchanged for the duration of the study. Sixteen participants met the selection criteria, including two women taking oral contraceptives and one woman who waspost-menopausal. The study was approved by the CSIRO Human Research Ethics Committee (HREC11/05) and the University of Adelaide Human Research Ethics Committee (H-033-2011). All participants gave written informed consent. This trial was registered with the Australian and New Zealand Clinical Trials Registry (Unique Identifier: ACTRN12611000583943). URL http://www.anzctr.org.au/trial_view.aspx?ID=343019

Study Methodology

In a randomised cross-over design, participants attended the clinical research unit on two mornings separated by at least one full day and consumed a high sodium meal (HSM) containing 65mmol Na or a control meal (LSM) containing 5mmol Na. Both meals contained 130mg potassium (3.3mmol). Subjects were randomly assigned to treatment order by using a numbered random-allocation sequence generated by a person independent to the study (CLINSTAT software; Martin Bland, York, United Kingdom). Participants were required to fast from 10pm the night before (no food, water only) and refrain from alcohol, smoking, vigorous exercise and caffeine in the 24hours prior to each study. On arrival, body height was measured at baseline to the nearest 0.1 cm with a stadiometer (SECA, Hamburg, Germany) while the participants were barefoot. Body weight was measured to the nearest 0.05 kg with calibrated electronic digital scales (AMZ 14; Mercury, Tokyo, Japan) while the participants were wearing light clothing and no footwear.

Blood pressure and vascular measurements

Seated blood pressure (BP) was measured with an automated sphygmomanometer (SureSigns V3; Philips, North Ryde, Australia) while fasting at Visit 1 and 2. After 5 minutes of rest four consecutive BP measurements were taken 1 minute apart. The first reading was discarded, and the mean of the next 3 consecutive readings with SBP readings within 10 mm Hg and DBP readings within 5 mm Hg of each other were taken as the fasting measurement. Additional measurements were made if required. The AIx was estimated by radial applanation tonometry using the SphygmoCor blood pressure analysis system (AtCor Medical, Sydney, Australia) as previously described [182]. Three consecutive measurements were performed. The intraobserver CV for AIx in our hands was 12.8% on the basis of data for healthy individuals (n = 12) who were tested on 2 separate occasions [111]. A fasting venous blood sample was taken for measurement of serum electrolytes, plasma osmolality and plasma nitrate/nitrite, ET-1, CRP, ANP and AVP. Fasting baseline parameters were assessed between 0800 and 0845 after which participants consumed 250ml soup within 5 minutes. Subsequent blood sampling (seated), BP and AIx and thirst were assessed at 30, 60, 90, 120 minutes after consuming the soup meal. Participants were not allowed to drink during the 2.5hour study protocol.

Serum electrolytes and plasma hormones

Blood for serum was collected in vacutainer tubes with no additives, kept at room temperature and sent to a certified commercial laboratory (IMVS, Adelaide, South Australia) for measurement of electrolytes, osmolality and CRP. Blood for plasma was collected in vacutainer tubes with EDTA for nitrate/nitrite, ET-1 and ANP and lithium heparin for AVP, stored on ice and centrifuged within 15 minutes of collection at 3000rpm for 10 minutes at 4 ^{o}C. The spun plasma was then stored at -80 ^{o}C. Nitrate/nitrite, ANP and

AVP were measured after the completion of the study. ANP samples were analysed by a commercial laboratory (ProSearch International Australia Pty Ltd, PO Box 515, Malvern, Victoria, Australia). Plasma AVP was measured by radioimmunoassay as previously described [183, 184]. The inter-assay and intra-assay coefficients of variation were less than 8% and the limit of detection was approximately 1 pmol/l. Plasma nitrate/nitrite levels were measured in duplicate using a commercially available enzyme immunoassay kit (Nitrate/nitrite Colorimetric Assay Kit, Cayman Chemical Company Ann Arbor, MI). After filtration using 30-kD microfuge ultrafilters (Nanosep 30k Omega Centrifugal Device, PALL Life sciences Ann Arbor, MI, USA), 40 µL of plasma was diluted with 200 µL assay buffer and mixed with 10µL enzyme cofactor and 10µL nitrate reductase After the plasma had been kept at room temperature for 3 hours to convert nitrate to nitrite, total nitrate was measured at 540 nm absorbance following reaction with Griess reagent (sulfanilamide and naphthalene– ethylene diamine dihydrochloride). The intra-assay CV was 2.7% and the inter-assay CV 3.4% and the limit of detection was approximately 2.5µM. Plasma ET-1 levels were measured in duplicate using a commercially available enzyme immunoassay kit (Human Endothelin-1 Immunoassay Kit, R&D System, Inc Minneapolis, MN) according to the manufacturer's instructions. The intra-assay CV was 4.6% and the inter-assay CV 6.5% and the limit of detection was approximately 1.0pg/ml.

Thirst visual analogue scale

Thirst was assessed at the time of blood sampling using a well-validated 10cm visual analogue scale as previously described [185]. Participants were asked the question "*How thirsty do you feel?"* and asked to indicate a vertical line on the scale between *"no thirst"*

at 0cm and *"very severe thirst"* at 10cm to represent their thirst. The thirst rating was defined as the distance (in mm) of the subject's mark from 'no thirst' at 0cm.

Statistical Analyses

Based on a previously study of sodium loading we had 80% power (α = 0.05) to detect a mean difference in serum sodium of 2.1 mmol/l in a cross-over design with 16 participants [186]. Preliminary analyses were conducted to assess normality using the Kolmogorov-Smirnov test and inspection of histograms and Q-Q plots. Paired samples t-test was used to compare fasting variables at baseline between treatments. Repeated measures ANOVA (with meal and time as within-subject variables) were used to assess the effect of intervention on outcomes over time. Gender was included as a between subject factor because of the possible influence of menstrual cycle status in women on ANP and AVP. Pearson's correlation was used to assess association between variables. Analyses were performed with IBM SPSS Statistics (version 20) for Windows (SPSS Inc, Chicago, IL) A Hill function was used to describe the AIx data generated during each 120-minute meal study [187]. For each set of AIx data, "population" parameter values for the Hill function were computed using an iterative two-stage method based on using the population mean at each iteration as a prior for improving the individual parameter estimates [188]. Population kinetic analysis takes into consideration inter-subject variability to estimate kinetic parameters for a group or population of individuals. The PopKinetics software (The Epsilon Group, Charlottesville, VA, USA) was used to fit the Hill function to the AIx data:

$$AIx = a + b\frac{t^n}{t^n + k^n}$$

where a is the baseline AIx, $a + b$ is the maximum AIx, t is time, k is the value of t where the function is 50% of its maximal value, and n, the Hill coefficient, determines the slope of the Hill function at k. Significance was set at $P < 0.05$. All data are Mean±SD unless otherwise stated.

4.4 RESULTS

Subjects

Sixteen participants completed the protocol. There were no significant differences between any fasting clinical and biochemical variables between treatments (Table 1).

Biochemical parameters

The high sodium meal increased serum sodium concentration within 60minutes compared with the low sodium meal (HSM 141±1.3 mmol; LSM 139.6±1.3 meal x time interaction p=0.008). Serum chloride (HSM 106.7±2.7mmol; LSM 104.3±1.8 mmol; meal x time interaction p=0.002) and osmolality (HSM 294±3.9mOsmol/kg; LSM 291±4.2mOsmol/kg; meal x time interaction p=0.046) were increased within 90 minutes compared with the low salt control meal (Figure 1). Potassium concentration increased in response to both meals with no significant difference between treatments (meal x time interaction p=0.253) (Figure 2).

Plasma nitrate and nitrite concentration was not significantly different between meals (meal effect p =0.81; time effect p=0.45; meal x time interaction p=0.50). Plasma ANP and AVP were not significantly different between treatments (Figure 2). This did not change when gender was added into the model as a between subject factor. There were no significant differences between treatments for ET-1 (meal effect P=0.64; time P=0.29;

meal x time interaction P=0.45) or CRP (meal effect P=0.35; time P=0.2; meal x time interaction P=0.36).

There was no significant correlation between plasma nitrate/nitrite and any other electrolyte, osmolality or BP variables (p>0.05). There was no significant correlation observed between change in any blood pressure variables from baseline and change in sodium, potassium, chloride or osmolality from baseline.

Augmentation Index

AIx increased following both meals. The change in AIx was significantly greater following the HSM compared with the LSM (HSM 4.5 % ±1.0 vs. LSM 2.3% ± 0.8 p=0.012). There was no significant difference between other parameters in the model (Table 2)

Blood pressure

There was no significant difference in SBP, DBP, MAP or HR at baseline (Table 1). A significant effect for time was observed for DBP and HR (DBP: P =0.034; HR: P=0.009). No significant effect of meal or meal x time interaction was observed for any BP variable (SBP: meal effect p=0.15; meal x time interaction p=0.37; DBP: meal effect p=0.15; meal x time interaction p=0.68; MAP: meal effect p=0.59; meal x time interaction p=0.41; HR: meal effect p=0.18; meal x time interaction p=0.51).

Thirst

Thirst was significantly greater with the high sodium meal over time compared with the low sodium meal (meal x time interaction p=0.003) (Figure 3). There was no significant correlation between change in AVP and change in thirst (HSM r = -0.38 p =0.15; LSM r = 0.14 p = 0.61)

4.5 DISCUSSION

This study demonstrated that a meal containing 65mmol sodium raised postprandial sodium by 1.5mmol/l in a group of healthy normotensive adults. We have previously shown that administration of an similar sodium load in a group of healthy normotensive individuals impaired postprandial flow-mediated dilatation, a nitric oxide-dependant response, within 60 minutes [133]. We hypothesised that the mechanism responsible for this observation would be a rise in postprandial serum sodium and a concomitant decrease in NO production. Studies in vitro have suggested this as a physiologically plausible mechanism in the postprandial state [119, 124]. A number of studies have shown that chronic sodium loading decreases NO bioavailability among patients who were hypertensive or sodium sensitive [109, 117]. The dietary sodium load in these studies was substantially higher than in the present study. It may be the sodium load was not sufficient to induce alterations in plasma nitrate/nitrite within the 2-hour postprandial period. Two in-vitro studies have demonstrated a significant reduction in nitric oxide bioavailability and nitric oxide synthase activity when plasma sodium was increased [117, 119]. The magnitude of change in sodium in these studies was in the range of 5-10mmol/l, which is greater than the maximum change observed in the current study.

However, concentrations of nitrate/nitrite did not change after the high sodium meal despite the significant rise in serum sodium observed in the present study suggesting that other mechanisms are involved. Other investigators observed a similar rise in serum sodium concentration and osmolality following a meal containing 100mmol of sodium [186]. They also observed a rise in BP which was not replicated in the present study.

We found that arterial stiffness, as measured by augmentation index , was increased to a greater extent (approximately 2% greater increase) following the high sodium meal. Previous longer term studies have shown that arterial stiffness is higher with increased sodium intakes but to our knowledge this is the first time augmentation index responses have been described after a high sodium meal [49]. Contrary to our hypothesis these changes are not explained by changes in nitric oxide bioavailability as nitrate/nitrite was not different between treatments, despite the association between nitric oxide, sodium and endothelial cell stiffness demonstrated in vivo [119]. Other possible mechanisms that may explain these effects could be endothelium-independent alterations in vascular smooth muscle cells caused by a higher sodium intake which were not examined in the current study [189]. The renin-angiotensin-aldosterone system is suppressed by a high sodium intake [99]. In a study in athletes aldosterone concentration decreased by 36.5% after ingestion of sodium citrate with no change in renin [190]. In our study vascular compliance as assessed by augmentation index was worse after the high salt meal with no effect on BP. However we did not analyse renin and aldosterone in the study but a reduction in aldosterone would not be expected to account for the vascular changes. There were no significant differences in plasma ET-1 following the meals. These results contrast with previous findings of chronic high sodium intake that demonstrate increased aortic ET-1 expression suggesting that ET-1 does not change acutely [141].

Compensatory mechanisms stimulated when serum sodium is raised and osmolality increases include fluid movement from the intracellular to the extracellular intravascular space, which if large enough may be accompanied by an increase in BP. However we did not observe any differences in BP in response to the meals, nor was there any correlation

between BP and nitrate/nitrite concentration. This is in contrast to a recent sodium loading study that reported a 1mmol increase in plasma sodium which was associated with an increase in SBP of 1.91mmHg over a 4hour postprandial period [186].

We also studied the response of vasoactive hormones to the high and low sodium meals as a potential mechanism for the conduit vessel vasodilatation we reported previously [133]. Following meal ingestion we observed a parallel decrease in ANP that occurred within 30 minutes following both meals with no significant differences between meals. ANP concentrations returned to fasting levels within the 2-hour period. Previous studies have also shown ANP levels to be unaffected by oral sodium-loading or intravenous saline infusion [180]. One study showed a transient, but significant increase in plasma ANP at 30minutes following a 100mmol sodium load compared with 5mmol sodium control meal, but levels returned to fasting values within 60 minutes [130].

Secretion of AVP, primarily stimulated by increased plasma osmolality, acts as a vasoconstrictor at high concentrations and is also stimulated by thirst. Despite the observed increase in osmolality with the high sodium meal we did not observe a rise in AVP nor was there a significant difference in AVP between the two meals over time. Participants were fasted, which may account for a higher AVP concentration at the time of baseline assessments. We also measured thirst, using a validated visual analogue scale which was significantly increased with the high sodium meal [185]. However, there was no relationship between the change in thirst and change in AVP.

This study used an amount of sodium-chloride typical of that in current foods and single meals consumed in developed countries [191]. However, to produce a change in serum sodium concentration of the magnitude observed in vitro studies it may not be

physiologically possible with oral sodium loading alone, without adverse effects (e.g. nausea and vomiting). We did not attempt to control dietary sodium or nitrate intake during the wash-out period, as we believed that the randomised design of the study would account for differences in habitual food intake.

Limitations of the study include the number of subjects although more than adequate for serum sodium and osmolality was relatively low for the vascular measures. The measurement period of 2 hours was relatively short and a longer collection period would have provided more information on serum sodium and osmolality. Dietary sodium or nitrate intakes were not controlled during the wash-out period which may have influenced the outcome. Participants were asked to replicate their food intake prior to the second study day referring to a 24hr recall which was taken on the first study day.

In conclusion, our results demonstrate that postprandial serum sodium and augmentation index are significantly increased after a meal containing 65mmol Na, which may, in part, explain increased cardiovascular disease with increased dietary salt intake. A rise in serum sodium of this magnitude does not appear to have any effect on nitrate/nitrite concentration over a 2-hour postprandial period in normotensive adults. ANP and AVP did not change in response to oral salt loading so they may not play a role in postprandial vascular responses. Therefore, the mechanism of the effects of salt loading on postprandial vascular function in healthy normotensive adults warrants further investigation.

Author Responsibilities

KMD designed the protocol, conducted the study, analysed the data and wrote the manuscript. PMC and JBK designed the study, contributed to interpretation of the data and critically reviewed the manuscript. LMB contributed to study design, interpretation of the data and critically reviewed the manuscript. PHRB contributed to statistical analysis, interpretation of the data and critically reviewed the manuscript.

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Disclosures

None of the authors had any conflict of interest in relation to this manuscript.

Table 1 : Fasting variables between participants [1]

	Low Salt Meal	High Salt Meal	p
Weight (kg)	71±3	72±3	0.10
SBP (mmHg)	116±3	113±3	0.17
DBP (mmHg)	73±2	71±2	0.11
MAP (mmHg)	87±2	85±2	0.14
HR (bpm)	59±2	58±2	0.13
Augmentation Index (%)	26±3	25±4	0.40
Plasma nitrate/nitrite (µmol/l)	19.6±2.0	22.2±3.0	0.51
Plasma ANP (pmol/l)	18.4±4.9	19.1±6.5	0.50
Plasma AVP (pmol/l)	2.3±0.4	2.6±0.3	0.32
Plasma Endothelin-1 (pg/ml)	1.1±0.1	1.3±0.2	0.37
Serum sodium (mmol/l)	139.6±0.3	139.5±0.4	0.84
Serum potassium (mmol/l)	4.4±0.1	4.4±0.1	0.95
Serum chloride (mmol/l)	104.7±0.6	105.0±0.6	0.53
Serum osmolality (mosmol/l)	291.0±1.1	291.1±1.0	0.91
Serum CRP (mg/dl)	1.1±0.5	0.7±0.3	0.34

[1]Data are Mean±SEM

n=16 (9 female, 7 male)

Abbreviations

ANP, atrial natriuretic peptide; *AVP,* vasopressin, *CRP, C-reactive protein, DBP,* diastolic blood pressure; *HR*, heart rate*; MAP*, mean arterial pressure*; SBP*, systolic blood pressure

Table 4.1 Model to describe the postprandial changes to Augmentation Index following a high salt meal and a low salt meal[1]

	High Salt Meal	**Low Salt Meal**	**P value**
Baseline AIx (%)	24.53 (3.37)	25.42 (2.93)	0.33
Change in AIx (%) over time	4.45 (1.04)	2.34 (0.84)	0.012
[4]Time (min) at which maximum change in AIx (%) observed	65.50 (3.69)	59.94 (6.12)	0.28
The Hill coefficient (a measure of the rate of change in AIx at time k)	10.95	11.68	N

[1]Data are Mean±SEM

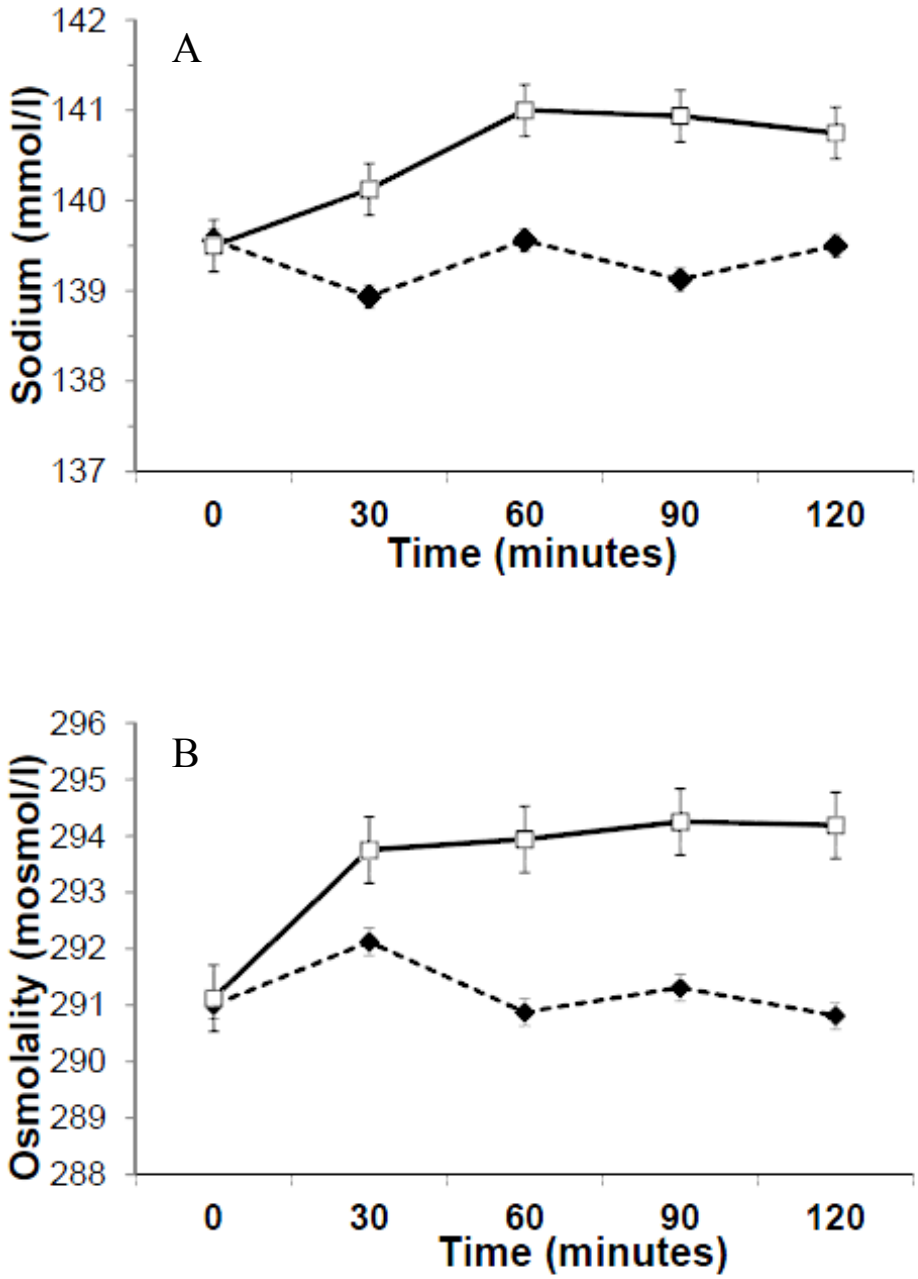
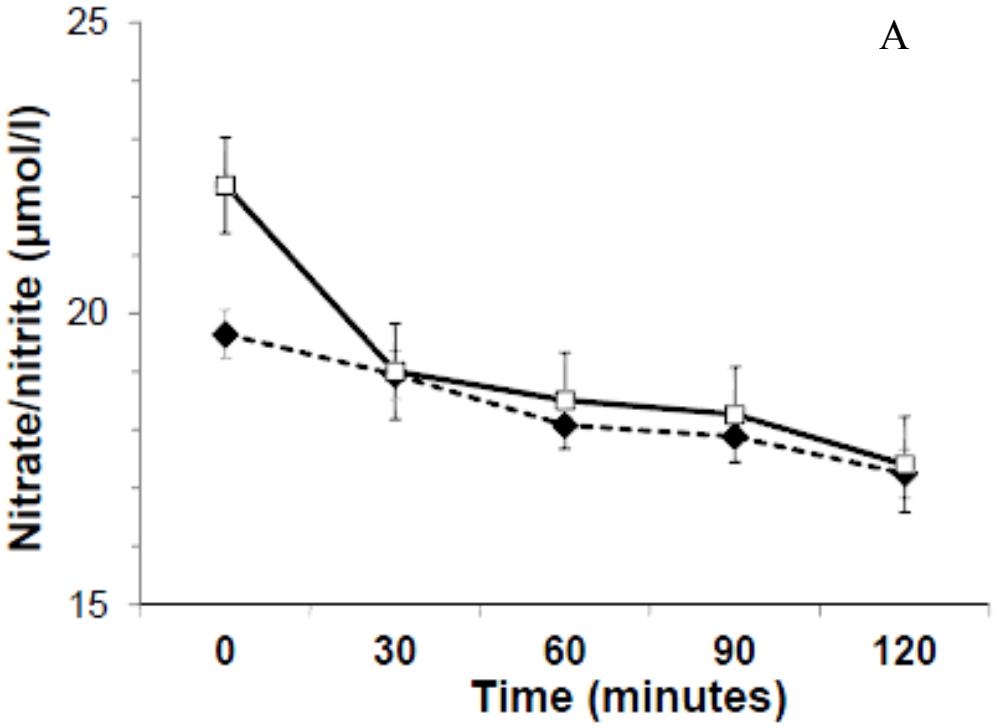


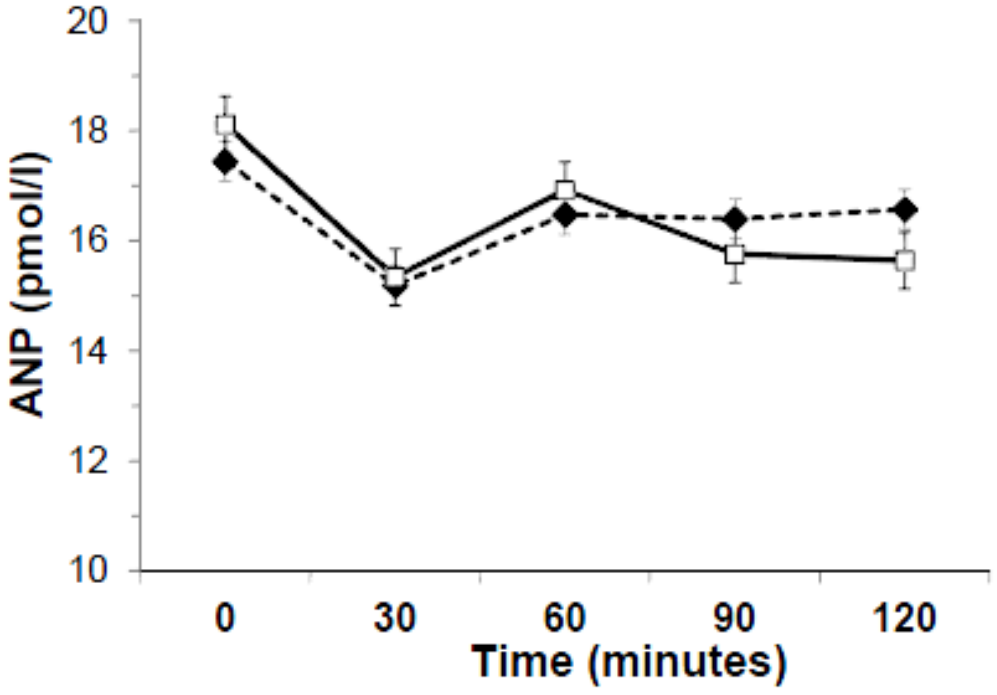
Figure 4.1: Mean (±SEM) serum a) sodium and b) osmolality concentration at fasting and in response to consumption of low salt meal (--♦--) and high salt meal (–□–)

Repeated measures ANOVA:

Sodium: meal p =<0.0001; time p=0.007; meal x time interaction p=0.008

Osmolality: meal p=0.021; time p=0.115; meal x time interaction p=0.046


A
25
20
15
Nitrate/nitrite (µmol/l)
0
30
60
90
120
Time (minutes)


20
18
16
14
12
10
ANP (pmol/l)
0
30
60
90
120
Time (minutes)

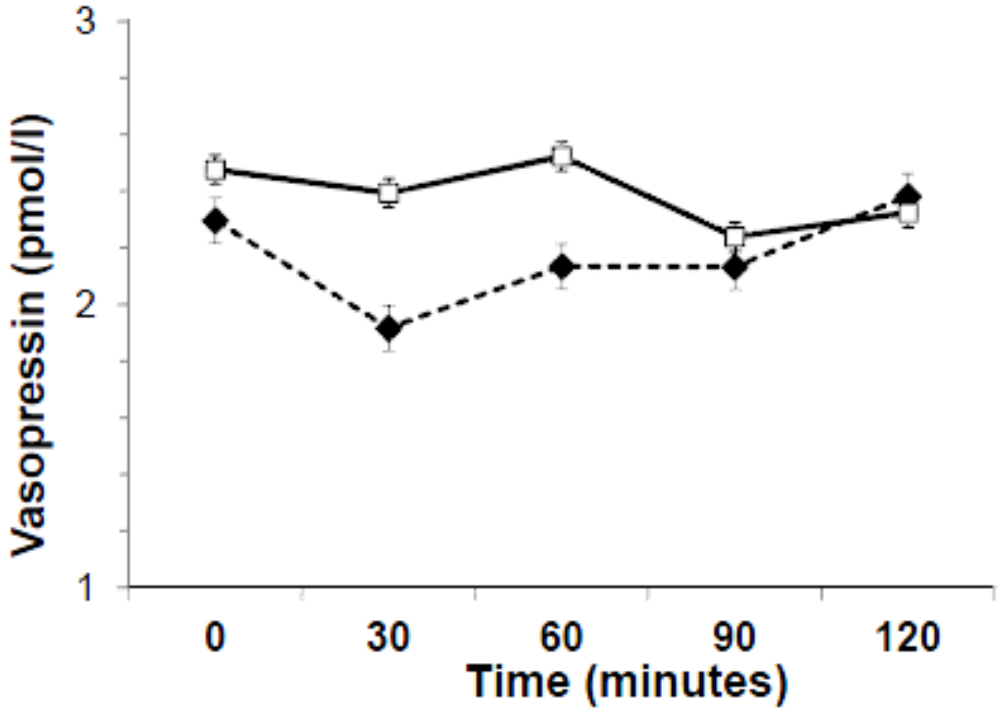


Figure 4.2: Mean (±SEM) a) Plasma nitrate/nitrite, b) ANP and c) vasopressin concentration at fasting and in response to consumption of low salt meal (--♦--) and high salt meal (–□–)

Repeated measures ANOVA

Nitrate/nitrite: Meal p=0.812; Time p =0.45; Meal x time interaction p=0.50

ANP: meal p= 0.961; time p =0.079; meal x time interaction p=0.682

Vasopressin: meal p =0.391 time p=0.841 meal x time interaction p=0.481

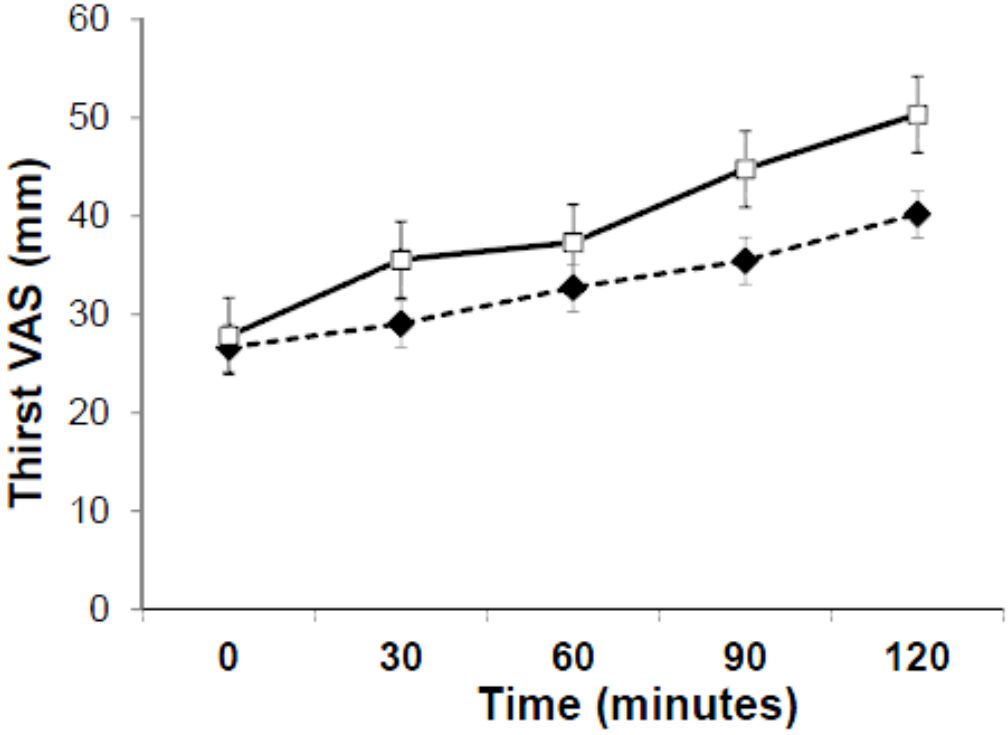


Figure 4.3: Mean (±SEM) thirst at fasting and in response to consumption of low salt meal (--♦--) and high salt meal (–□–)

Repeated measures ANOVA

Meal: p=0.064; Time: p = 0.128; Meal x Time interaction p=0.003

Chapter 5: DISCUSSION

5.1 OVERVIEW

The focus of thesis was to investigate how modifying dietary salt intake impacts vascular function and explore mechanisms underlying these effects. The overall aims of this thesis were to examine whether a small reduction in dietary salt intake can improve vascular function (Chapter 2) and to explore the effects of a high salt meal on post-prandial vascular function (Chapter 3 and 4).

Previous research has shown large reductions in salt intake can improve vascular function and this may be mediated by alterations in NO. However it is uncertain whether a modest reduction in salt intake from current levels of consumption to 6g salt per day can have the same benefits on vascular function in normotensive patients. It is also unknown whether the high reported levels of salt in some processed foods have any adverse effects on post-prandial vascular function when consumed in a single meal.

The results of these studies contribute to evidence that salt may have non-blood pressure dependent mechanisms by which it contributes to cardiovascular disease risk and atherosclerosis progression. In the broader context the results support the view that strategies to reduce population intakes of salt through the food supply, may benefit the cardiovascular health of adults beyond blood pressure reduction alone.

5.2 KEY FINDINGS

5.2.1 CHONIC EFFECTS OF MODEST SALT REDUCTION ON VASCULAR FUNCTION

Previous research has shown that short-term benefits of dietary salt reduction to 4.2 – 6.0g per day has beneficial effects on endothelial function [114] [111]. However no previous studies have evaluated benefits of modest salt reduction from usual intakes to current upper

limits in overweight and obese normotensive adults at risk for cardiovascular disease. It is also not known whether any short term effects observed from modifying salt intake on vascular function persist in a chronic dietary intervention beyond 1 month duration.

Chapter 2 demonstrated that modest salt reduction of 3g per day improves flow-mediated dilatation after 2 days, an effect that persisted after 6 weeks. These findings confirm that a smaller salt reduction (2.5g salt) than has been previously studied has benefits on vascular function. In addition this adds to our knowledge of the time course of vascular effects of salt during a chronic intervention. The 1% mean increase in FMD (2.09% absolute) observed with this small salt reduction after 6 weeks suggests that an individual's risk for cardiovascular events may be considerably lowered with such a level of salt reduction. An improvement in FMD as little as 1% is associated with a 10% reduced risk of cardiovascular events and all-cause mortality [192]. This supports some of the published data around salt and cardiovascular disease related mortality, which suggests small reductions in salt intake (1-3g per day) result in a 20% risk reduction in morbidity and mortality associated with cardiovascular disease [193]. It is uncertain whether the beneficial effects of modest salt reduction on FMD persist beyond the 6 week intervention period in the current study. A prospective randomised controlled trial should be carried out to address this.

We also found the change in UNa:Cr after 6 weeks was significantly correlated with the change in vascular function. These changes in FMD were not related to changes in BP. We also found that nitroglycerin-mediated dilatation was significantly increased after salt reduction. These results suggest that endothelium-independent mechanisms are also involved in the responses observed. Some evidence suggests reduced responsiveness to NO donors in smooth muscle cells with increased salt intake, which may explain these findings [126]. Overall these finding supports previous research into this area, which in general has demonstrated that dietary salt plays a role in modulating vascular function [114] [111] [108] [109].

A secondary aim of Chapter 2 was to determine mechanisms of the effects of salt reduction on vascular function. The reduced salt intervention lowered ET-1 after 6 weeks but did not change plasma concentration or urinary nitrate/nitrite excretion. Previous studies have detected differences in plasma nitrate/nitrite in response to greater magnitudes of salt reduction than in our study [116] [118] [110] [114] [109].

In summary the original contribution of this study is knowledge that a small reduction in salt intake has beneficial effects on vascular function. This builds on previous studies in the area that have studied larger changes in salt. The results in Chapter 2 suggest a role for ET-1 in mediating these effects, but given endothelium independent response to nitroglycerin also improved with salt reduction, we cannot rule out other mechanisms for the effects observed. Taken with other evidence, it appears the vascular effects of salt are multi-factorial so this area needs further research to confirm some of these mechanisms involved.

5.2.2 ACUTE EFFECTS OF SALT LOADING ON VASCULAR FUNCTION

In Chapter 2 we observed that beneficial effects of modest salt reduction on endothelial function were rapid and occurred within 2 days. This is consistent with many short-term studies of salt loading under much more extreme conditions of salt loading and depletion [108]. Contemporary patterns of dietary salt consumption support the fact that it is not uncommon to consume large amount of salt (in the form of sodium) in a single meal [67] [194] [195]. So we decided to explore whether a meal containing a physiologically relevant salt load could have immediate effects on vascular function. Previous studies have demonstrated large changes in salt intake short-term (4-7days) can alter vascular function and our findings in Chapter 2 suggest these changes may occur more rapidly than previously thought. Therefore the aim of this study was to determine if a high salt meal impairs post-prandial endothelial function compared with a low salt control meal. We demonstrated that a high salt meal acutely impairs post-prandial endothelial function within 30 minutes of consuming the meal and that endothelial function had not returned to baseline after 2 hours.

These findings suggest contemporary dietary salt consumption; through the high levels present in many processed and convenience foods has adverse effects on vascular function immediately after a meal. It is not known if this has long-term consequences for CVD risk, however postprandial hyperlipidemia and hyperglycemia which are associated with CVD risk are also associated with adverse effects on vascular function over this post-meal period [158] [159], so the role of salt on this aspect of vascular function requires further clarification.

Following on from the novel findings in Chapter 3, we sought to explore further the mechanisms by which a single meal containing a salt load can acutely alter vascular function. In chronic studies [114] and Chapter 2, it has been demonstrated that the effects of modifying salt on vascular function may be mediated by endothelium derived factors – NO, ADMA and ET-1. However this had not previously been investigated in the post-prandial phase. Based on our findings in Chapter 3 that a high salt meal impairs post-prandial endothelial function, the aim of this study was explore further the effects of high salt meal on post-prandial NO, ADMA and ET-1. In this study we measured augmentation index- a rapid, reproducible, non-invasive, validated marker of arterial stiffness rather than FMD.

The role of vasoactive humoral factors (ANP and vasopressin) was also explored. These may be altered by a physiological oral salt load, and may explain our observations in Chapter 3. The findings of Chapter 4 demonstrated that arterial stiffness is increased post-prandially in response to a high salt meal compared with a low salt meal, thus confirming findings of the adverse effects of a high salt meal on endothelial function in Chapter 3.
The magnitude of increase in AIx following the high salt meal was 18% absolute. This is could potentially lead to an increased risk of cardiovascular events. Vlachopoulos et al [43] found that 10% absolute increase in AIX is associated with a 31% increase in cardiovascular events and 38% increase in all-cause mortality.

However we did not observe changes in total plasma nitrate/nitrite, ET-1, ADMA, ANP or vasopressin in response to the high compared with low salt meal, so we cannot confirm that that these factors play a role in the mechanisms underlying post-prandial vascular dysfunction in response to the salt load in the current study. In summary the original contribution of these studies is knowledge of the post-prandial vascular effects of salt, which may have important implications for CVD health given excessive dietary salt consumption well beyond physiological requirements is a widespread issue.

5.3 STUDY LIMITATIONS

There was substantial difficulty in recruitment and retention of participants to the study in Chapter 2. Approximately 50% of subjects screened who entered into the study withdrew before completion (Figure 2.1). Whilst reasons for withdrawal did not appear to be directly related to dietary modification it is known that drop out in dietary clinical trials may be due to factors such as perception that the intervention will not be of benefit [196]. This may be relevant to this study as our participants were normotensive and did not stand to gain major benefits in BP lowering with only a small salt reduction. In addition, there may be multiple other potential obstacles in implementing a chronic dietary salt intervention in a free-living setting. Previous studies have investigated perceived barriers to successfully reducing salt intake. It is perceived that a reduced salt diet may affect the palatability, enjoyment, convenience and cost of food choices [197] [198]. It is also suggested that a multi-faceted intervention with motivational interviewing would be beneficial in implementation and aiding compliance in successfully reducing salt intake [197]. Despite providing comprehensive dietetic counseling and education and resources all these factors may have played a role in lack of retention of participants in the study [199]. The participant burden was also considerable as the protocol also included multiple 24hr urine collections, 24hr blood pressure monitoring and 3-day weighed food records. However we did not explore the influence of these barriers on participant's ability to comply or remain in the study. These factors should be explored further to aid planning and implanting future dietary salt reduction interventions to ensure maximum compliance. An individualised approach to developing intervention meal plans, using baseline dietary data may be a useful, but

resource intensive strategy [200] in facilitating translation of reduced salt interventions into the community.

Dietary compliance was measured using multiple 24-hr urinary sodium excretion and 3-day weight food records (Chapter 2) and 24-hr dietary recall (Chapter 3). 24-hr urinary sodium excretion is recognised as the gold standard for assessing dietary salt intake, but it has been reported that up to six samples are required to accurately determine salt intake [201]. Intake may also be underestimated with this method where there are increased sweat losses of salt during warmer weather. This is a possibility in Chapter 2 as we recruited two cohorts over a 2-year period from February-August and July-December. A recent study has also revealed that even at constant salt intake, 24hr sodium excretion fluctuates with a weekly rhythm, resulting in sodium storage, which has implications for reliability of 24-hr urinary sodium excretion as the gold-standard measurement for estimating dietary salt intake [202]. Other compliance markers were 3-day weighed food records and 24hr recalls. A major limitation of these methods is the issue of misreporting and measurement error that occurs, particularly in overweight and obese participants who may under-report intake [203].

Guidelines have been established to attempt to minimise methodological differences that exist in FMD assessment [204] [26]. Nonetheless direct comparison of similar intervention studies remains difficult due to methodological differences. The studies in this thesis assessed the maximum brachial artery diameter during a three-minute post-occlusion period to determine FMD. Many studies and groups record brachial diameter at 60 seconds, however maximum dilatation can occur anywhere from 45-90seconds post-occlusion. In fact it has been reported that this may underestimate the FMD response [205]. Differences in cuff occlusion location also have implications for the hyperemic response [27] [206].

The shear stress response is not widely reported in studies that employ FMD methodology, despite its recognised importance [207]. A limitation in Chapter 2 and 3 is that we did not

measure or correct FMD responses for flow rate. In the literature it has been suggested that FMD% response should be normalised to reactive hyperaemic shear stress response.

The rapid degradation of NO poses a difficulty for the sensitive measurement of NO in human plasma [208]. It is also disputed whether changes in total plasma nitrate/nitrite, the method for NO determination in the current study, is a useful marker of endogenous NO production because of variations in dietary sources of nitrates. A recent study demonstrated plasma nitrite rather than nitrate reflects eNOS activity [208]. Another study has shown an inverse association between plasma nitrite and endothelial function, which also suggests measurement of plasma nitrite alone, may be a more suitable biomarker [209]. We did not quantify dietary intake of nitrates and nitrites. It is possible the contribution of high nitrate foods to increases in plasma nitrate/nitrite concentrations during the usual salt intervention is an explanation for the outcomes observed in these studies.

5.4 FUTURE RESEARCH DIRECTIONS

Studying the effects of salt on vascular function is an important area of research, especially with population-wide salt reduction strategies being implemented worldwide with the aim of reducing the burden of CVD. The World Health Organisation has also set a target to reduce global salt intake to less than 5g per day by 2025. Future work related to the area of salt in CVD needs to determine what the longer-term effects of salt reduction are on vascular function to fully understand how it effects clinical disease progression and CVD endpoints. In addition it will be important to determine how individual dietary salt reduction can be sustained long-term, so this should also be incorporated in future work in this area.

Specific experiments that could further clarify the mechanisms of salt loads on post-prandial vascular function observed in Chapter 3 and 4 include intravenous saline infusions to observe effects on the vasculature of wider range of sodium concentrations beyond what can be achieved with an oral salt load. We observed a 2mmol change in plasma sodium in response to a high salt meal, however in vitro studies suggest as much as a 10mmol change in plasma sodium may be required to observe effects on endothelial NO production [119].

In addition to this, investigating the vascular responses of intra-arterial infusion of vasopressor (e.g. noradrenaline) and vasodilator (e.g. acetylcholine) agents in response to varying salt concentrations would clarify further the direct effects of salt on the microcirculation and the role of neurohormonal agents in mediating this response.

It will also be of importance to elucidate the effects and the mechanisms of benefit of salt reduction within specific patient groups at risk for CVD. Patients with T2DM, hypertension and also salt-sensitive patient groups may have different vascular responses to salt reduction and potentially different underlying mechanisms in response to salt which has implications for advice given to patients about benefits of salt reduction and CVD risk within these groups.

Other nutrients are known to have positive effects on blood pressure and vascular function and CVD risk. Dietary potassium may even mitigate the adverse effect of dietary salt on vascular function. Dietary patterns in real life encompass complex combinations of many nutrients and potentially interactions of these combinations of nutrients at the endothelial level. Future work should determine the potential of cardioprotective dietary patterns on vascular function to provide evidence that can be readily translated to public health messages in the community.

5.5 CONCLUSIONS

These findings suggest that both small changes in total daily salt intake and mealtime salt intakes can modify vascular endothelial function. We demonstrated a modest achievable reduction in salt intake improves vascular function after 2 days that was maintained after 6 weeks. To date this is the longest dietary salt intervention to assess effects on vascular function. The implication from this research is that even small reductions in salt intake from current levels are beneficial for vascular function. However more research is required to confirm the mechanisms involved. It is widely documented that many processed foods frequently consumed are high in salt, but this is the first evidence that consuming a high salt meal has immediate post-meal effects on vascular function. These results provide

important information about how contemporary patterns of dietary salt consumption may alter early processes underlying cardiovascular disease.

This work adds to understanding of the role of dietary salt intake on vascular pathophysiology and mechanisms of atherosclerosis. The overall findings of this work contributes to evidence underlying public health recommendations for CVD risk management with greater mechanistic evidence to substantiate the population dietary salt reduction messages which could be implemented easily by health professionals in the general community. The information may also contribute pressure to reduce salt in the food supply in order to support moves to reduce population burden of CVD.

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APPENDICIES

Appendix 1: PUBLISHED PAPER

CHAPTER 3: Published paper

Dickinson KM, Clifton PM, Keogh JB. Endothelial function is impaired after a high salt meal in healthy subjects, American Journal of Clinical Nutrition, 2011; 93; 500-505.

Dickinson, K., Clifton, P. & Keogh, J. (2011) Endothelial function is impaired after a high salt meal in healthy subjects.
American Journal of Clinical Nutrition, v. 93(3), pp. 500-505