

TITLE

Salt sensitivity: genetic and physiological markers and its effects on salt taste perception and intake

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LIVERPOOL HOPE UNIVERSITY

Salt sensitivity: genetic and physiological markers and its effects on salt taste perception and intake

Thesis submitted in accordance with the requirements of
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Philosophy

Leta Pilic

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Abstract

Salt sensitivity of blood pressure (BP) is an independent cardiovascular disease (CVD) and mortality risk factor, present in both hypertensive and normotensive population. Better understanding of this phenotype in healthy individuals may lead to more effective prevention of hypertension and CVD. Salt sensitivity is genetically determined and it may affect the relationship between salt taste perception and salt intake. This thesis, for the first time, comprehensively explored the associations between genetics, salt sensitivity of BP, salt taste perception and salt intake as well as the potential of using genetic information in salt sensitivity biomarker development.

The study population comprised young to middle-aged, healthy adults. Salt sensitivity was defined as the change in BP after seven days of low-salt (51 mmol sodium/day) and seven days of high-salt diet (308 mmol sodium/day). Salt taste perception was identified using British Standards Institution sensory analysis method (BS ISO 3972:2011). Salt intake was assessed with a validated food frequency questionnaire and two 24-hour dietary recalls based on the 5-step multiple pass method. DNA was genotyped for single nucleotide polymorphisms (SNPs) in the *SLC4A5*, *SCNN1B* and *TRPV1* genes coding for sodium and ion channels and transporters. Protein levels were measured from urinary exosomes with the focus, for the first time, on methods readily used in clinical setting, such as enzyme-linked immunosorbent assay (ELISA). Results showed that the participants with AA genotype of the rs7571842 (*SLC4A5*) exhibited the highest increase in BP (Δ SBP = 7.75 mmHg, $p = 0.002$). There was no association between genetics and salt taste perception as well as genetics and salt intake. No associations were observed between salt sensitivity of BP, salt taste perception and salt intake. These results warrant further investigation in a larger sample size study. Nevertheless, preference for salty taste or awareness of health risks related to increased salt intake may be a driver of salt intake in younger and healthy population and warrants further investigation. The involvement of *SLC4A5* in salt sensitivity of BP, together with functional effects of the investigated SNPs, makes it a candidate for genetic and physiological marker of salt sensitivity. The ELISA measurement of its expression from urinary exosomes may serve as a method of choice in a clinical setting, if further optimised.

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Publications and Conference Presentations

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Abbreviations

ABPM	Ambulatory blood pressure monitoring
ACE	Angiotensin-converting enzyme
ADMA	Asymmetric dimethyl arginine
AHA	American Heart Association
AMPM	Automated multiple pass method
ANP	Atrial natriuretic peptide
APOA	Apolipoprotein A
APOE	Apolipoprotein E
AQP	Aquaporin
ATP	Adenosine triphosphate
AUC	Area under the curve
BMI	Body mass index
BP	Blood pressure
BSI	British Standards Institution
CA-VI	Carbonic anhydrase
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
cRNA	Complementary ribonucleic acid
CV	Coefficient of variation
CVD	Cardiovascular disease
CVP	Circumvallate papillae
Dahl R	Dahl salt-resistant rat
Dahl S	Dahl salt-sensitive rat
DASH	Dietary Approaches to Stop Hypertension
DBP	Diastolic blood pressure
DTC	Direct to consumer
ELISA	Enzyme-linked immunosorbent assay
ENaC	Epithelial sodium channel
ET-1	Endothelin-1
FFQ	Food frequency questionnaire

GFR	Glomerular filtration rate
GWAS	Genome wide association study
HCl	Hydrochloric acid
HNF4A	Hepatocyte nuclear factor 4 alpha
HRP	Horseradish peroxidase
IHD	Ischaemic heart disease
Ile	Isoleucine
ISO	International organisation for standardisation
KCl	Potassium chloride
LD	Linkage disequilibrium
MAF	Minor allele frequency
MAP	Mean arterial pressure
mRNA	Messenger ribonucleic acid
miRNA	Micro-ribonucleic acid
MTHFR	Methylenetetrahydrofolate reductase
MUFA	Monounsaturated fatty acid
MVB	Multivesicular body
N	Normality
Na	Sodium
NaCl	Sodium chloride
Na-K-ATP-ase	Sodium potassium pump
NaOH	Sodium hydroxide
NBCe1	Sodium bicarbonate cotransporter 1
NBCe2	Sodium bicarbonate cotransporter 2
NCC	Thiazide sensitive sodium chloride cotransporter
NDCBE	Sodium dependent chloride bicarbonate exchanger
NDNS	National diet and nutrition survey
NHE	Sodium hydrogen exchanger
NICE	National Institute for Health and Care Excellence
NIH	National Institutes of Health
NKCC2/Na-K-2Cl	Sodium-potassium-chloride cotransporter
NO	Nitric oxide
OD	Optical density

OR	Odds ratio
PABA	Para aminobenzoic acid
PAT1	Putative anion transporter 1
PCR	Polymerase chain reaction
PHE	Public Health England
PODXL	Podocalyxin-like protein 1
PP	Pulse pressure
PRA	Plasma renin activity
PTC	Phenylthiocarbamide
PUFA	Polyunsaturated fatty acid
RAAS	Renin angiotensin aldosterone system
RBF	Renal blood flow
RNI	Reference nutrient intake
rNTS	Rostral nucleus of solitary tract
RR	Relative risk
SACN	Scientific Advisory Committee on Nutrition
SBP	Systolic blood pressure
SCNN1(A/B/C)	Amiloride sensitive sodium channel
SEM	Standard error of the mean
SFA	Saturated fatty acid
SLC	Solute carrier
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system
SS BP	Salt sensitivity of blood pressure
STDT	Salt taste detection threshold
STRT	Salt taste recognition threshold
TEI	Total energy intake
TGF- β	Transforming growth factor β
TRPV1	Transient receptor potential cation channel, subfamily V, member 1
TSG101	Tumor susceptibility gene 101 protein
USDA	United States Department of Agriculture
UVA	University of Virginia

Val

VEGP

WHO

Valine

von Ebner's gland protein

World Health Organisation

Chapter 1 Introduction

1.1. Hypertension

1.1.1. Definitions and epidemiology

High blood pressure (BP), also referred to as hypertension, is one of the main preventable causes of premature morbidity and mortality in the UK (National Institute for Health and Care Excellence (NICE), 2011). Worldwide, approximately 54% of stroke and 47% of ischaemic heart disease (IHD) events can be attributed to hypertension (Lawes et al., 2008) and hypertension is strongly related to vascular and overall mortality (Lewington et al., 2002).

BP is normally distributed in the population and, according to the NICE, the natural cut-off point above which hypertension would exist cannot be defined. The risk of cardiovascular disease (CVD) increases continuously and with each 2 mmHg increase in systolic blood pressure (SBP) there is a 7% increase in risk of mortality from IHD and a 10% increase in the risk of mortality from stroke (NICE, 2011). Approximately a third of deaths attributed to BP occur in individuals with BP lower than the hypertensive range (Appel, 2017).

However, the following guidance has been developed to enable easier diagnosis of hypertension with the following cut-offs:

- Stage 1 hypertension: SBP 140 mmHg or higher and diastolic BP (DBP) 90 mmHg or higher,
- Stage 2 hypertension: SBP 160 mmHg or higher and DBP 100 mmHg or higher,
- Severe hypertension: SBP 180 mmHg or higher and DBP 110 mmHg or higher (NICE, 2011).

The prevalence of hypertension for adults in England in 2015 was 31% among men and 26% among women. The Health Survey for England shows that the population average BP in England has fallen over the last decade by almost 3 mmHg, however remaining at the same level over the last three years. In addition, the most recent data available shows that the estimated prevalence of undiagnosed hypertension in general practitioners practices in 2014/15 ranged from 3.8% to 20.4% with an interquartile range of 11% to 13% (Evans and Hughes, 2016). The prevalence of hypertension in other UK countries reflects the one in England (Department of Health Northern Ireland, 2016; Scottish Government, 2016; Welsh Government, 2016).

In addition to the country-specific statistics, recent systematic analyses of population-based studies report on global disparities in hypertension prevalence. Data from 135 population-based studies in 968 419 adults from 90 countries shows that in 2010, 31.1% of the world's adults had hypertension; 28.5% in high-income countries and 31.5% in low- and middle-income countries (p for difference in prevalence = 0.001). From 2000 to 2010, the age-standardised prevalence of hypertension decreased by 2.6% in high-income countries, but increased by 7.7% in low- and middle-income countries (Table 1.1). This can be attributed to disparities in hypertension awareness, treatment and control between high-income and low- and middle-income countries. From 2000 to 2010, the proportions of awareness (58.2% vs. 67.0%), treatment (44.5% vs. 55.6%), and control (17.9% vs. 28.4%) increased substantially in high-income countries. During the same time period, the proportions of awareness (32.3% vs. 37.9%) and treatment (24.9% vs. 29.0%) increased much less and the proportion of control even slightly decreased (8.4% vs. 7.7%) in low and middle-income countries (Mills et al., 2016). Moreover, according to data from 154

countries with 8.69 million participants, over the past 25 years (from 1990 to 2015) the rate of SBP of at least 110 to 115 mmHg increased from 73 119 to 81 373 per 100 000 persons, and SBP of 140 mmHg or higher increased from 17 307 to 20 526 per 100 000 persons. The estimated rate of annual deaths associated with SBP of at least 110 to 115 mmHg increased from 135.6 to 145.2 per 100 000 persons, and for SBP of 140 mmHg or higher increased from 97.9 to 106.3 per 100 000 persons (Forouzanfar et al., 2017).

Table 1.1 Age-specific and age-standardised prevalence estimates and absolute numbers of men and women with hypertension in high-income and low- and middle-income countries in 2010 (Adapted from Mills et al., 2016).

Age (years)	Prevalence %				Absolute numbers in millions			
	High-Income Countries		Low- and Middle-Income countries		High-Income Countries		Low- and Middle-Income countries	
	Men	Women	Men	Women	Men	Women	Men	Women
20-29	10.7	4.3	15.2	10.4	10.0	3.7	77.1	50.6
30-39	18.5	9.1	22.1	17.4	17.4	8.2	90.2	69.6
40-49	31.0	22.0	31.2	30.6	29.0	20.5	108.0	103.9
50-59	48.5	41.0	43.0	47.2	40.4	35.9	106.1	115.7
60-69	60.8	60.9	55.3	61.9	36.4	40.8	76.4	90.6
≥70	73.6	77.5	65.6	74.7	41.1	65.5	62.4	88.5
Overall	31.6	25.3	31.7	31.2	174.2	174.7	520.1	518.8

Comparison of national survey data in England, USA and Canada, on hypertension prevalence and stroke and IHD mortality, revealed that the mean SBP was the highest in England in all age groups between 20 and 79 years (30% vs. 19.5% in Canada and 29.1% in the USA) (Joffres et al., 2013).

Finally, in the UK, the clinical management of hypertension is one of the most common interventions in primary care accounting for approximately £1 billion

in drug costs alone in 2006 (NICE, 2011). Overall costs due to hypertension related disease such as stroke, coronary heart disease, vascular dementia and chronic kidney disease are reaching up to £2.1 billion per year (Public Health England (PHE), 2017). Worldwide, it is estimated that 10% of health expenditures are consumed for hypertension treatment (Joffres et al., 2013).

From the above described it is evident that hypertension still presents a major public health issue in the UK. Considering a continuous increase in the risk of CVD with an increase in BP, together with substantial costs attributed to treatment of hypertension, it can be concluded that the efforts should be directed towards primary prevention of hypertension. This can be achieved through hypertension risk factor modification which will be discussed in the following sections of the thesis.

1.1.2. Risk factors

Since the focus of this thesis will be salt-sensitive hypertension as a distinct phenotype, the risk factors will be explained in more detail in sections that follow and only a short overview will be presented in this section.

Demographic, dietary and lifestyle risk factors

Hypertension is a complex trait associated with a number of non-modifiable and modifiable risk factors. Among the non-modifiable risk factors, age, family history and race are the most researched to date. The evidence suggests that older populations, individuals of African descent and the population with confirmed

family history of hypertension are at higher risk of developing hypertension (Barlassina et al., 2002; Vasan et al., 2002; Hertz et al., 2005).

Considering its preventable nature, many modifiable environmental risk factors such as diet, physical inactivity, toxins or psychosocial factors, have been reported to date (Forman et al., 2009; Appel, 2017). Out of these diet plays the most important role in BP homeostasis (Appel, 2017). Both observational studies and intervention trials have shown a reduction in BP with lower body mass index (BMI) and alcohol intakes together with higher physical activity levels and intake of food rich in potassium (Appel, 2017). A meta-analysis of 25 randomised controlled trials showed that a weight loss of 5.1 kg, by means of energy restriction, increased physical activity, or both, resulted in 4.4 mmHg reduction in SBP and 3.6 mmHg reduction in DBP (Neter et al., 2003). The trials included in the meta-analysis were of eight to 260 weeks of duration, included both hypertensive and populations with normal BP of different ethnicities and both sexes, 37 to 66 years of age. The meta-analysis also included trials with participants taking antihypertensive medications. Considering the heterogeneity in the study populations, meta-analysis was also performed stratifying according to age, sex, race, hypertension status and weight loss. Analyses were repeated with a multivariate model to adjust for potential confounders (age, sex, initial BP, change in body weight and duration of intervention). Larger BP reductions were observed in populations with an average weight loss > 5 kg than in populations with less weight loss, both for SBP [-6.24 mmHg (95% CI, -8.06, -4.41) vs. -2.44 mmHg (95% CI, -4.38, -0.49)] and DBP [-4.97 mmHg (95% CI, -6.62, -3.31) vs. -1.97 mmHg (95% CI, -3.71, -0.21)]. BP reductions were also larger in populations who were taking antihypertensive medication than in untreated populations, both for SBP [-7.00 mmHg (95% CI, -

10.02, -3.98) vs. -3.77 mmHg (95% CI, -5.33, -2.22)] and DBP [-5.49 mmHg (95% CI, -8.06, -2.93) vs. -2.97 mmHg (95% CI, -4.39, -1.55)]. Limited statistical power did not allow to draw conclusions about the potential differences according to race. More recent data suggested that a modest weight reduction resulted in lower prevalence of hypertension by approximately 20% in overweight, normotensive individuals (The Look AHEAD Research Group, 2010).

Together with weight loss, increased potassium intake is also associated with lower BP. A meta-analysis of randomised controlled trials reported that potassium supplementation (a mean increase of 51 mmol/24h) was associated with a mean change in SBP of -2.42 mmHg (95% CI, -3.75, -1.08) and a mean change of -1.57 mmHg (95% CI, -2.65, -0.50) in DBP when exploring the overall results of 27 trials. There was no difference in BP response to potassium intervention according to age, sex, hypertension status, initial body weight, 24-hour sodium and potassium excretion as well as according to an increase in 24-hour potassium excretion. Considering that the 80% of the trials did not report on BMI, the effect of BMI could not be controlled for which may have affected the results (Geleijnse et al., 2003).

Contrary to the effects of weight loss and increased potassium intake, there is a detrimental dose-response effect of alcohol consumption on BP. A meta-analysis of 15 randomised trials showed that a median reduction in alcohol consumption of 76% lowered SBP by 3.3 mmHg and DBP by 2 mmHg in both hypertensive and normotensive individuals. There was no heterogeneity among studies ($p > 0.50$ for SBP and $p > 0.25$ for DBP). Furthermore, pooled estimates of the alcohol reduction effect among subgroups of trials, defined according to participant and study design

characteristics such as sample size, study design, duration and type of intervention, BP measurement, hypertension status and the use of antihypertensive medication, did not differ (e.g. duration of the intervention $p = 0.81$ for SBP and $p = 0.85$ for DBP; hypertension status $p = 0.54$ for SBP and $p = 0.35$ for DBP). Nevertheless the trials consisted of predominantly male population and heavy drinkers (≥ 3 drinks/day) which limits generalisation of these results in female populations and in persons with low to moderate alcohol consumption (Xin et al., 2001). A more recent meta-analysis of 36 trials reported that reduction in SBP (mean difference -5.50 mmHg, 95% CI, $-6.70, -4.30$) and DBP (-3.97 mmHg, 95% CI, $-4.70, -3.25$) was strongest in participants who drank six or more drinks per day if they reduced their intake by about 50%. No effect was observed in people who drank two drinks or fewer [SBP reduction: -0.18 mmHg (95% CI, $-1.02, 0.66$); DBP: 0.61 mmHg (95% CI, $-0.04, 1.26$)]. Similar to what was reported by Xin et al. (2001), the data on women was scarce with only three trials including female participants (Roerecke et al., 2017).

The evidence presented in this section indicates that weight loss, increased intake of potassium and decreased alcohol intake may be suggested as approaches in prevention and treatment of hypertension. Nevertheless, further research is needed to explore the potential beneficial effects of alcohol reduction in women. Moreover, other dietary risk factors, such as increased salt intake, should be taken into consideration. For this purpose, a better understanding of the methods to measure salt intake is required.

1.2. Methods of measuring salt intake

Methods of measuring dietary intake of salt can be divided into: 1) dietary methods such as food recalls, food diaries or food frequency questionnaires (FFQs) and 2) urinary biomarker methods. These include 24-hour urine sodium excretion (i.e. total daily sodium intake) and casual sample sodium excretion which can then be converted to predicted 24-hour urinary sodium excretion. The latter, that is the 24-hour urinary sodium excretion, is considered as the “gold-standard” and the method of choice by the World Health Organisation (WHO), the Pan-American Health Organisation and the United States Centres for Disease Control and Prevention (Conkle and van der Haar, 2016). It is used to measure population salt intakes and changes over time in national dietary surveys such as the UK National Health and Nutrition Survey (NDNS) (Department of Health, 2016).

Such measurement, if accurate, reflects intake reliably as approximately 90% of ingested sodium is excreted in the urine over the same period. Variable losses also occur through sweat and faeces, and have been estimated to be around 10% under normal conditions (McLean, 2014). Moreover, excreted urine captures both discretionary and non-discretionary salt intake and does not rely on the food composition databases that may not reflect the changes or differences in the salt content of processed food by a manufacturer, a brand or salt added in different recipes (Conkle and van der Haar, 2016). Despite it being considered the “gold standard” method, sodium intake at an individual level varies highly on a daily bases depending on the food consumed. Variance in sodium excretion within individuals can be as much as three times larger than variance between individuals (Dyer et al., 1994). When the amount of sodium intake is decreased or increased and then held

constant, it takes about three days for the amount of sodium excreted in urine to equal intake (Cogswell et al., 2015). This suggests that only one collection may not be appropriate to assess sodium intake at an individual level and that multiple collections should be performed for accurate measure of person's sodium intake. If the research aim is not to assess individual sodium intake, but a mean population intake, single collection may be sufficient if sample size is large to account for between individual variability in sodium intake. Elliott and Brown (2007) suggest that as long as 24-h urine is collected across seasons and on different days of the week to balance variability in day-to-day sodium intake, mean group sodium intake is unlikely to be biased.

Furthermore, this method may introduce bias due to the high participant burden of collection, underestimation due to missed or lost urine, and under- or over-collection due to incorrect timing. It is also laborious for researchers and different methods of assessing the completeness of collections must be employed (McLean, 2014). The most common of which are urinary creatinine excretion and para-amino benzoic acid (PABA) recovery. The creatinine excretion relies on the notion that creatinine excretion is fairly constant in an individual and depends on age, body weight and sex. When observed creatinine excretion is less than expected based on a person's age, body size, or sometimes sex, the urine is judged to be potentially incomplete. However, creatinine excretion may vary in relation to muscle mass and dietary intake, especially meat consumption, and may therefore not be an accurate indicator of the completeness of collection (Conkle and van der Haar, 2016). Nevertheless, it is still widely employed in research (John et al., 2016). Another method widely used is PABA recovery. PABA is completely absorbed and 93% is excreted in urine within five hours of administration. Participants are required to

consume three 80 mg doses, one with each meal. Urinary PABA is then measured and a 24-h urine collection with 85% –110% of PABA recovered is considered complete. When compared to PABA recovery, John et al. (2016) suggest the use of a creatinine index < 0.7 based on sex and body weight as the most sensitive method for ruling out incomplete 24-hour urine specimens post collection.

Instead of collecting 24-hour urine sample, a causal urine sample can be collected to measure sodium excretion and intake. It is suggested that studies collecting causal urine sample usually have higher response rates than studies employing 24-hour urine collections (Cogswell et al., 2015). Despite this advantage, causal urine sample sodium excretion needs to be converted into 24-hour excretion by using different predictive equations (Kawasaki et al., 1993; Tanaka et al., 2002). When comparing sodium concentrations from a single causal sample to 24-hour urine collections, eight studies included in a systematic review by Ji et al. (2012) reported that the correlation coefficients between the two methods ranged between 0.17 and 0.73, with the mean coefficient of 0.44. These results suggest that causal urine sodium excretion may not predict 24-hour excretion accurately and may consequently not be accurate in determining dietary sodium intake. Moreover, sodium excretion and concentration vary across the day, which further limits the accuracy of measuring sodium intake if a collection period of less than 24 hours is used (Stow and Gumz, 2011). Finally, urinary biomarker methods to measure sodium intake do not provide information on different dietary sources of sodium necessary to inform public health interventions as opposed to dietary methods (Mclean, 2014).

Among dietary methods, FFQs are used to measure sodium intake, most

commonly in epidemiological studies. For example, a meta-analysis by Strazzullo et al. (2009), examining the association between sodium intake and CVD, reports that four out of 13 studies included in the review used FFQs to assess sodium intake. FFQs are useful as they assess intake over a longer period and potentially overcome problems associated with the high day-to-day variability of intake (McLean, 2014). Moreover, they are usually used to rank individuals according to their intake rather than measuring absolute intake. Nevertheless, poor agreement has been reported between the sodium intake obtained with FFQ and 24-hour urine excretion measurement (Day et al., 2001; Freedman et al., 2015). Day et al. (2001) report on the correlation coefficient of 0.13 between the two methods. Similarly, Freedman et al. (2015) report that sodium intake assessed by FFQ was on average 30% less than that measured in 24-hour urine collections. One of the reasons for poor agreement may be the fact that often it is not possible to quantify discretionary salt intake when using a FFQ, which indeed may result in underestimation of the actual intake. In countries where non-discretionary salt intake accounts for the majority of the total intake, such as the UK, this may be to a lesser extent when compared to countries where discretionary salt use accounts for 70-80% of the total intake such as China (Anderson et al., 2010). Furthermore, salt intake is highly correlated with energy intake and is therefore, not surprising that FFQs tend to underestimate the intake of this nutrient. To overcome this limitation, it is suggested that sodium intake is energy adjusted. This may account for the effect of potential confounders associated with energy intake such as BMI, sex and physical activity levels (McLean, 2014). Indeed, Freedman et al. (2015) report attenuation factors for sodium intake obtained with FFQ compared to urinary biomarker of 0.10. For sodium density (mg sodium/1000 kcal) attenuation factors were markedly higher than those for absolute sodium, with

an average of around 0.35, suggesting that energy adjusted sodium intake may capture the actual intake more accurately.

A more accurate method to measure salt intake appears to be 24-hour dietary recall. Freedman et al. (2015) report that sodium intake tends to be underestimated by 5-10% with a 24-hour recall as compared to 30% with a FFQ. Moreover, multiple 24-h dietary recalls are the preferred method for population level or group intake estimates (Rhodes et al., 2013). With this notion, Rhodes et al. (2013) aimed to compare the estimated sodium intake from foods from self-reported dietary recalls with 24-h urinary excretion in the United States Department of Agriculture (USDA) Automated Multiple-Pass Method (AMPM) Validation Study. The two 24-h dietary recalls were conducted by trained interviewers using USDA's 5-step AMPM and sodium intakes obtained with this dietary method were compared to two 24-hour urinary sodium excretions. Both the recalls and urine collections were performed on the same day. The results showed that the mean sodium, calculated as the ratio of reported dietary sodium intake from 24-h recall to 24-h urinary sodium, was 0.93 for men and 0.90 for women, suggesting the USDA AMPM is a valid method for assessing dietary sodium intake (Rhodes et al., 2013; McLean, 2014). This method has also been developed into an online system, Automated Self-Administered 24-h Recall, which has been validated in a feeding study with 81 adults (Kirkpatrick et al., 2014). Despite its advantages, the accuracy of both 24-hour recalls and FFQs depends on the quality of food composition databases. The majority of the sodium in the UK diet comes from packaged and processed foods. The amounts of sodium in these foods may be highly variable which creates difficulties in compiling accurate food composition databases (Freedman et al., 2015). Additionally, the amount of sodium added during cooking at homes, restaurants or catering outlets is highly

variable which may result in incorrect estimations of true sodium intake (Conkle and van der Haar, 2016).

In summary, there are evident advantages and limitations of each of the methods used to measure sodium intake. While dietary methods tend to underestimate true intakes, which may be attenuated by using energy adjusted sodium intake, urinary biomarker methods may present high participant burden and do not provide information on dietary sources of sodium. Nevertheless, 24-hour urine sodium excretion is considered as the method of choice and, if used to estimate individual sodium intake, multiple collections are recommended depending on feasibility. On the other hand, multiple 24-hour dietary recalls may be appropriate for group estimates, depending on the quality of food composition database to calculate sodium intake. If only a dietary method is used in research, ideally it should be validated against a 24-hour urine biomarker method in population of interest.

1.3. Salt intake as the key hypertension risk factor

Despite the evidence linking hypertension with described dietary and lifestyle risk factors, high salt (i.e. sodium chloride; the terms salt and sodium will be used interchangeably where 1 g of salt corresponds to 400 mg or 17.1 mmol sodium) intake stands out as the most prominent environmental hypertension risk factor.

One of the largest studies to date, exploring the association between salt intake and BP is Intersalt. This standardised, cross-sectional, epidemiologic study was conducted in 10079 men and women from 32 countries (52 research centres) around the world, aged between 20 and 59 years. Within centre analysis revealed

significant positive associations between SBP and sodium intake (pooled SBP/sodium coefficient of 0.0354 mmHg/mmol sodium ($p < 0.0001$)), measured by 24-hour urine sodium excretion, after adjustment for BMI, alcohol consumption, and potassium excretion. In across centre analysis, a significant, positive and independent linear correlation was reported between 24-hour urinary sodium excretion and SBP (SBP/sodium coefficient = 0.0030, $p < 0.001$). Moreover, the increase in BP was higher with increasing age (linear regression coefficients ranging from 0.0014 to 0.0021 mmHg/year/mmol sodium ($p < 0.01$ and $p < 0.001$, respectively) (The Intersalt Cooperative Research Group, 1988). Despite the reported findings, one of the limitations of the study was the measurement of sodium intake which included only one 24-hour urine sample for sodium excretion. Collecting only one sample may have resulted in regression dilution bias due to day to day variance in sodium excretion within individuals which may be as much as three times larger than variance between individuals (Dyer et al., 1994). Nevertheless, since Intersalt, there have been a number of studies showing similar results. The largest of the dose-response trials was a Dietary Approaches to Stop Hypertension (DASH) - Sodium trial, exploring the effects of DASH diet, together with reduced sodium intake, on BP. This randomised crossover trial investigated the BP modifying effects of three different salt intakes (4, 6 and 8 g/day) in two different diets, typical American and DASH and comprised of 412 adult participants. The typical American or Western diet was rich in red meat, dairy products, processed and artificially sweetened foods, and salt, with minimal intake of fruits, vegetables, and whole grains. Conversely, the DASH diet was rich in in fruits, vegetables, low fat dairy and it included mostly whole grains, lean meats, fish and poultry, nuts and beans. The duration of each of the dietary sodium interventions was 30 days and salt

intake was assessed by the means of urinary sodium excretion. The results showed a significant decrease in SBP with reducing dietary salt intake on both typical Western and DASH diet. These results suggest that, irrespective of other dietary factors, a reduction in salt intake results in a decrease in BP. However, the low-salt DASH diet produced greater reductions in SBP (further 2.2 mmHg) than the low-salt Western diet supporting the notion that DASH diet may be recommended as a measure to reduce BP, alongside salt reduction (Sacks et al., 2001). Despite this being the largest randomised controlled trial to date, the duration of interventions was only 30 days and the effects of prolonged adherence to low-salt diet on BP response should be explored. In addition, one 24-hour urine sample at the end of each intervention periods may not be adequate to control for dietary salt intake.

The BP lowering effects of reduced salt intake were confirmed by systematic reviews and meta-analyses of randomised trials conducted by He and MacGregor (2004) and He et al. (2013). The most recent one reviewed the effects of a modest, long-term (four weeks to three years duration) reduction in salt intake in 20 trials conducted in hypertensive and 11 trials conducted in normotensive population. The median reduction in urinary sodium excretion in hypertensive populations was -78 mmol (4.6 g/day of salt), ranging from -53 to -117 mmol (3.1 to 6.9 g/day of salt) with the pooled estimates of changes in BP being -5.06 mmHg (95% CI, -5.81, -4.31) for systolic and -2.70 mmHg (95% CI, -3.16, -2.24) for diastolic. In populations with normal BP the median net change in 24-h urinary sodium was -74 mmol (4.4 g/day of salt), ranging from -40 to -118 mmol (2.4 to 6.9 g/day of salt) with the corresponding pooled estimates of changes in BP of -2.03 mmHg (95% CI, -2.56, -1.50) for systolic and -0.99 mmHg (95% CI, -1.40, -0.57) for diastolic. Despite the observed overall reduction in BP due to reduction in salt intake, the

median duration of the interventions was only four weeks and the effects of longer term sodium reduction should be explored. The authors reported high heterogeneity across studies which may be due to differences in the age of participants, different ethnic groups included, BP levels and the duration of salt reduction intervention. Further sensitivity analysis was not performed due to low number of trials included and availability of data which suggests these results should be interpreted with caution and replicated with studies of a more homogenous design. Nevertheless, the authors imply that current recommendations to reduce salt intake from 9-12 to 5-6 g/day will have a major effect on BP, but a further reduction to 3 g/day will have a greater effect and should become the long term target for population salt intake (He et al., 2013).

Contrary to these results, O'Donnell et al. (2014) reported that, when compared to sodium excretion of 4 to 5.99 g/day (10 to 15 g salt/day), estimated sodium excretion of 7 g/day or more (17.5 g salt/day) was associated with an increased risks of a major cardiovascular event (OR, 1.16, 95% CI, 1.01, 1.34), death from cardiovascular causes (OR, 1.54, 95% CI, 1.21, 1.95), and stroke resulting in death or hospitalization (OR, 1.29, 95% CI, 1.02, 1.63). Similar was reported when comparing sodium excretion of 4 to 5.99 g/day to an estimated excretion of less than 3 g/day (7.5 g salt) suggesting that lower intakes of salt may also be detrimental to health. However, hypertension at baseline modified the association between estimated sodium excretion and cardiovascular events suggesting it may not be clear if hypertension preceded low sodium intake or vice versa (i.e. reverse causality). In addition, sodium intake was estimated by collecting one spot urine sample which may not accurately estimate true intake and thus the conclusions should be interpreted with caution. Similar to above-described, Graudal et al. (2017), in their

recent systematic review of randomised control trials, with meta-analysis, reported a small reduction in BP (1%) in a normotensive population on a low-sodium diet (approximately 3.8 g salt/day) compared to the high-sodium diet (approximately 12 g salt/day) whereas higher reduction in BP (3.5%) was reported in populations with hypertension. The authors also reported on slightly increased renin, aldosterone, cholesterol, triglyceride, adrenalin and noradrenalin levels as a consequence of a low-sodium diet. Graudal et al. (2017) concluded that there was no evidence for favouring sodium reduction below 100 mmol/day or 5.8 g salt/day in general population. However, this review included data of 185 interventions studies with high heterogeneity in study duration ranging from four to 1100 days and sodium intake ranging from 66 mmol/day (3.8 g salt) to 201 mmol/day (12 g salt). In addition, the aim of certain studies included in the review was the assessment of sodium loading or sodium depletion effects on BP and consequently hormone and lipid levels. This may have resulted in a short-term increase in hormone levels, such as renin and aldosterone, on a low- salt diet but does not reflect the longer term effects of sodium reduction. Moreover, the heterogeneity across studies exploring hormone and lipid levels expressed by I^2 was high (e.g. I^2 for aldosterone = 98%) suggesting these variables should be explored further in studies with a more uniform design.

Considering the evidence available and presented in this section there appears to be a positive association between salt intake and high BP with some conflicting recommendations for maximal daily intake in general population. One of the most prominent limitations across studies is the actual measurement of dietary salt intake which may have contributed to discrepancies in results. Moreover, potential effect of genetic variability among study participants was not considered which may have also

contributed to discrepancies in conclusions and recommendations and will be explored in this thesis.

1.3.1. Health effects of high salt intake beyond BP

Regarding the health implications of high salt intake, it expands beyond BP. In a study conducted on a sample of 458 children (age, ≥ 4 years) and 785 adults who participated in the NDNS and had valid weight and height measurement as well as complete 24-hour urine collection, 24-hour urinary sodium excretion was higher in overweight and obese individuals. Interestingly, a 1 g/day increase in salt intake was associated with an increase in the risk of obesity by 28% (OR, 1.28; 95% CI, 1.12, 1.45; $p = 0.0002$) in children and 26% (OR, 1.26; 95% CI, 1.16, 1.37; $p < 0.0001$) in adults, after adjusting for age, sex, ethnic group, household income, physical activity, energy intake, and diet misreporting, and in adults with additional adjustment for education, smoking, and alcohol consumption. Higher salt intake was also significantly related to higher body fat mass in both children ($p = 0.001$) and adults ($p = 0.001$) after adjusting for age, sex, ethnic group, and energy intake. The authors suggest how salt intake may be a risk factor for obesity independent of energy intake (Ma et al., 2015).

With respect to vascular health, research in animals suggests how high salt intake in pregnancy may cause adverse foetal programming. The offsprings of Sprague-Dawley rats fed a high-salt diet (8% Na) had significantly higher wall thickness of central (aorta, carotid), muscular (mesenteric) and intrapulmonary arteries compared to the offsprings of mothers fed a low-salt diet (0.15% Na),

irrespective of the post-weaning diet and BP. These results correlated with elevated levels of marinobufagenin and asymmetric dimethyl arginine (ADMA), promoting vasoconstriction, as well as increased oxidative stress (Piecha et al., 2012). Similar was reported in humans, where seven days of a high-salt diet (300-350 mmol/day) resulted in decreased endothelium dependent dilation, independently of changes in BP (DuPont et al., 2013).

In addition to these adverse health effects, high salt intake has been associated with an increased risk of gastric cancer. A systematic review and a dose-response meta-analysis of prospective cohort studies showed that excess dietary salt intake may increase risk for gastric cancer significantly (RR: 1.11, 95% CI, 1.05, 1.16). Moreover, dose-response analysis revealed that the risk of gastric cancer increased by 12% per 5 g/day increment of dietary salt intake (RR: 1.12, 95% CI, 1.02, 1.23). The results were similar across different populations studied. The authors suggest how salt intake may not be carcinogenic *per se*, however excess salt is present in brine, cured or pickled vegetables, processed meat, salt fish and may act as an irritant to the gastric mucosa, causing atrophic gastritis, increased DNA synthesis, and cell proliferation. In addition, high salt intake may potentiate *H. pylori*-associated carcinogenesis by inducing proliferation, colonisation, and glandular atrophy (Fang et al., 2015).

The above-described suggests that the health effects of increased salt intake expand beyond its effects on BP, further supporting the importance of better understanding this dietary behaviour which will be one of the aims of this thesis.

1.3.2. Salt intake in the UK

Dietary intake of salt can be divided into discretionary (salt added to food during cooking) and non-discretionary (salt added during the processing and manufacturing of foods). It is estimated that the majority of dietary salt comes from manufactured foods (~ 85%) and a smaller proportion (~ 15%) is added during cooking (Scientific Advisory Committee on Nutrition (SACN), 2003). The top 10 contributors of salt intake in the UK are bread, bacon and ham, pasta, rice, pizza and other cereals, vegetables (not raw) and vegetable dishes, chicken and turkey dishes, savoury sauces, pickles, gravies and condiments, cheese, sausages, beef and veal dishes, biscuits, buns, cakes, fruit pies and pastries (Department of Health, 2016). SACN recommends that daily intake for adults should not exceed 6 g of salt or 2400 mg sodium (SACN, 2003). The reference nutrient intake (RNI) suggested by SACN is 4 g of salt or 1600 mg sodium/day. The data from the NDNS show that the mean salt intake in the UK is, as estimated by 24-hour urinary excretion, 8.0 g/day for adults aged 19 to 64 years. This is 33% greater than the SACN recommendation. Men have a mean daily intake of 9.1 g/day and women a mean daily intake of 6.8 g/day. The distribution of sodium excretion/estimated salt intake varies widely, ranging from 0.8 g/day to 24.2 g salt/day (Department of Health, 2016).

Regarding the efforts to reduce salt intake, the UK salt reduction programme was launched in 2003. The programme encouraged reformulation of foods and was supported by a campaign to raise awareness on the health effects of high salt intake and offer guidance to the public (Tedstone, 2016). Despite its initial success and a slow but steady decrease in salt consumption until 2011, an increase in the mean salt intakes in the UK can be observed since 2012 (Table 1.2), suggesting that further

efforts, and a potential change in approach, are needed to reduce salt intake in the UK population (Department of Health, 2016)

Table 1.2 Number of participants (N), arithmetic means and standard errors (SE) of estimated salt intake (g/day) between 2005-06 and 2014. Data from Sodium survey for England (Department of Health, 2016).

Survey year	Combined			N	Men		N	Women	
	N	Mean (g/day)	SE		Mean (g/day)	SE		Mean (g/day)	SE
2005-06	445	8.8	0.18	187	10.0	0.29	258	7.6	0.17
2008-09	688	8.4	0.16	301	9.3	0.23	387	7.5	0.24
2009-10	109	8.1	0.38	50	9.5	0.60	59	6.8	0.49
2010-11	109	7.6	0.31	56	9.1	0.43	53	6.1	0.40
2011-12	725	8.4	0.21	325	9.7	0.32	400	7.2	0.18
2012-13	155	7.8	0.41	60	8.6	0.73	95	7.0	0.35
2014	689	8.0	0.25	298	9.1	0.33	391	6.8	0.24

1.4. Salt sensitivity

The link between dietary salt intake and hypertension is well established and it has been shown that a reduction in salt intake lowers BP (Poulter et al., 1990; He et al., 2013). However, it has also been noted that BP of individuals responds differently to dietary salt intake, with some exhibiting an increase in BP with increasing dietary salt, while in others no significant difference in BP is observed (Weinberger et al., 1986). This phenomenon is called salt sensitivity which can therefore be defined as an increase in BP in response to high dietary salt intake. There are a number of definitions and methods for determining salt sensitivity with one of the major concerns being the reproducibility of the method used (Felder et al., 2013). These will be explained in the later sections. Considering the discrepancy in the methods used by different researchers, determining the prevalence of this phenomenon is problematic. Nevertheless, Weinberger et al. (1986) have estimated

salt sensitivity to be present in 51% of hypertensive and 26% of normotensive population with the incidence of hypertension being higher in salt-sensitive individuals than in salt-resistant individuals (Barba et al., 2007). Moreover, salt sensitivity is considered to be an independent cardiovascular and mortality risk factor. Weinberger et al. (2001) have followed the cohort initially diagnosed for salt sensitivity for 25 years and have shown that normotensive salt-sensitive individuals have a cumulative mortality rate similar to that of hypertensive ones, whereas salt-resistant normotensive individuals have an increased survival rate ($p < 0.0001$) (Figure 1.1).

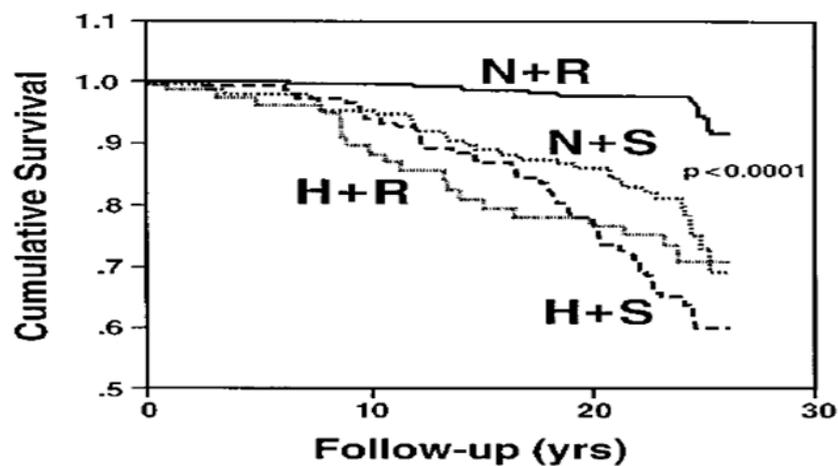


Figure 1.1 Kaplan-Meier survival curves for normotensive salt-resistant participants (N+R), normotensive salt-sensitive participants (N+S), hypertensive salt-resistant participants (H+R), and hypertensive salt-sensitive participants (N+S) over the follow-up period. As noted, the N+R group had an increased survival (Weinberger et al., 2001).

A high prevalence of hypertension in the UK and worldwide, with the intake of salt still exceeding the recommendations, suggests there is a need for a more

effective and appropriate approach in prevention of hypertension and CVD. In this context, if salt sensitivity is detected and better understood in young and healthy individuals it may result in more successful prevention of hypertension and CVD (Iatrino et al., 2016).

1.4.1. Salt sensitivity diagnosis

As stated previously, there are number of definitions and methods of diagnosing salt sensitivity, putting the reproducibility of the research on this phenomenon into question. The main protocols used to diagnose salt sensitivity to date are: 1) the outpatient protocols relying on the dietary sodium manipulation and 2) the inpatient “quick” protocols entailing oral and intravenous administration of salt and diuretics. In more detail, the latter method has first been introduced by Weinberger and his group (Weinberger et al., 1986) where patients were administered a saline infusion on one day and restricted sodium intake and oral diuretic the second day. Patients exhibiting a fall in mean arterial pressure (MAP) of more than 10 mmHg were considered to be salt-sensitive and ones with a fall of less than 5 mmHg as salt-resistant. In the recent appraisal of the methods to test for salt sensitivity, Kurtz et al. (2017) note how this method does not measure BP response to changes in salt intake, but the BP-lowering effects of a diuretic in patients that have previously received salt-loading. In addition, this method does not provide good reproducibility. Studies testing its reproducibility have reported that only half of the population classified as salt-sensitive in the first stage of the study has been classified as salt-sensitive in the repeated test (Weinberger and Fineberg, 1991; Strazzullo et al., 2001). Moreover, this protocol was reported to have an accuracy of

only 65% to 75%, thus a different method is suggested for salt sensitivity diagnosis (Sharma et al., 1994; Galletti et al., 1997; Kurtz et al., 2017).

More recent research suggests a dietary method whereby individuals are placed on a low-salt diet followed by a high-salt diet. This is an outpatient protocol and its typical duration is two weeks. BP response to a dietary salt intake is directly measured and this method is suggested to better reflect an actual change in BP due to a change in salt intake. Moreover, it is considered as a “gold standard” method (Elijovich et al., 2016). In a dietary protocol used in the GenSalt genome wide association study (GWAS), individuals received a low sodium diet (3 grams of salt or 51.3 mmol of sodium/day) for seven days. Subsequently they received a high sodium diet (18 grams of salt or 307.8 mmol of sodium/day) for an additional seven days. Patients were then classified as salt-sensitive or salt-resistant according to changes in their BP from low- to high-salt periods (The GenSalt Collaborative Research Group, 2007). This dietary method is reproducible and is considered as the most reliable to date (Gu et al., 2013; de Leeuw and Kroon, 2013). Supporting the use of a dietary method to test for salt sensitivity, the American Heart Association (AHA) suggests that the high-salt phase of the protocols should have a daily intake of sodium of at least 250 mmol for about a week, followed by an equal period of low-salt intake of ≤ 50 mmol of sodium, with salt sensitivity assessed by the change in BP from the end of the high-salt period to the end of the low-salt period (Elijovich et al., 2016). Cut-offs suggested to classify normotensive participants as salt-sensitive is a MAP change from 3 to 5 mmHg and for hypertensive participants MAP change from 8 to 10 mmHg. Order and administration of different salt diets may vary per study objective (Kurtz et al., 2017). Even though salt sensitivity is more often expressed as a categorical variable, some investigators have expressed

salt sensitivity as a continuous variable representing the change in BP (Δ BP) in response to dietary salt (Melander et al., 2000).

Despite its high reproducibility, the dietary protocol to test for salt sensitivity requires substantial time and resources and is not feasible in a clinical setting. However, the use of genetic markers still cannot be advocated due to the lack of genetic association studies of a large sample size investigating salt sensitivity as well as the lack of studies validating the newly identified genetic markers. This can, to a great extent, be attributed to the lack of a quicker and simpler method of testing for salt sensitivity.

With this notion, there have been attempts to develop a surrogate method, which would replace the cumbersome dietary protocol. Gildea et al. (2013a) have used urine-derived renal proximal tubule cells from patients already characterised for salt sensitivity with the reproducible dietary method (Carey et al., 2012) and have found a correlation between the degree of salt sensitivity and dopamine-1 receptor plasma membrane recruitment and angiotensin II-stimulated intracellular calcium concentration in response to intracellular sodium. The authors suggested this method as a surrogate for dietary method. The same group has also reported the association of 45 urinary exosomal miRNAs associated with patients' BP response to dietary sodium manipulation (Gildea et al., 2013b). In addition, two miRNAs have been reported to be differently expressed between salt-sensitive and salt-resistant hypertensive patients in China, diagnosed for salt sensitivity using an acute loading protocol (Qi et al., 2017). Even though these methods hold promise in discovery of a salt sensitivity biomarker, neither of the two described has been followed-up to date, leaving a clear space for further attempts in discovery and validation of such

biomarkers. The AHA encourages the attempts in discovery of salt sensitivity biomarkers from an easily accessible tissue such as urine (Elijovich et al., 2016).

1.4.2. Salt sensitivity risk factors

Similar to hypertension, salt sensitivity is associated with a number of demographic and environmental factors. The following sections will explore its demographic correlates together with the relationship between diet, lifestyle and salt sensitivity.

Racial disparities in salt sensitivity

Salt sensitivity is suggested as a potential reason for increased hypertension prevalence and CVD risk in black compared to white populations (Luft et al., 1991). Indeed, salt sensitivity is estimated to be present in 73% of hypertensive and 36% of normotensive black individuals compared to the reported 51% and 26% in white populations (Weinberger et al., 1986). Similarly, a more pronounced BP response to a dietary salt intake manipulation in black compared to white population was reported in a study by He et al. (1998) where 71 white and 33 black patients with hypertension followed a high sodium diet (350 mmol/day) for five days followed by five days of a low sodium diet (10 mmol/day). The mean fall in SBP in black participants after low sodium diet was 22 mmHg compared to 16.6 mmHg in white participants ($p < 0.001$). Similar was observed for DBP (black participants -10.1 mmHg vs. white participants -6.1 mmHg, $p < 0.001$). The adherence to diets was assessed with two 24-urine samples for sodium and creatinine excretion which is deemed appropriate considering the duration of diets. The effect of sodium loading

on BP was assessed with a preferred dietary method. In addition, diets were prepared by the research staff and consumed in the Metabolic Unit kitchen. Even though the number of black participants was lower compared to white, which may have affected the results, similar results were reported by other researchers. Campese et al. (1991) explored BP response to dietary salt intake manipulation (nine days of 20 mmol sodium/day followed by 14 days 200 mmol sodium/day) in 17 black and nine white patients with essential hypertension. Participants provided a 24-urine sample on each intervention day, for measurement of creatinine, sodium, and potassium excretion. The aim of this procedure was to account for within individual variability in sodium excretion and capture sodium intakes when sodium balance is achieved. However, it is not clear how the completeness of collections was determined. Out of 26 participants, 11 were considered salt-sensitive according to a MAP increase of at least 10 mmHg on a high-salt diet compared to the MAP on a low-salt diet. All salt-sensitive participants were black which is in line with, already stated, higher salt sensitivity prevalence in black populations. In contrast, Wright et al. (2003) reported similar salt sensitivity prevalence in white and black normotensive and hypertensive postmenopausal women (salt-sensitive 51% vs. 53.5% and salt resistant 30% vs. 26.3%). However, in hypertensive group, increase in MAP, after seven days of 200 mmol of sodium, was 12.6 vs. 8.2 mmHg in black compared to white women ($p < 0.01$) and for SBP it was 23 mmHg vs. 14.8 mmHg ($p < 0.01$). Even though the authors did not calculate required sample size *a priori*, it was larger than in previous studies described in this section ($n = 199$) and the distribution of participants between the two race groups and hypertension status was similar. In addition, the randomised design and dietary method to diagnose salt sensitivity should be

considered as strengths of the study. The generalisability of the results, however, is limited to postmenopausal women.

Furthermore, the prevalence of hypertension appears to be lower in black populations living in their indigenous areas (e.g. age adjusted hypertension prevalence in persons aged 35-64 years living in Nigeria was 13.5%) than in those living in Western countries (age adjusted hypertension prevalence in persons aged 35-64 years living in the US was 44%) (Cooper et al., 2005). The latter are probably adhering to a typical Western diet with high dietary salt intakes, thus exhibiting high BP responses to salt.

In summary, considering that the studies reported here and available in the literature focus predominantly on exploring the mechanisms of salt sensitivity and not its prevalence, the choice of the method may not always be the most appropriate to determine the latter. In addition, the sample size may also not be adequate. Nevertheless, a greater BP response to an increase in dietary salt intake can be observed in black participants. Considering the potential effects of race it should be noted that genetics may play an important role in development of salt sensitivity in both black and white populations, however it may have a more pronounced role in the former group. Svetkey et al. (1996) concluded that 26% to 84% of the variability in MAP and 26% to 74% of the variability in SBP response to sodium loading in black populations can be explained by genetic factors. The role of genetics in development of salt sensitivity will be discussed in the sections that follow.

Demographic correlates of salt sensitivity other than race

Similar to race, sex differences are reported in risk of hypertension and CVD mortality. Men are usually considered to be at greater risk of CVD considering their higher BP compared to women (August and Oparil, 1999; Reckelhoff, 2001). In contrast, female sex is found to be an important predictor of salt sensitivity (He et al., 2009). Bursztyrn and Ben-Dov, (2013) estimated salt sensitivity from ambulatory BP monitoring indices for 2064 patients and found increased mortality risk in men (RR 1.96, 95% CI, 1.07, 3.62) but not in women (RR 1.02, 95% CI, 0.51, 2.07). However, salt sensitivity was more common in women (16%) than in men (10%) ($p = 0.0001$). Oestrogen in women appears to act protectively before menopause and may be an explanatory factor for increased salt sensitivity but lower overall mortality in women. It is especially after menopause that changes in renal sodium handling, oxidative stress, and hypertension occur, most likely due to an imbalance between nitric oxide (NO) and angiotensin II. A study in normotensive women in whom menopause was induced surgically confirmed the protective effect of female sex hormones on salt sensitivity, independent of aging. Results of the study showed that the prevalence of salt sensitivity doubled four months after the surgery, even though hypertension did not develop. The authors pointed to the decrease of oestrogen and the resultant lack of modulation of the renin angiotensin aldosterone system (RAAS) and NO system as the potential mechanisms involved in postmenopausal salt-sensitive hypertension (Scuteri et al., 2003; Hernandez Schulman and Raij, 2006). Nevertheless, age is also a strong predictor of salt sensitivity (Luft et al., 1991; Weinberger and Fineberg, 1991; Overlack et al., 1993; Chen, 2010). In normotensive population younger than 30 years, 65% of the population was considered salt-resistant after following a quick intravenous protocol to determine salt sensitivity

and 35% salt-sensitive or indeterminate (if a decrease in MAP after sodium depletion was between 6 and 9 mmHg participants salt sensitivity status was considered as indeterminate). In the 50 years and older normotensive population, 77% were salt-sensitive or indeterminate and 23% salt-resistant. A similar shift was seen in the hypertensive participants (Weinberger and Fineberg, 1991). However, the proportion of participants with indeterminate salt sensitivity status was not stated which may affect the interpretation of these results. Nevertheless, in a more recent study whereby salt sensitivity was determined with a preferred dietary method, there was a dose-response relationship between age and SBP responses to low-salt and high-salt interventions. In the younger than 35 years group the SBP increase after high-salt diet was 4.31mmHg (95% CI, 3.75, 4.86), in the age group 35-44 years it was 5.73 mmHg (95% CI, 5.28, 6.19) and the age group 54 and older the increase in SBP was 7.36 mmHg (6.83, 7.89) ($p < 0.0001$), suggesting an increased BP sensitivity to salt in older populations (Chen, 2010).

To summarise, black race, older age and female sex appear to be demographic risk factors for salt sensitivity. In females, oestrogen acts protectively on BP before menopause which may be one of the explanations for higher prevalence of salt sensitivity but lower CVD risk in females. Taking into account the importance of detecting salt sensitivity in younger and healthy populations, then besides race, demographic risk factors such as age and sex, may not be relevant. Other factors, such as genetics, should be explored in context of salt sensitivity in younger populations.

Dietary and lifestyle correlates of salt sensitivity

A number of nutrients have been associated with salt sensitivity. These may either attenuate or exacerbate salt-sensitive changes in BP. Potassium is one of the nutrients suggested to act protectively on salt-sensitive rise in BP. It exerts its beneficial effect on salt-sensitive BP primarily through its natriuretic properties, action on RAAS, endothelial function and vascular structure. Some of the studies dating back to 1970s show that potassium supplementation decreases BP and plasma renin activity (PRA) and promotes natriuresis in normotensive or participants with essential hypertension (Brunner et al., 1970). With regards to endothelial function, Kanbay et al. (2013) reported a positive effect of dietary potassium on endothelial production of transforming growth factor (TGF- β) during high salt intakes. TGF- β is a growth factor produced in the endothelium and other tissues, and is considered to have a role in BP regulation. Increased production of this factor leads to vasoconstriction and an increase in BP (Zacchigna et al., 2006). NO produced by the endothelium may be a mediator in TGF- β synthesis, especially during a high salt diet, exerting its protective effects on salt-sensitive BP (Ying and Sanders, 2003). In a normotensive adult Chinese rural population, potassium supplementation (4.5 g/day, KCl) reduced BP in salt-sensitive but not salt-resistant participants (SBP in salt-sensitive on a high-salt diet 119.7 ± 1.7 mmHg vs. potassium supplementation 108.2 ± 1.6 mmHg, $p < 0.05$; SBP in salt-resistant on a high-salt diet 109.5 ± 1.7 mmHg vs. potassium supplementation 104.6 ± 1.2 mmHg, $p > 0.05$). The authors suggested increased ADMA levels and increased NO production observed in salt-sensitive but not salt-resistant participants as a mechanism for BP reduction (Fang et al., 2006). Further beneficial effects of potassium supplementation on vascular function were reported in a Chinese population. In a study population of

normotensive and mildly hypertensive salt-sensitive men and women, short term BP variation and vascular function expressed as ambulatory arterial stiffness index improved after potassium supplementation. A high-salt intervention (307.7 mmol sodium/day for seven days) significantly increased BP, ambulatory arterial stiffness index, symmetric ambulatory arterial stiffness index ($p < 0.001$) and vasoconstrictor peptide, endothelin (ET-1), levels ($p < 0.05$). These effects were more pronounced in salt-sensitive population [e.g. low salt salt-sensitive SBP (113 mmHg, 95% CI, 105, 121) vs. salt-resistant 112 mmHg, 95% CI, 103, 125); high-salt salt-sensitive SBP (133 mmHg, 95% CI, 121, 141) vs. salt-resistant (119mmHg, 95% CI, 109, 133)]. Sixty mmol of potassium chloride daily during a week on a high salt diet (18 g of salt or 307.7 mmol sodium/day) resulted in improved SBP levels in both groups: salt-sensitive (125 mmHg, 95% CI, 113, 131) vs. salt-resistant (115 mmHg, 95% CI, 105, 127) ($p < 0.05$) (Liu et al., 2013). Similar effects of potassium supplementation were observed in a study by Wang et al. (2015) who employed the same dietary protocol as Liu and colleagues, also in Chinese population. BP levels increased from the low-salt intervention to the high-salt intervention (SBP 109.1 ± 12.2 vs. 116.4 ± 17.2 mmHg, $p < 0.05$), and decreased from the high-salt intervention to the high-salt plus potassium supplementation intervention (SBP 116.4 ± 17.2 mmHg vs. 107.8 ± 13.0 mmHg, $p < 0.05$; DBP 77.3 ± 8.7 mmHg vs. 72.4 ± 9.0 mmHg, $p < 0.05$). In addition, BP levels in salt-sensitive participants after high-salt diet were significantly higher than in salt-resistant participants (SBP salt-sensitive 135.6 ± 19.4 mmHg vs. salt-resistant 111.4 ± 12.6 mmHg, $p < 0.05$; DBP salt-sensitive 83.6 ± 6.4 mmHg vs. salt-resistant 75.7 ± 8.6 mmHg, $p < 0.05$). Besides studies conducted in Chinese populations, research suggests that black populations have particularly low potassium intakes and urinary potassium excretion (Wong et al., 2003). In this

context, Morris et al. (1999) tested the hypothesis that salt sensitivity may be more frequent in black populations compared to white populations, but only when dietary potassium is deficient. The study was conducted in 38 healthy normotensive men (24 blacks, 14 whites) who ate a basal diet low in sodium (15 mmol/day) and marginally deficient in potassium (30 mmol/day) for six weeks. Throughout the last four weeks, sodium was loaded (250 mmol/day); throughout the last three, potassium was supplemented (as potassium bicarbonate) to either mid- or high-normal levels of 70 and 120 mmol/day. When dietary potassium was 30 mmol/day, salt loading induced a mean increase in BP only in black participants ($p < 0.001$), and salt sensitivity occurred in most blacks but not whites (79% vs 36%, $p < 0.02$). In the 10 black participants in whom dietary potassium was supplemented for seven days to a high-normal intake (120 mmol/day), SBP and DBP decreased (-7.7 ± 6.6 mmHg, $p < 0.001$; 5.2 ± 5.5 mmHg, $p < 0.01$). The salt-induced increase in MAP became less than that observed in blacks supplemented to 70 mmol/day ($p < 0.01$) confirming the above-stated hypothesis (Morris et al., 1999).

Even though research suggests beneficial effects of potassium on BP and the studies employ a preferred dietary method of testing for salt sensitivity, the majority is conducted in Chinese populations and therefore, further studies in other populations are required. Moreover, only Morris et al. (1999) explore the effects of different daily doses of potassium supplementation. Optimal, BP reducing, potassium intake in salt-sensitive populations should be explored in studies supplementing with a range of daily potassium doses.

Together with protective effects of potassium, beneficial effects of calcium supplementation have been suggested in salt-sensitive populations. In a double blind,

randomised, crossover study, normotensive and hypertensive black and white participants were given either calcium carbonate (1.5 g/day, corresponds to 600 mg of calcium) or a corresponding placebo for eight weeks, with two week placebo lead-in (Weinberger et al., 1993). Interestingly, when looking at the study population as a whole, no significant difference in BP was observed with calcium supplementation. However, when stratifying the participants according to their salt sensitivity status, it was the salt-sensitive group that exhibited statistically significant decrease in BP after supplementation ($p < 0.05$). Similar beneficial effects of calcium on BP were observed in a hypertensive sub-set of adult, predominantly white population. For the first study week, all participants consumed a high-salt diet (225 to 250 mmol sodium/day). This diet contained either 350 to 400 mg calcium (designated high salt/low calcium, $n = 12$) or 1000 mg calcium (designated high salt/high calcium, $n = 9$). Calcium was provided by dairy products and calcium carbonate. The second week participants consumed a low-salt (10 mmol sodium/day), low-calcium (350 to 400 mg/day) diet with unchanged potassium intake throughout the intervention. Compared to low-salt SBP, high-salt diet increased the BP but the increase was greater in participants receiving high-salt diet with low calcium compared to high-salt and high calcium ($+ 14.6 \pm 3.9/ +8.2 \pm 1.7$ mmHg vs. $+7.5 \pm 1.9/+2.5 \pm 1.4$ mmHg; SBP/DBP, both $p < 0.05$), suggesting beneficial effects of calcium supplementation on salt-sensitive increase in BP (Rich et al., 1991).

Even though these results suggest beneficial effects of calcium in relation to salt sensitivity of BP, the method of distinguishing between salt-sensitive and salt-resistant individuals in the study by Weinberger et al. (1993) (intravenous rapid protocol) may not be accurate for this specific purpose. Additionally, Rich et al.

(1991) provided their participants with dairy products as one of the sources of calcium. These foods may be rich in other nutrients, such as protein or fat, which may also affect BP. As such, the contents of the diets should have been carefully described for each study phase which was not the case in this study. It can, therefore, be concluded that further studies should be conducted to explore the suggested effects of calcium on salt-sensitive BP. Such studies should employ a dietary method of diagnosing for salt sensitivity and control for dietary intake of, not only calcium, sodium and potassium, but also other macronutrients which may affect BP.

Contrary to studies exploring the BP lowering effects of potassium and calcium, research exploring potential beneficial effects of magnesium intake is scarce. Moreover, the studies do not evaluate the effects of magnesium supplementation, but predominantly the difference in intra- and extra-cellular levels of magnesium between salt-sensitive and salt-resistant populations. In this sense, Resnick et al. (1994) conducted a randomised trial in 19, predominantly white, individuals. They followed a low-salt diet for two months (< 50 mmol sodium/day) and high-salt diet (> 200 mmol sodium/day) during a different two month period in a crossover fashion. Salt sensitivity was defined as more than 5% increase in DBP on a high-salt diet compared to low-salt diet. Magnesium levels were significantly lower ($p < 0.05$), and sodium levels significantly higher ($p < 0.05$) in salt-sensitive versus salt-resistant participants on high-salt diet. Furthermore, an inverse association was found between changes in BP on a high-salt diet and magnesium levels when looking at the study population as a whole ($r = -0.757$, $p < 0.001$) (Resnick et al., 1994). These results suggest that salt sensitivity may be mediated by magnesium depletion and further studies should explore potential effects of magnesium supplementation on salt-sensitive changes in BP. These studies should explore

different doses of magnesium supplementation to define optimal concentrations of this nutrient for reduction in BP. Despite a long duration of dietary intervention which is suggested by AHA (Elijovich et al., 2016), further studies should define salt sensitivity according to change in MAP instead of DBP, as it encompasses changes in both SBP and DBP and can therefore reflect the overall BP change more accurately.

In addition to potassium, calcium and magnesium, higher intake of protein rich foods has been suggested to act protectively on BP. In this sense, Rebholz et al. (2012) support the partial substitution of dietary carbohydrate with protein in the lowering of high BP. Arginine is suggested as the component with the most pronounced protective effects. This semi-essential amino acid found in soy, meat, fish, lentils, beans, whole grains and nuts, but also formed endogenously in kidney and liver, is a substrate for NO production (Rajapakse and Mattson, 2013). In this sense, only a few studies have been conducted with the aim of exploring the effects of arginine supplementation on salt-sensitive changes in BP in humans. Campese et al. (1997) conducted a study on 21 hypertensive (salt-sensitive and salt-resistant) and five normotensive, predominantly male African Americans. Throughout the study, the participants ingested the same basic diet containing constant amounts of protein (1.3 g/kg body weight), calories (30 kcal/kg body weight), calcium (800 mg/day), and potassium intake (80 mEq/day), while their sodium intake varied (20 mmol/day for six days followed by 250 mmol/day for additional six days). On the last day of the high-salt diet, participants received 500 mg/kg of body weight arginine intravenously for 30 minutes. Under the condition of high sodium intake, SBP, DBP, and MAP were significantly greater ($p < 0.01$) in salt-sensitive than in salt-resistant patients (155 ± 7.4 mmHg, 92.6 ± 2.7 mmHg, 113.8 ± 3.8 mmHg vs. 138 ± 3.7 , 85.7

± 2.1 and 103.3 ± 2.4 mmHg, respectively). After 30 min of L-arginine, the fall in MAP was significantly greater ($p = 0.02$) in salt-sensitive (-11.5 ± 2.2 mmHg) than in salt-resistant patients (-3.7 ± 1.5 mmHg) suggesting beneficial effects of arginine especially in salt-sensitive individuals. Similar was observed in 23 Japanese salt-sensitive and salt-resistant mildly hypertensive patients who were following a low-salt (50 mmol sodium/day) for a week followed by a high-salt diet (340 mmol sodium/day) for subsequent week (Higashi et al., 1996).

Considering a very limited number of studies, more research is needed to confirm protective effects of arginine or protein intake on salt-sensitive BP. The results of the presented studies should be replicates in larger cohorts and further studies are needed to explore the effects of arginine rich foods, and not intravenous supplementation, on BP. Finally, such studies should control for intake of food rich in nitrates (as NO precursors) as this may affect BP response to dietary interventions and was not conducted in described studies.

As opposed to the protective effects of described nutrients, alcohol may exert harmful effects on salt-sensitive BP. Di Gennaro et al. (2000) examined the link between chronic alcohol consumption and the association of salt sensitivity with arterial hypertension. The study participants were 30 detoxified alcoholics (being detoxified for six to 12 months) and 30 teetotaler controls. Participants underwent a dietary intervention study receiving a 55 mmol sodium/day diet for seven days, followed by a 260 mmol sodium diet for an additional seven days and were classified as salt-sensitive or salt-resistant. Salt sensitivity was determined based on six different criteria using different cut-off points for the changes in MAP. In alcoholics, alteration in sodium intake was followed by greater changes in both SBP and MAP

(measured with ambulatory blood pressure monitor), which rose by 10.6 ± 2.2 mmHg and 7.3 ± 1.5 in alcoholics versus 4.7 ± 1.4 and 3.9 ± 1.0 mmHg controls, respectively ($p < 0.03$ for SBP and $p < 0.05$ for MAP). Moreover, salt sensitivity was more prevalent in alcoholics than in controls (70 vs. 37%), regardless of BP levels. The RAAS response to different sodium intake was blunted in the detoxified alcoholics group. The BP and PRA response to different salt intakes observed in the study population were similar to responses normally observed in salt-sensitive hypertensives. Two years later, the same research group reported that in early withdrawing alcoholics exposed to a normal sodium intake (150 mmol), there was a positive sodium balance ($+288.6 \pm 45.6$ mmol; $p < 0.0001$) and rise in MAP ($+11.8 \pm 2.9$ mm Hg; $p = 0.001$), similar to the responses of study population of long-term detoxified alcoholics (Di Gennaro et al., 2002). These studies suggest an association of salt sensitivity with alcohol intake, however limited number of participants suggests further research is needed on the topic together with research exploring the association between alcohol intake and salt sensitivity in general population. Similar study design should, however be employed, considering that DiGennaro and colleagues chose a preferred dietary method to diagnose salt sensitivity and employed a 24-hour BP measurement which is suggested by the AHA (Elijovich et al., 2016).

Furthermore, research suggests an association between caffeine intake and BP. Caffeine is most commonly consumed in coffee, tea or soft drinks and it was suggested that it elevates BP acutely after ingestion without long-term effects (Hartley et al., 2000). Rakic et al. (1999), for example, reported that caffeine did not increase 24-hour BP, measured with an automated BP monitor in 22 normotensive participants, whereas the opposite effect was observed in 26 participants with

hypertension (54 to 89 years old). More specifically, in the hypertensive group, rise in mean 24-hour SBP was greater by 4.8 ± 1.3 mmHg ($p = 0.031$) and increase in mean 24-hour DBP was higher by 3.0 ± 1.0 mmHg ($p = 0.010$) in coffee drinkers (five cups of instant coffee for two weeks + caffeine free diet) than in abstainers (no coffee consumption in addition to caffeine free diet for two weeks). There were no significant differences between abstainers and coffee drinkers in the normotensive group for 24-hour, daytime, or night time SBP or DBP, suggesting it is only in individuals with hypertension that caffeine exerts its detrimental effects on BP. However, the authors point out that coffee has a number of substances and it cannot be excluded that substances other than caffeine produced this effect. Nevertheless, it was reported recently how chronic caffeine intake prevented the development of salt-sensitive hypertension in Dahl salt-sensitive (Dahl S) rats. It was through promoting urinary sodium excretion, which was associated with inhibition of renal tubular epithelial sodium channel (ENaC) (Yu et al., 2016). Considering that only one study focused on the effects of caffeine on salt-sensitive BP and the lack of research in humans, the effects of caffeine on salt sensitivity should be explored further.

Finally, another potential lifestyle correlate of salt sensitivity is participation in physical activity. A large dietary feeding study was conducted among 1906 individuals who were 16 years of age or older and living in rural northern China. Participants underwent a seven day low sodium intervention (51.3 mmol sodium/day) followed by a seven day high sodium intervention (307.8 mmol sodium/day) to determine salt sensitivity. Usual physical activity during the past 12 months was assessed at baseline using a standard questionnaire. The multivariable-adjusted (age, sex, BMI, educational level, cigarette smoking, alcohol consumption, and baseline urinary excretion of sodium and potassium) OR for high salt sensitivity

of SBP was 0.66 (95% CI, 0.49, 0.88) for persons in the highest quartile of physical activity compared with those in the lowest quartile (Rebholz et al., 2012b). The study participants were, however, from rural China where physical activity levels are higher than in Western countries which limits the generalisability of these findings. These results should therefore, be replicated in other populations to further explore this association.

In summary, potassium appears to be the most strongly associated with salt sensitivity of BP. The fact that the majority of studies were conducted in Asian populations suggests that further research is needed in other populations. As for other nutrients and lifestyle factors presented in this section, further randomised controlled trials are needed. Such trials should employ a dietary method of diagnosing for salt sensitivity, controlling for diet adherence with multiple 24-hour urinary sodium excretion measurements and 24-hour BP monitoring. Besides sodium and potassium, studies should control for other dietary factors that may be associated with salt sensitivity and affect BP, something that is not yet a common practice. Finally, an adequate sample size should be reported to ensure one can draw appropriate conclusions.

1.4.3. Salt sensitivity mechanisms with focus on ion transport

The biological background of salt sensitivity is complex and not entirely elucidated (Campese, 1994; Ando and Fujita, 2012). There are number of mechanisms that appear to be involved in salt sensitivity presented in Figure 1.2.

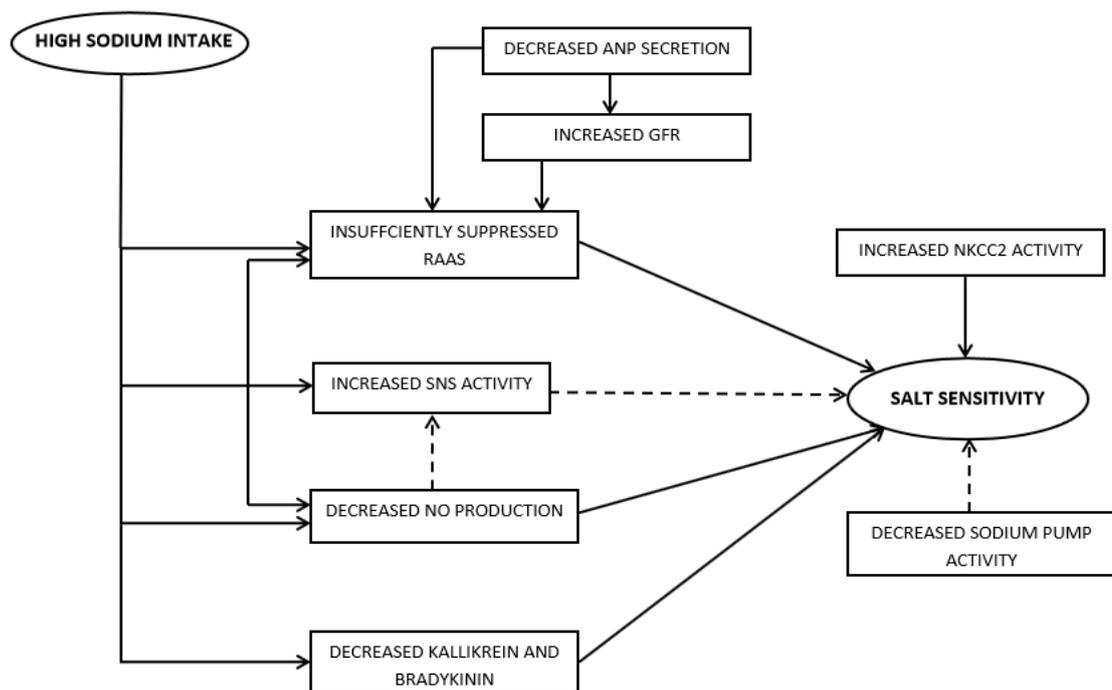


Figure 1.2 Schematic overview of the systems associated with salt sensitivity. Dashed arrows represent potential pathways contributing to hypertension that warrant further investigation in salt-sensitive populations. Asymmetric dimethyl arginine (ADMA), atrial natriuretic peptide (ANP), blood pressure (BP), glomerular filtration rate (GFR), sodium-potassium-chloride cotransporter (NKCC2), nitric oxide (NO), renin angiotensin aldosterone system (NO), sympathetic nervous system (SNS).

Despite a clear interplay of mechanisms involved in salt sensitivity, literature to date suggests that impaired ion transport is one of the most prominent ones affecting salt-sensitive changes in BP (Ando and Fujita, 2012). Ray et al. (2016) state how almost all monogenic hypertensive syndromes involve decreased urinary sodium excretion by the kidney, highlighting the importance of the kidney in regulating BP. This suggests that dysregulation of renal sodium handling likely contributes to non-Mendelian hypertension. Indeed, the kidneys play a major role in the reabsorption of dietary salt and the regulation of body fluid volume (Fels et al., 2010). All nephron segments play a role in the regulation of sodium homeostasis and

BP where different proteins are involved in sodium transport (Yang et al., 2017). For example, the thick ascending limb of Henle's loop has been suggested as a part of the kidney with the most prominent effects on BP in certain populations. Aviv et al. (2004), in their comprehensive review, suggest enhanced activity of the sodium potassium chloride (Na-K-2Cl) cotransporter in the thick ascending limb of Henle's loop as the major factor contributing to the high prevalence of salt sensitivity in black populations. In the distal tubule, the thiazide sensitive sodium chloride cotransporter (NCC) has been shown to be upregulated in Dahl S rats on a high-salt diet (Fanestil et al., 1999). Moreover, the ENaC, formed of three sub-units (α , β , γ) and located in the apical membrane in distal tubule and the collecting duct cells, has been associated with a specific form of hypertension, Liddle's syndrome. This form of salt-sensitive hypertension is caused by mutations in, primarily, the β and γ sub-unit of this channel resulting in its upregulation and an increased sodium reabsorption in these nephron segments (Caretto et al., 2014). In the collecting duct, another channel, the transient receptor potential cation channel subfamily V member 1 (TRPV1) may act as ENaC antagonist suggesting that its activation suppresses ENaC activity and sodium reabsorption in this part of the nephron (Li et al., 2014, Figure 1.3).

However, it is in the proximal tubule where approximately 70% of sodium is being reabsorbed, a process mediated by various sodium transporters (Curthoys and Moe, 2014) such as the sodium hydrogen exchanger (NHE) located in the apical membrane together with sodium bicarbonate cotransporter 1 (NBCe1) and sodium potassium pump/Na⁺-K⁺-ATPase located in the basolateral membrane (Figure 1.3). More recently, another sodium bicarbonate cotransporter, NBCe2 has been characterised in the apical membrane of the renal proximal tubule cell where it is

suggested to play a role in sodium reabsorption and BP regulation (Gildea et al., 2015).

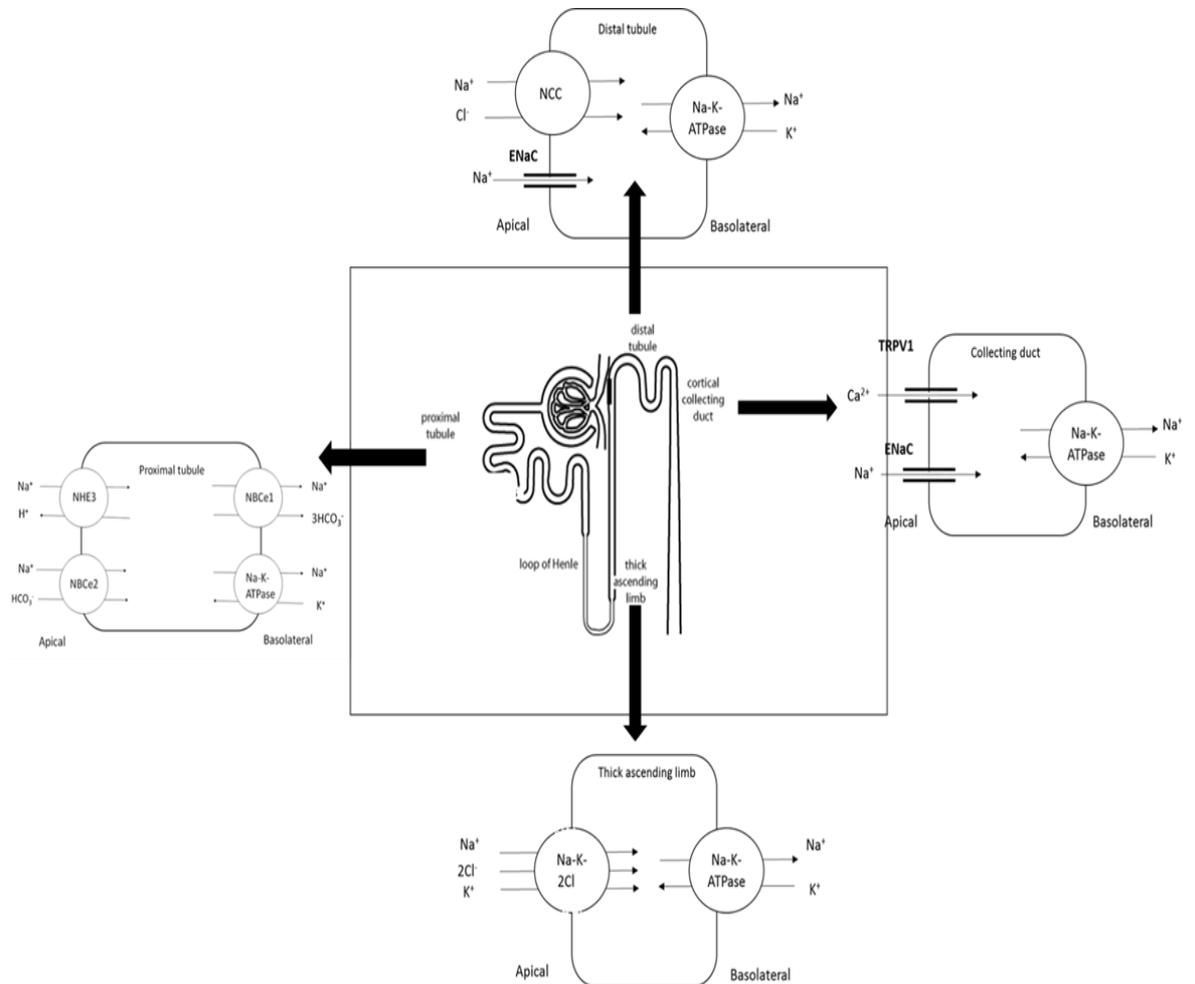


Figure 1.3 Sodium transporters in the kidney (adapted from Horita et al., 2011 and <https://courses.washington.edu/conj/bess/sodium/sodium.htm>). Sodium bicarbonate cotransporter 1 (NBCe1) encoded by the *SLC4A4* gene, sodium bicarbonate cotransporter 2 (NBCe2) encoded by the *SLC4A5* gene, sodium hydrogen exchanger (NHE3) encoded by the *SLC9A3* gene, sodium potassium pump (Na⁺-K⁺-ATPase) encoded by the *ATP1A1* gene (alpha-1 isoform), sodium potassium chloride cotransporter (Na-K-2Cl) encoded by the *SLC12A1*, sodium chloride cotransporter (NCC) encoded by the *SLC12A3*, epithelial sodium channel (ENaC) encoded by the *SCNNIA*, *SCNNIB*, *SCNNIG* genes and the transient receptor potential cation channel subfamily V member 1 (TRPV1) encoded by *TRPV1* gene. Activation of TRPV1 appears to inhibit ENaC mediated sodium reabsorption. The focus of this thesis will be on the *SLC4A5*, *SCNNIB* and *TRPV1* genes with the rationale provided in the following sections.

It has been suggested that the control of sodium transport through renal epithelium is at the entry side (apical membrane) of the cell. Thus the transporters located on this side may have a more pronounced role in the aetiology of salt-sensitive hypertension. It is mainly the overexpression of these transporters that is associated with increased reabsorption of sodium and salt-sensitive increase(s) in BP, suggesting a clear genetic background (Capasso et al., 2005; Yang et al., 2017).

1.4.4. Genetics of salt sensitivity

Hypertension is a complex polygenic disease and BP is considered to be a heritable trait with 30-70% of its variability suggested to be due to genetic variation (Doris, 2011). Ever since the human genome has been mapped in 2003, there has been a number of GWAS conducted to identify the genetic basis of hypertension (Cabrera et al., 2015; Ji et al., 2017). However, to date, GWAS have identified genes that influence only 2% of BP variability with little focus on salt sensitivity (Wain et al., 2011). Furthermore, these studies have focused on distinct single nucleotide polymorphisms (SNPs) as disease markers. Single SNPs generally have poor predictive value for health outcomes and small effect sizes which may be one of the reasons for such a large discrepancy between heritability estimates and the actual variance in BP explained by genetic factors (Do et al., 2012).

Indeed, heritability tends to be overestimated. However, there may be genetic variants associated with BP phenotypes that have not yet been identified in GWAS. Furthermore, posttranscriptional and posttranslational modifications as well as complex gene-gene interactions may affect the final outcome and account for the

”missing heritability” (Lin et al., 2015). All the above-described may explain poor predictive value of single SNPs which, as stated, usually have effect sizes that are too small to be used as risk predictors individually.

For this reason, nowadays, genetic profiles based on a number of genetic variants are used for prediction of complex diseases such as hypertension and CVD (Padmanabhan, 2013). Nevertheless, even when using multiple markers, these genetic risk scores do not seem to improve genetic CVD risk prediction. Simulation studies assuming mean relative risks of 1.1 to 1.2 for CVD risk variants estimate that 100 genetic variants would explain only 1.0% to 9.1% of the variance of CVD (van der Net et al., 2009). Therefore, there is a need to identify further SNPs associated with hypertension and CVD that would account for the majority of the genetic variance. Furthermore, gene-diet interactions are to be further elucidated.

In this sense, although the research is moving in direction of exploring complex gene-gene interactions, certain individual SNPs may potentially be useful when predicting physiological responses (e.g. BP) to dietary interventions. In an investigation of 181 CVD patients, it was reported that a specific interaction between the 677C→T polymorphism, in the gene encoding the folate-metabolizing enzyme, and riboflavin played a novel role in BP. In this randomised intervention study hypertensive participants homozygous for the methylenetetrahydrofolate reductase (*MTHFR*) TT allele exhibited the highest decrease in BP after 16-week riboflavin supplementation compared to CT or CC genotype (from 144/87 to 131/80 mmHg; $p < 0.05$ SBP; $p < 0.05$ DBP) (Horigan et al., 2010). In a 4-year follow up study, Wilson et al. (2012) obtained similar results. At follow-up in 2008, as in 2004, patients with the TT genotype had higher SBP ($p < 0.01$). Riboflavin

supplementation, as in 2004, produced an overall decrease in SBP (-9.2 ± 12.8 mmHg; $p = 0.001$) and DBP (-6.0 ± 9.9 mmHg; $p = 0.003$) in their cohort of TT individuals. *In vitro* studies suggest that this SNP is functional as homozygous mutant (TT) genotype produces an MTHFR enzyme with decreased activity. The B-vitamin riboflavin is required as a cofactor for MTHFR and the decreased enzyme activity evident in individuals with the TT genotype results from the loss of its riboflavin cofactor (Guenther et al., 1999). This in turn can facilitate oxidative breakdown of tetrahydrobiopterin, an essential NO cofactor, which would reduce NO bioavailability and increase BP in TT carriers (Antoniades et al., 2006), highlighting biological plausibility for the interaction between the *MTHFR* and riboflavin. Grimaldi et al. (2017) suggest that evidence of this type of interaction may be used to develop an appropriate genotype-based dietary recommendation which may reduce or eliminate the potential negative consequences associated with the specific genotype. Consequently, it may be targeted to specific groups of people, as shown in a study by Wilson et al. (2012) and, with respect to salt sensitivity, highlights the need for comprehensively exploring genetic markers associated with this phenotype, together with their functional effects and potential involvement in diet-modulated BP pathways.

Regarding genetic predisposition to salt sensitivity, considering the lack of GWAS, the majority of genetic association studies exploring genetic susceptibility to salt sensitivity are candidate gene studies. Both in white and black populations, *SLC4A5* has been reported as the gene involved in salt-sensitive BP response (Carey et al. 2012; Taylor et al., 2009). The *SLC4A5* gene codes for a transmembrane protein which functions as an electrogenic cotransporter of bicarbonate and sodium. This cotransporter helps to maintain the homeostasis of intracellular pH by

cotransporting three bicarbonate anions for each sodium cation independently of chloride (Sassani et al., 2002; Figure 1.3). In a study population of 108 African American women, the ones heterozygous for the rs8179526 (C/T) who had sodium intake above the recommended 2,300 mg/day had lower SBP (~ 125 mmHg) than those who consumed less than recommended (SBP ~ 140 mmHg). In a linear mixed model analysis of the gene x sodium intake interaction effect on BP, this SNP was a significant predictor of SBP ($p = 0.007$), after controlling for age, BMI, and the use of hypertensive medication. Moreover, for the rs10177833, women with CA or AA genotype had higher SBP ($p = 0.030$ and $p = 0.046$, respectively) compared to the women homozygous for the C allele. This analysis however did not include control for confounding variables (Taylor et al., 2009). Since the study population comprised of only African American women, it is difficult to generalise these findings to other populations.

Furthermore, the *SLC4A5* was associated with BP phenotypes in Caucasians; five SNPs in the *SLC4A5* gene showed significant associations with pulse rate in Whites (Barkley et al., 2004). Also in white population, rs6731545 and rs7571842 were significantly associated with resting and sub-maximal exercise pulse pressure (PP) ($0.0004 < p < 0.0007$ and $0.002 < p < 0.003$, respectively). Furthermore, rs6731545 was associated with sub-maximal-exercise SBP and rate pressure product (both $p = 0.002$) (Stütz et al., 2009).

The association between the *SLC4A5* and salt sensitivity was confirmed in two separate cohorts. In 55 hypertensive and 130 normotensive white participants from the University of Virginia (UVA) discovery cohort and 211 white hypertensive participants from the HyperPATH cohort, SNPs in the *SLC4A5* gene, rs7571842 and rs10177833, were significantly associated with salt sensitivity (Carey et al., 2012).

This study will be explained in more detail in Chapter 2 on genetic predisposition to salt sensitivity.

With respect to genome wide analyses, the GenSalt study, the only GWAS on salt sensitivity to our knowledge, explored susceptibility genes that influence individual BP responses to dietary sodium and potassium intake (The GenSalt Collaborative Research Group, 2007). In their study population of 3153 Chinese Han adults, a number of SNPs associated with salt sensitivity has been identified. A great number of these variants are in genes coding for proteins involved in sodium transport. Multiple common SNPs in the *SCNN1G* gene, coding for the γ -subunit of the ENaC, were significantly associated with BP response to low-sodium intervention (rs4073930, $p = 1.7 \times 10^{-5}$; rs4073291, $p = 1.1 \times 10^{-5}$; rs7404408, $p = 1.9 \times 10^{-5}$; rs5735, $p = 3.0 \times 10^{-4}$; rs4299163, $p = 0.004$; and rs4499238, $p = 0.002$) even after correcting for multiple testing. For example, under an additive model, the minor allele G of SNP rs4073291 was associated with 1.33 mmHg lower SBP reduction during low-sodium intervention. This study showed that the ENaC genes play important roles in salt-sensitive BP regulation in the Han Chinese population (Zhao et al., 2011). Furthermore, other GenSalt studies have shown the association of the ENaC variants with BP in the same population (Yang et al., 2014; Liu et al., 2015).

In addition to ENaC, this GWAS identified common variants in the solute carrier 4 (SLC4) gene family. The SLC4 membrane transporter proteins are involved in the kidney acid-base regulation, intracellular pH maintaining and the balance of cation composition (Romero, 2005; Romero et al., 2013). There are currently five mammalian sodium-coupled bicarbonate transporter genes in the SLC4 family:

SLC4A4 (encodes NBCe1), *SLC4A5* (encodes NBCe2), *SLC4A7* (encodes NBCn1), *SLC4A8* (encodes NDCBE) and *SLC4A10* (encodes NBCn2/NCBE). Guo et al. (2016) reported that the *SLC4A4* SNP rs4254735 was significantly associated with DBP response to low-sodium intervention ($p = 5.05 \times 10^{-4}$), with mean (95% CI) response of -2.91 ($-3.21, -2.61$) and -0.40 ($-1.84, 1.05$) mmHg for genotype AA and AG, respectively. Individuals with GG genotype for this SNP have not been detected in this study. In addition, BP responses to high-sodium intervention significantly increased with the number of minor C alleles of the *SLC4A4* rs10022637. Mean SBP responses among those with genotypes TT, CT, and CC were 4.62, 5.94 and 6.00 mmHg ($p = 1.14 \times 10^{-4}$); mean DBP responses were 1.72, 3.22 and 3.94 mmHg ($p = 2.26 \times 10^{-5}$), and MAP responses were 2.69, 4.13 and 4.61 mmHg ($p = 2.07 \times 10^{-6}$), respectively (Guo et al., 2016).

The above-described studies suggest an association between genes coding for sodium transport proteins and salt sensitivity of BP. These results warrant further investigation and replication in different populations. In addition, the evidence from *in vitro* studies suggests that the *SLC4A5* rs7571842 and rs10177833 may be functional.

In this sense, genetic association studies are a valuable tool in providing insight into the physiological role of specific genes by identifying links to particular phenotypes or diseases. However, the findings require duplication and ideally functional validation (Love-Gregory et al., 2011). SNPs can affect phenotype by exerting its functional effects on: 1) protein structures, by changing single amino acids; 2) transcriptional regulation, by affecting transcription factor binding sites in promoter or intronic enhancer regions; and 3) alternative splicing regulation, by

disrupting exonic splicing enhancers or silencers (Yuan et al., 2006).

The majority of known disease causing SNPs lie in the gene coding regions and the distinction can be made between the non-synonymous SNPs which alter an amino acid in a protein, resulting in a different protein structure (mis-sense, non-conservative change), a non-sense change that results in a premature termination of the amino acid sequence (non-sense), or a mis-sense (conservative) SNP which alters an amino acid in a protein to one with similar structural characteristics. Synonymous SNPs, however, do not result in a change in amino acid. Coding SNPs may also disturb the binding sites of an exonic splicing enhancer or a silencer (Yuan et al., 2006).

Non-coding SNP, on the other hand, may affect the transcription factor-binding site of the gene which can affect the level, location, or timing of gene expression. In addition, a SNP at the 'splicing site' may break the consensus splicing site sequence resulting in a different form of a protein (Yuan et al., 2006).

Regarding salt sensitivity, the GenSalt research group reported on the functional validation of the ENaC variants explored previously in their study population (Zhao et al., 2011; Yang et al., 2014; Liu et al., 2015). Out of 16 non-synonymous variants identified previously, the results revealed five gain-of-function and two loss-of-function variants. Furthermore, several α -subunit extracellular domain variants altered channel inhibition by extracellular sodium (Na^+ self-inhibition). One variant (αA334T) decreased and one (αV481M) increased cell surface expression. None of the variants was, however, associated with salt sensitivity of BP suggesting their role in regulating sodium dependent BP pathways may be limited (Ray et al., 2016).

Regarding the functional effects of the *SLC4A5* SNPs, Felder et al. (2016) conducted an experiment by using proximal tubule cells isolated from a spot urine sample of four homozygous carriers of the rs10177833 A allele, which was previously suggested as the risk allele, and six homozygous carriers of the C allele, already diagnosed for salt sensitivity (Carey et al., 2012). They reported that the *SLC4A5* rs10177833, an intronic SNP, caused an increase in transcription factor hepatocyte nuclear factor 4 alpha (HNF4A) binding to the *SLC4A5* gene. This in turn resulted in an increase in *SLC4A5* mRNA, its protein product (NBCe2) levels, and increased NBCe2-mediated bicarbonate and sodium transport under conditions of elevated intracellular sodium. This increase was evident only in homozygous A allele carriers suggesting that this SNP may be involved in diet-modulated BP pathways (Figure 1.4). However, due to limited sample size these results should be replicated in a larger cohort.

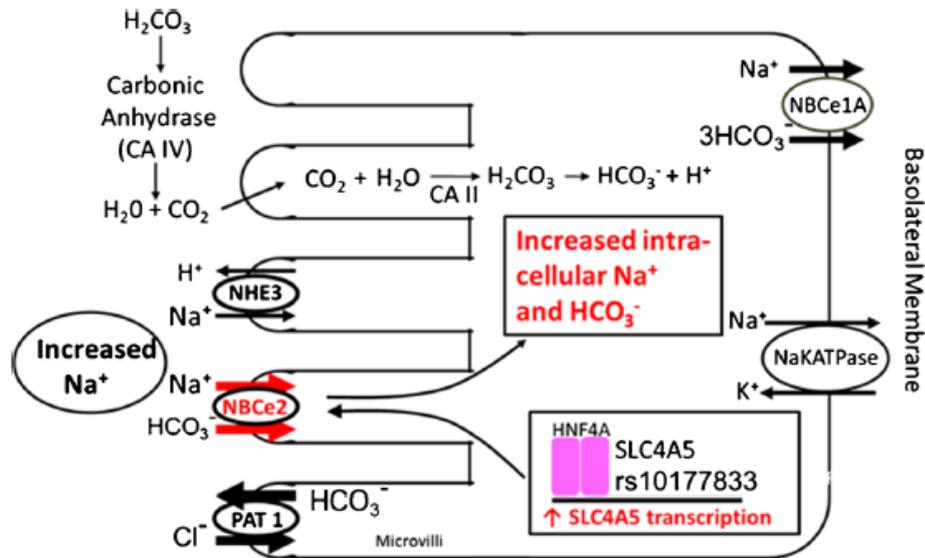


Figure 1.4 The mechanism causing the increase in sodium transport in renal proximal tubule cells carrying the *SLC4A5* rs10177833. In proximal tubule cells, the rs10177833 causes an increase in HNF4A binding to *SLC4A5* gene an increase in NBCe2 mRNA, NBCe2 protein expression, and increased NBCe2-mediated bicarbonate and sodium transport under conditions of elevated intracellular sodium. This is associated with an increase in PAT1 and a slight reduction in NBCe1 activity (Felder et al., 2016).

Putative anion transporter 1 (PAT1), sodium bicarbonate cotransporter 1 (NBCe1), sodium bicarbonate cotransporter 2 (NBCe2), sodium hydrogen exchanger (NHE3), sodium potassium pump ($\text{Na}^+\text{-K}^+\text{-ATPase}$) (Felder et al., 2016).

Other SNPs in the SLC genes, reported in the previous section, have not been investigated regarding their functionality limiting the ability to draw any conclusions about their role in BP related biological pathways. Further research is needed to better understand the potential functional effects of SNPs associated with salt sensitivity.

Finally, salt sensitivity is a clear example of gene-diet interactions where genetic susceptibility to salt-sensitive hypertension alone does not result in increased BP or hypertension. An environmental factor, increased dietary salt intake, is, together with genetic predisposition, necessary for phenotypic expression of salt sensitivity. The first animal genetic model of salt sensitivity, Dahl S rat was

introduced by Lewis Dahl in 1960s. Dahl administered triiodothyronine to Sprague-Dawley rats and selected the ones with the highest response to a high-salt diet. These rats were then mated with equally responsive siblings. After few generations, a salt-sensitive strain with consistent hypertensive response to high-salt diet was created. Moreover, Sprague-Dawley rats without a hypertensive response to high-salt diet were inbred to produce a salt-resistant strain. The results of their experiments showed consistently that the BP of salt-resistant rats remained the same when they were switched from a control diet to a high-salt diet. Salt-sensitive rats, however exhibited a deadly increase in BP. Salt-sensitive rats that were maintained on a control diet did not develop hypertension, showing that the appropriate diet can prevent the development of hypertension even in susceptible individuals (Bashyam, 2007).

In humans, a similar response can be observed, although much more complex, due to a more heterogeneous genetic background compared to inbred Dahl rats, and the presence of both salt sensitivity protective alleles and the ones predisposing to salt sensitivity. Nevertheless, salt sensitivity is a phenotype that, among other, depends on the dietary salt intake (Figure 1.5). Therefore, the latter should be explored in context of salt sensitivity together with its genetics and physiology.

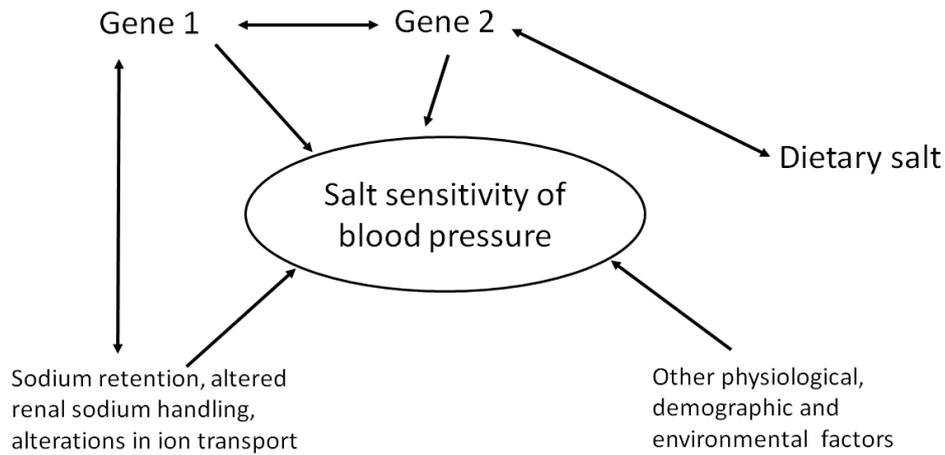


Figure 1.5 Gene-gene and gene-diet interactions as factors contributing to salt sensitivity. As a complex phenomenon, salt sensitivity is associated with numerous physiological, environmental, demographic and genetic factors (adapted from Strazzullo et al., 2000).

In summary, genes involved in ion transport across kidney seem to be associated with salt-sensitive changes in BP in white, black and Chinese populations and code for some of the key transporters regulating BP and sodium homeostasis. Even though single SNPs would generally serve as poor predictors of health outcomes, it is not to exclude that certain genetic variants may be useful in predicting BP response to dietary salt intake. This may especially be the case if these SNPs are functional and if their involvement in diet-modulated biological pathway is plausible. In this sense, the *SLC4A5* rs7571842 and rs10177833 provide a sound avenue for further research in context of salt sensitivity.

1.5. Salt taste perception as a driver of salt intake

Considering the contributions of dietary salt intake towards a development of salt-sensitive hypertension, the following sections will explore salt taste perception

as a main driver of salt intake, together with its genetics. Different approaches in measuring salt taste perception/sensitivity will also be discussed.

1.5.1. Organoleptic properties of salt

Palatability appears to be a main driver of salt intake. However, evidence is controversial with attempts to investigate its role as the main factor affecting salt intake reaching conflicting conclusions. For example, a number of studies show that salt palatability is modulated by bodily sodium availability. Sodium appetite is, together with thirst, considered as the only innate mechanism of acquiring a certain nutrient. Nevertheless, the majority of studies reporting on the sodium appetite have been conducted in animals (Leshem, 2009). In humans, there is modest evidence for sodium palatability induced by physiological needs. Research shows that haemodialysis or natriuretic treatment increase sodium palatability (Beauchamp et al., 1990; Leshem and Rudoy, 1997). Moreover, in intense exercise, where significant amounts of sodium are excreted through sweat, research more consistently shows physiologically elicited sodium palatability (Kanarek et al., 1995; Leshem et al., 1999). However, it was also indicated that palatability is not a simple reflection of need state but acts to promote intake through a distinct hedonic system, by seeking the specific taste of sodium chloride (Yeomans et al., 2004). Palatability is largely affected by taste sensitivity, that is, the sensorial threshold for a specific taste. Salt taste sensitivity is the capacity to identify the flavour of salt and salt taste sensitivity threshold can influence salt appetite or salt food preference (Nilsson, 1979). Taste sensitivity has been shown to affect dietary intake and is influenced by genetic factors (Dias et al., 2013).

1.5.2. Methods of measuring salty taste sensitivity - salt taste thresholds

Salt taste sensitivity can be assessed with a variety of techniques. Among the methods of evaluating taste sensitivity, threshold measurements can be especially useful due to individual differences in taste acuity (Giguère et al., 2016).

Considering that the taste thresholds are most commonly assessed by tests whereby participants are administered with aqueous solutions of a specific taste stimulus (a prototypical salty taste stimulus is sodium chloride), salt taste detection threshold (STDT) is defined as the solution at which the participants clearly indicate it as different from water, but not necessarily recognising the type of stimulus. On the other hand, salt taste recognition threshold (STRT) commonly defined as the lowest concentration of a sodium chloride solution that is consistently identified by the participant as having a salty taste (British Standards Institution (BSI), 2011). In addition to thresholds measurement, subjective ratings of intensity for suprathreshold stimuli or differential thresholds (the minimum concentration difference necessary to determine that two suprathreshold stimuli are different) can also be employed in research (Bartoshuk, 1978).

One of the first reported methods for measuring taste thresholds, and still used to date, is the Harris-Kalmus method developed to classify participants according to their ability to taste phenylthiocarbamide (PTC) and determine the bitter taste recognition threshold. Participants were first asked to taste a number of serial dilutions of PTC made up in water. Starting from the higher dilutions and working down, the participants were given the solutions until the definite taste could be perceived. This step provided an approximate value for the threshold. The participants were subsequently presented, in random order, with eight solutions, four

of which contained water and four containing a solution determined in stage one. The participants were asked to distinguish between the water and a solution containing PTC. The lowest concentration at which participants were able to consistently distinguish between water and the solution containing the stimulus was considered as the threshold (Harris and Kalmus, 1949). Since then, researchers have used this or a modified version of this method to measure thresholds for different tastes, including salty taste.

Another commonly used method is a 2-alternative forced-choice detection threshold method based on Wetherill and Levitt (1965) and Levitt (1971) work on sequential estimation of points on a psychometric function. In brief, participants receive a one taste solution and one blank in random order. Participants are then instructed to determine which sample tastes stronger. Starting with the best estimate of threshold, a 4-down, 1-up rule can be used: concentration increases one step after each incorrect response and decreases one step after four consecutive correct responses at the same concentration. An increase in concentration that follows a decrease (or a decrease that followed an increase) is termed a reversal. Testing continues until participants accrue five reversals. The threshold is then calculated as the average of the concentrations at which the last four reversals occurred. Both above-described methods can, however, prove to be complicated for untrained participants and require a large amount of time to complete (Giguère et al., 2016).

The International Organisation for Standardisation (ISO) also provides guidelines for measurement of taste thresholds and these have been used in research on salt taste sensitivity (International Standard Organisation, 2002; British Standard Institution, 2011). These methods are more simple and/or can be adapted into a more

simple and rapid methods to test salt taste thresholds (Giguère et al., 2016).

Despite presenting only the most commonly methods of measuring taste thresholds, there are marked differences both within the methods used in current literature, such as the concentration of the salty taste stimulus (sodium chloride) or the exposure of the stimulus (whole mouth or local applications), and between the methods as already described. In addition, the measures do not necessarily correlate with each other (Giguère et al., 2016) and there is no consensus which method should be used to measure salt taste sensitivity. Thus, it is on the researchers to decide which method to employ in their work.

1.5.3. Genetics of salt taste perception

The gustatory system in mammals comprises taste cells, afferent gustatory nerves and brain structures involved in central processing of taste. The cascade of reactions begins with the taste receptor cells located in taste buds which are located within gustatory papillae in the tongue. Mature taste receptor cells interact with taste stimuli via taste receptor proteins when the signal is transduced via gustatory nerves to the brain to elicit the perception of a specific taste (Figure 1.6) (Bachmanov et al., 2014).

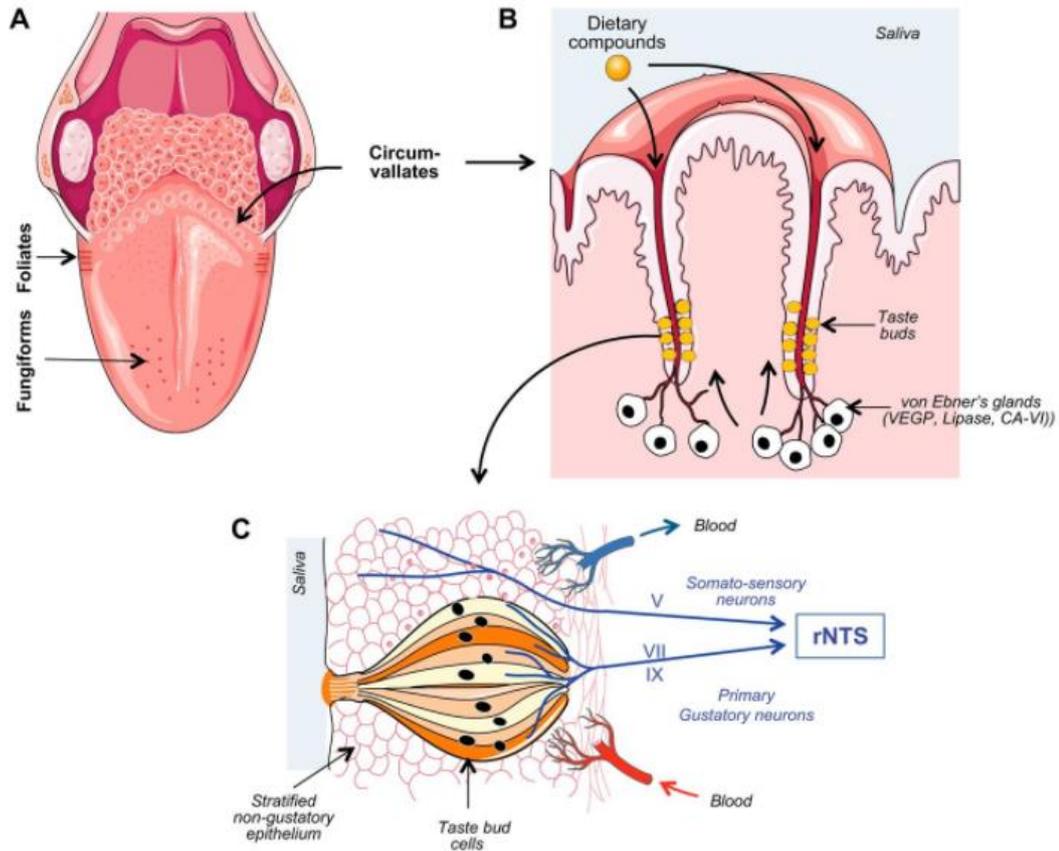


Figure 1.6 Peripheral taste system (Besnard et al., 2016). A: localization of different types of gustatory papillae onto the human tongue. B: sagittal section of a circumvallate papillae (CVP) with a typical dome-shaped structure and the anatomical relationship with the von Ebner's glands. C: schematic representation of a taste bud. CA-VI, carbonic anhydrase; rNTS, rostral nucleus of solitary tract; VEGP, von Ebner's gland protein; V, trigeminal endings; VII, afferent fibers of the chorda tympani nerve; IX, afferent fibers of the glosso-pharyngeal nerve (Besnard et al., 2016).

One of the first proposed salty taste receptors in the tongue was the ENaC, also expressed in the kidney, involved in transepithelial sodium transport. This protein is inhibited by amiloride, a potassium-sparing diuretic, and therefore forms a part of the taste response which is amiloride-sensitive. Amiloride-insensitive part of taste receptors is not suppressed by this substance. As described in the context of BP regulation, ENaC in humans is formed of several subunits: α , β , γ , and/or δ . Each ENaC subunit has two transmembrane domains and is encoded by a separate gene.

Humans have four ENaC channel subunits, α , β , γ , and δ , encoded by the non-voltage-gated sodium channel 1 genes *SCNN1A*, *SCNN1B*, *SCNN1G*, and *SCNN1D*, respectively. Mice and rats lack the ENaC δ subunit and therefore have only three ENaC subunits encoded by *Scnn1a*, *Scnn1b*, and *Scnn1g* (Bachmnov et al., 2014). The α ENaC confers a low-amplitude, amiloride-sensitive sodium current, whereas β - and γ -subunits are required for the maximal channel activity (Shigemura et al., 2008). In mice, a variation in the gene coding for α subunit resulted in a stronger amiloride induced inhibition of chorda tympani response to sodium chloride. These results suggest that the variation in the α ENaC subunit affects amiloride sensitivity of the ENaC and that ENaC is involved in amiloride-sensitive salt taste responses in mice (Shigemura et al., 2008).

In humans, variation in individuals' ability to taste salt, may be a possible explanation of the variable salt intake. Genetic variation in bitter and sweet taste receptors may affect the ability to taste sweet and sour (Garcia-Bailo et al., 2009). Regarding the genetic variation affecting salt taste perception in humans, only one study reports the association of SNPs rs239345 and rs3785368 in the *SCNN1B* gene coding for β subunit of ENaC with salt taste perception in adult Caucasian population (Dias et al., 2013). The results will be explained in detail in Chapter 3.

Regarding the amiloride-insensitive part of salt taste receptor, one of the candidates is TRPV1 (transient receptor potential cation channel, subfamily V, member 1; formerly named vanilloid receptor subtype 1, or capsaicin receptor). This receptor transduces painful thermal stimuli and is also activated by capsaicin, therefore is considered to be mainly involved in nociception. However, a TRPV1 variant was proposed to function as an amiloride-insensitive salt taste receptor in

rodents. It has been reported that *trpv1* knockout mice lack sodium chloride chorda tympani nerve responses in the presence of amiloride, whereas control mice displayed normal levels of nerve innervations (Lyall et al., 2004). This indicates that by disrupting the function of ENaC and TRPV1 channels concurrently, one may eliminate chorda tympani mediated salt taste (Dias et al., 2013). However, *trpv1* knockout mice do not have deficiencies in behavioural taste responses to salt (Ruiz et al., 2006; Treesukosol et al., 2007).

In humans, the rs8065080 in the *TRPV1* gene has been found to modify the salt taste perception in the Toronto Nutrigenomics and Health Study (Dias et al., 2013). Details of this study will be explained in Chapter 3 of this thesis.

Even though there are, most likely, more genes involved in salt taste perception (Figure 1.7), the three SNPs in *SCNN1B* and *TRPV1* gene are, to date, the only genetic variants reported in literature to be associated with salt taste perception/sensitivity in humans. These proteins are also expressed in kidney, therefore showing the potential link between salt sensitivity of BP and salt taste perception.

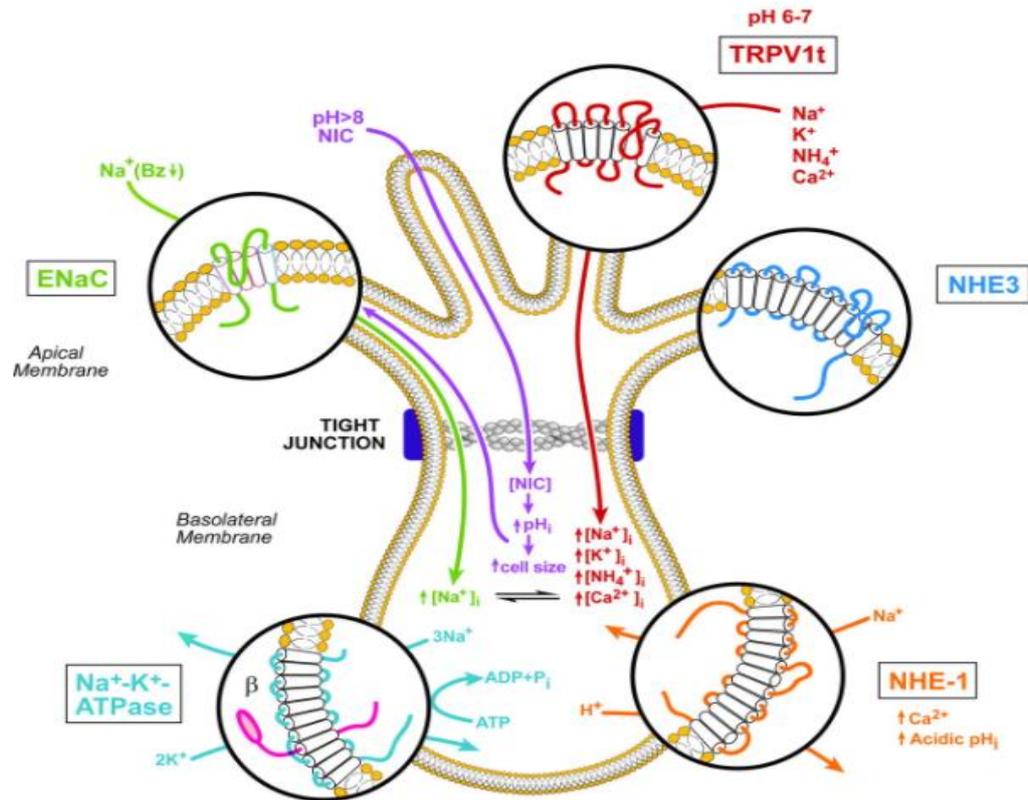


Figure 1.7 Proposed model for sodium transport in fungiform taste receptor cells and salt taste transduction in the anterior tongue. Amiloride-sensitive epithelial sodium channel (ENaC), transient receptor potential variant salt taste receptor-1 (TRPV1t), basolateral sodium hydrogen exchanger 1 (NHE-1); apical sodium hydrogen exchanger 3 (NHE-3). The exit of sodium from taste receptor cells occurs by the basolateral sodium-potassium pump (Na-K-ATPase). An additional sodium transport mechanism involves the NHE-1. The apical NHE-3 seems to be quiescent in taste receptor cells (Lyall et al., 2007).

In addition to salt taste receptors expressed lingually, it has recently become clear that many sensory receptors, including taste receptors, play key roles in organs and tissues traditionally thought of as “nonsensory”. Sour taste receptors can be found in the spinal column regulating pH, bitter taste receptors regulate bronchodilation in the lung in response to certain inhalants and sweet taste receptors regulate glucose transport in the gut. In proximal and distal segments of the renal tubule there are distinct sensory mechanisms/proteins which can detect the concentration of the bicarbonate ion and tune ion (sodium) transport processes

accordingly. One of these proteins is encoded by a member of the SLC family of transporters (Pluznick and Caplan, 2015).

With this notion, it is justifiable to hypothesise that the SNPs in genes involved in sodium transport may also affect salt taste perception and salt intake together with BP.

1.6. The relationship between salt taste sensitivity, dietary salt intake and blood pressure

As suggested in previous sections, there is a genetic predisposition to both salt sensitivity of BP and salt taste sensitivity, which may be due to the action of genes coding for sodium transport proteins expressed both in kidneys and lingually. In this sense, a link between salt taste perception and BP is suggested. Moreover, research suggests an association between salt taste sensitivity and salt intake, albeit inconclusive. Studies exploring these relationships will be evaluated in this section, first focusing on the association between BP and salt taste sensitivity, followed by the studies exploring the link between salt taste sensitivity and salt intake. Finally, research on the association between all the three mentioned variables will be discussed.

The most recent study investigating the association between salt taste perception and BP has been conducted in 421 healthy adolescents aged 14-19 years in Brazil. The adolescents received nine sodium chloride solutions (4 mmol/l, 8 mmol/l, 15 mmol/l, 30 mmol/l, 60 mmol/l, 120 mmol/l, 250 mmol/l, 500 mmol/l and 1000 mmol/l) in increasing concentrations until the taste was correctly

identified. The cut-off concentration for normal taste sensitivity threshold was set at 30 mmol/l. Participants that were able to taste salt at concentrations higher than the cut-off were considered as having an increased salt taste sensitivity threshold (Nilsson, 1979). DBP was higher in adolescents with high threshold compared to the ones with the normal salt taste threshold (73.3 ± 9.89 vs. 70.7 ± 10.6 mmHg, $p = 0.017$) whereas no difference in SBP was reported (123.9 ± 13.7 vs. 121.9 ± 13.8 mmHg, $p = 0.168$) suggesting an association between DBP and salt taste sensitivity in adolescents (Kirsten and Wagner, 2014). Even so, BP measurements were performed in duplicate on one day, with only a small interval between each of the measurements. This may have affected the results as DBP measurement is associated with a greater degree of error than the SBP. This error may be attenuated if several BP measurements are performed, especially on different days. Nevertheless, the association between BP and salt taste sensitivity was also reported in Nigerian 10 to 17 year olds. The authors reported a significant ($p = 0.0103$) but weak association ($r = 0.1439$) between salt taste sensitivity and SBP (Okoro et al., 1998).

Together with studies conducted in younger populations, several studies have investigated above-described associations in adults. A recent explored the associations between BP and parameters related to salt intake such as salt taste thresholds and salt preference in adult population, with both normal and high BP, in Korea and Myanmar. The authors reported higher salt taste detection (0.102 ± 0.108 vs. $0.046 \pm 0.026\%$, $p < 0.001$) and recognition thresholds (0.174 ± 0.163 vs. $0.103 \pm 0.115\%$, $p < 0.01$), preference for salty food, expressed as the preferred salt concentration in soup (0.44 ± 0.16 vs. $0.37 \pm 0.10\%$, $p < 0.001$), salt intake measured by a spot sodium excretion (158 ± 84.3 vs. 117 ± 62.1 mg/dl, $p < 0.01$), as well as SBP (137 ± 25.5 vs. 125 ± 15.6 mmHg, $p < 0.01$) and DBP (86.0 ± 14.9 vs. $76.8 \pm$

10.1 mmHg, $p < 0.001$) in a Myanmar population. SBP was positively associated with salt taste detection ($r = 0.242$, $p = 0.005$) and recognition threshold ($r = 0.179$, $p = 0.041$) but not with the actual salt intake ($r = 0.122$, $p = 0.164$) and salt preference ($r = 0.060$, $p = 0.494$) in the total study population. A limitation of the study may be considered a lack of exploration of the salt taste threshold and salt intake relationship. In addition, a spot urine sample is not appropriate to measure dietary salt intake due to circadian variations in sodium excretion, which may have affected the results of the correlation analysis (Cho et al., 2016).

Furthermore, in normotensive and hypertensive diabetic patients, STRT was significantly associated with MAP ($\beta = 0.125$, $p < 0.001$) which remained significant after adjustment for age, gender, family history of hypertension, BMI, antihypertensive medication and proteinuria. The study participants were presented with different concentrations of salt solutions ranging from 0-400 mmol/l. The first sodium chloride solution recognised by the participant as tasting different from distilled water was recorded as STDT. The concentration recognised as definitely salted was recorded as the taste threshold for salt recognition. The participants with the recognition taste threshold higher than 100 mmol/l were defined as being salt taste insensitive (Isezuo et al., 2008). Similarly, another study reported OR for hypertension of 2.47 (1.53, 3.99) in the female participants with impaired salt taste thresholds while no difference between the male participants with normal or impaired thresholds was observed. The study participants, 40 year old and older Japanese adults, were tested for their salt taste thresholds by tasting a strip saturated with different concentrations of sodium chloride and reporting on the taste. Although a correlation between BP and salt taste sensitivity was observed, classification of hypertension was based on the causal BP measurement which may have resulted in

outcome misclassification and affected the results (Michikawa et al., 2009). Similarly, Rabin et al. (2009) investigated an association between exercise induced hypertension and salt taste thresholds in 203 Brazilian adult men and women, predominantly Caucasian. They have defined the value for salt recognition in their normotensive study population as 15 mmol/l of sodium chloride and have reported a higher OR for exercise induced hypertension in participants with high salt taste thresholds; 6.71 (1.50, 29.99). The authors hypothesised that sodium intake mediated this relationship but did not measure it in their study population and could, therefore, not explore this hypothesis.

Even though there appears to be a relationship between salt taste sensitivity and BP in adolescents and adults, further studies are needed to explore this relationship. Such studies should, to ensure uniformity in study design, use the same method of measuring salt taste sensitivity (with the same NaCl concentrations) and repeated BP measurements.

Regarding the relationship between salt taste sensitivity and salt intake, Matsuzuki et al. (2008) found no association between the salt taste thresholds, assessed using a slip of paper impregnated with different salt concentrations, and sodium intake, assessed by spot urinary sodium excretion, in Japanese school children aged 10-12 years ($p = 0.481$ for boys and $p = 0.825$ for boys). As stated, a single spot urine collection may not be representative of the actual dietary sodium intake and these results should be confirmed in the same population by employing multiple 24-hour urinary sodium measurements. Contrary to these results, Kim and Lee (2009) suggested an association between salt taste sensitivity and salt taste preference. Korean children aged 12-13 years who reported liking for a Korean high-

salt soup/stew had a significantly higher detection threshold than those who did not (7.01 ± 4.44 vs. 4.96 ± 3.08 mm, $p = 0.029$). Considering that the actual salt intake was not measured, further studies are needed to explore this association.

Furthermore, Pangborn and Pecore (1982) did not demonstrate a relationship between salt intake and taste thresholds in 57 male and female young adults ($p < 0.05$). Possible explanation for a failure to demonstrate the association between thresholds and dietary intake may be the validity and reliability of the method to assess dietary salt intake. Their FFQ did not include any information on the portion sizes of food consumed and they conclude that the future studies should use a more accurate measure of salt intake.

From the above-described, methods of measuring salt intake may be suggested as a limiting factor. None of the studies presented above employed a 24-hour urinary sodium measurement or the methods suggested to be correlated with this “gold-standard” method. This may have resulted in conflicting results and is highlighting the need for employing more accurate methods of measuring dietary salt intake in future studies.

The final studies that will be presented in this section, explore the relationship between salt taste thresholds, salt intake and BP in a more comprehensive manner, taking into consideration all three variables. In 97 normal weight and overweight/obese children, salt taste threshold was positively correlated with SBP only in normal weight children ($r = 0.32$, $p = 0.03$) but no correlation was observed in overweight/obese children ($r = -0.20$, $p = 0.22$). The proportion of children with high thresholds was similar in both groups. Moreover, thresholds were not correlated with reported salt intake measured with an Automated Self-

Administered 24-Hour Recall system whereby mothers provided the information about the foods consumed at home and children reported on any food eaten outside of their homes (Bobowski and Mennella, 2015). Despite using a validated method of measuring salt intake, multiple 24-hour recalls would be advised in future studies to obtain a more accurate measure of salt intake. Moreover, children are prone to reporting error which should be considered as a limitation of the study and may have affected the results on the relationship between salt intake and salt taste thresholds.

In a Nigerian population, a higher sodium intake, assessed by 24-hour urinary sodium excretion, was reported in hypertensive compared to normotensive group of participants (36.6 ± 20 mmol/l/mmol creatinine vs. 14.8 ± 5.8 mmol/l/mmol creatinine, $p < 0.001$). Furthermore, there was a higher urinary sodium excretion in the participants with higher salt taste thresholds (30.3 ± 5 mmol/l/mmol creatinine vs. 19.5 ± 14 mmol/l/mmol creatinine, $p < 0.05$). (Azinge et al., 2011). Even though the authors employed a “gold standard” method of measuring salt intake, multiple measures would be advised to achieve more accurate results. Moreover, adjustment for creatinine excretion may not be appropriate. A more valid approach would be using the PABA method to exclude incomplete urine collections or predefined creatinine excretion cut-offs, reported to be correlated with PABA method. Similarly, Piovesana et al. (2013) investigated the relationship between salt taste thresholds, hypertension status and dietary salt intake in adult Brazilians which was evaluated through 24-hour urinary sodium excretion and self-reported measures (discretionary salt, sodium- FFQ, and 24-hour recall). Salt taste detection (8 ± 9 vs. 15 ± 2 mmol/l, $p = 0.001$) and recognition thresholds (13 ± 17 vs. 27 ± 16 mmol/l, $p < 0.001$) were higher in the population with hypertension compared to their healthy counterparts. Interestingly, regarding the association between salt taste perception

and salt intake, different results were obtained when taking into consideration different measures of salt intake. A weak positive correlation ($r = 0.28$, $p = 0.042$) was observed between STDT and salt intake measured with FFQ in hypertensive participants and STRT and FFQ salt intake ($r = 0.28$, $p = 0.043$) in normotensives. In total sample, detection threshold was negatively correlated ($r = -0.20$, $p = 0.04$) with 24-hour recall salt intake. Sodium intake measured with urinary sodium excretion, a method considered as the gold standard, was not significantly correlated with salt taste thresholds in the overall population ($r = -0.04$, $p = 0.654$) and when stratifying according to hypertension status (hypertensive population: $r = -0.11$, $p = 0.424$; normotensive: $r = -0.09$, $p = 0.511$) (Piovesana et al., 2013). However, only one 24-hour recall measurement and no control for completeness of urine collection present limitations of this study and the results should be replicated in a different study employing multiple 24-hour recalls (preferably weekend and weekday) and controlling for completeness of urine collections.

Finally, in a cross-sectional study of 2371 participants, the perception of salt taste, measured using a filter paper impregnated with sodium chloride, was related to the frequency of discretionary salt. The percentage of participants who reported never adding salt was higher in the group that had higher salt taste sensitivity (30.6%) compared to the groups with lower ability to taste salt (24.0%). There was no association between discretionary salt use and hypertension status (Fischer et al., 2012). Despite a large sample size, this study addressed only one aspect of salt intake (i.e. discretionary salt use). Further studies are needed to explore the association between salt taste sensitivity and total salt intake in this study population.

In summary, there is a clear discrepancy in the results of presented studies

and studies that do suggest an association between salt taste sensitivity, salt intake and BP report mostly on weak associations. In addition, these studies differ according to the study population and methodology. Further research is needed to elucidate the possible relationship between salt taste thresholds, BP and the actual salt consumption. Such research should rely on a more uniform design, employing 24-hour BP or multiple spot BP measurements conducted on different days, together with multiple 24-hour urine sodium measurements (controlling for completeness of collections) or validated 24-hour recalls. Finally, to the best of our knowledge, there are no studies exploring salt sensitivity as a distinct phenotype in the context of salt taste sensitivity and salt intake in humans, providing an avenue for future investigation. As discussed, better understanding of salt intake as the environmental contributor to salt sensitivity is necessary to inform future strategies in prevention of hypertension. Such strategies may be based on the concept of personalised nutrition.

1.7. Personalised nutrition approach in disease prevention and treatment

Personalised nutrition is an innovative concept that is aimed at identifying individual nutritional needs based on health status, genotype and/ or phenotype and then to provide healthy eating advice that is tailored to suit the individual (Stewart-Knox et al., 2015). Personalised nutrition advice based on individuals' genetic make-up, especially, is emerging as a promising approach in efforts to achieve more effective prevention of chronic disease. Such advice is based on the fields of nutrigenetics and nutrigenomics. Nutrigenetics focuses on the effects of genetic

variation on an individual's responsiveness to a particular diet or nutrient whereas nutrigenomics focuses on how specific nutrients or dietary constituents affect gene expression, protein and metabolite concentration (Ordovas and Mooser, 2004; Corthésy-Theulaz et al., 2005). The advancement in research in these two related disciplines is resulting in a shift from a more generalised "one size fits all" approach in disease prevention and treatment to a more personalised one, which may be more successful in prevention and early diagnosis of disease. Regarding the efforts to reduce disease burden Phillips (2013) states how, at a public health level, more attention must be given to modification of lifestyles of the general public to reduce risk of chronic disease. At a clinical level, however, individual patients with increased disease risk need to be identified so that their risk factors can be reduced. The author also acknowledges that the early identification of "at risk" individuals is of utmost importance. In addition, early diagnosis and prevention could enable earlier and targeted interventions such as implementation of healthy lifestyle changes in nutritional behaviour thus preventing or reducing disease development. A research about the underlying gene-nutrient interactions may provide the evidence base to determine whether more targeted nutritional advice is an appropriate public health approach (Phillips, 2013). According to Corella and Ordovás (2013), translational goals of this research include the widespread use of genetic profiles to guide physicians in the classification of patients according to their disease rather than to their symptoms and the use of those genetic profiles to implement targeted recommendations and therapies aimed to improve prevention. However, to achieve these goals, one of the crucial steps required is deeper and more comprehensive understanding of gene-environment interactions.

In this sense and as already stated, single SNP approach in prediction of

complex biological conditions such as hypertension may not be appropriate because of complex gene-gene and gene-environment interactions that all contribute to the final health outcome. Two studies, to date, have successfully recruited participants based on their genotype and obtained a response to dietary intervention similar to what was already reported in literature. In brief, in the Fish oil intervention and genotype (FINGEN) study, participants were recruited based on their apolipoprotein E (*APOE*) genotype with the aim of exploring the effect of sex and genotype on CVD health outcomes after fish oil intervention. The results showed that the greatest triacylglycerol-lowering responses (reductions of 15% and 23% after 0.7 g of fish oil and 1.8 g of fish oil, respectively) were evident in *APOE4* men (Caslake et al., 2008). Previous research suggested that *APOE4* individuals are particularly sensitive to dietary fat intake interventions (Minihane et al., 2000). In a similar fashion, Wilson et al. (2012) prospectively recruited participants according to their *MTHFR* 677C/T polymorphism, in a randomised trial exploring the effect of riboflavin supplementation (1.6 mg/day) on BP in hypertensives with the TT risk genotype. As expected and based on previous research (Horigan et al., 2010), analyses showed that riboflavin supplementation resulted in a significant decrease in both SBP ($p = 0.001$) and DBP ($p = 0.003$). The results suggested that riboflavin supplementation may be used as hypertension treatment in persons at risk of CVD with this specific genotype.

Considering only two studies reporting on such results, it is evident that further research with similar study design is needed in order to advance the field of genotype-based personalised nutrition.

Bearing in mind the potential limitations of single SNP approach, polygenic

risk scores have been suggested in prediction of complex conditions such as CVD. Müller et al. (2016), however suggest that these risk scores may not have a value beyond traditional risk factors and that further research is needed on how to best apply the knowledge obtained from GWAS. Nevertheless, the authors state that future research may also focus on assessments in which the investigation of traditional risk factors is more costly than the investigation of genetic risk factors, which is the case with salt sensitivity, and these issues will be discussed in Chapter 5.

Despite the mentioned drawbacks in the area of nutrigenetics, Grimaldi et al. (2017) acknowledge there is a growing demand for genotype-based dietary advice. To facilitate acceptance by the public and other stakeholders, such advice should be based on sound evidence. In this context, the experts in the field have developed a framework to assess scientific validity of a genotype-based dietary advice to limit the possibilities of premature application of nutrigenetic research. The assessment criteria for specific gene-diet interaction should be based on: study quality and the body of evidence available, together with biological plausibility of the interaction which would encompass the type of gene-diet interaction (which may be direct, intermediate or indirect) and the nature of the genetic variant (depending if the variant is causal or the mechanisms are unknown). In context of salt sensitivity, and based on these criteria, further research is needed into functional effects of genetic variants together with further trials exploring the gene-dietary salt interactions in relation to hypertension.

Ideally, together with the validation of evidence behind specific gene-diet interactions, it should be explored whether this would work in practice, that is,

would individuals change their dietary behaviour after receiving the information about their genetic risk and personalised dietary advice. The largest trial of this kind was a Food4Me pan European study exploring the effects of personalised dietary advice on change in dietary behaviour. The aim of the study was to explore if personalised dietary intervention is more effective than traditional dietary guidelines and if the change in dietary behaviour depends on the level of personalisation (level 1: personalised advice based on diet alone; level 2: personalised advice based on diet + phenotype such as lipid profile or similar; level 3: personalised advice based on diet, phenotype and genetics). After a 6-month follow-up, participants randomised to personalised intervention consumed less red meat [-5.48 g, (95% CI, -10.8, -0.09), $p = 0.046$], salt [-0.65 g, (95% CI, -1.1, -0.25), $p = 0.002$] and saturated fat [-1.14 % of energy, (95% CI, -1.6, -0.67), $p < 0.0001$], increased folate [29.6 mg, (95% CI, 0.21, 59.0), $p = 0.048$] intake and had higher Healthy Eating Index scores [1.27, (95% CI, 0.30, 2.25), $p = 0.010$] than those following a general healthy eating guidelines (control group). Addition of phenotype and genotype information did not enhance the effectiveness of the intervention suggesting there may be little benefit of providing genotype-based dietary advice (Celis-Morales et al., 2016). However, longer term effects of the intervention are not known and the message on genetic risk conveyed to participants did not include information on salt sensitivity risk. Additionally, it may not be the most appropriate to elicit behaviour change. Alongside this, the number of genetic variants included in the advice was limited and it did not include any of the salt-sensitivity related genes, highlighting the need for further research. Contrary to these findings, Nielsen and El-Sohemy (2014) suggest that this type of dietary advice may be effective in changing behaviour. The authors reported on reduced sodium intake after 12-month follow-up in participants receiving

a genotype-based dietary advice and carrying the salt sensitivity risk allele of the *ACE* gene, compared to the group receiving only general dietary recommendations with no genetic information (intervention group change in sodium intake: -287.3 ± 114.1 mg vs. control: 129.8 ± 118.2 mg, $p = 0.008$). Those who had the non-risk version of *ACE* did not significantly change their sodium intake compared to the control group (12-months: -244.2 ± 150.2 , $p = 0.11$). Nevertheless, the number of participants in the former group was lower ($n = 27$) than in the *ACE* risk group ($n = 63$) which could have resulted in a false negative finding. However, one of the limitations of this study was the use of the FFQ to measure salt intake which may be more appropriate in a larger epidemiological study and not when a 3- to 12-month change in dietary intake is to be observed.

With the aim of assessing the impact of communicating DNA based disease risk estimates on risk-reducing health behaviours and motivation to engage in such behaviours, Hollands et al. (2016) conducted a meta-analysis reviewing the current research on the topic. Pooled results of seven randomised controlled trials ($n = 1784$) assessing self-reported dietary behaviour showed no significant evidence of a benefit from DNA based risk communication (standardised mean difference 0.12, 95% CI, $-0.00, 0.24$, $p = 0.05$). Considering the date of publication, Food4Me results were not included, however they would probably strengthen the current evidence supporting no benefit of communicating genetic risk.

One of the key limitations of the studies included in this meta-analysis is self-reported measurement of dietary intake. The authors suggest that any additional randomised controlled trials are conducted using methodologically robust designs including valid measures of behaviour. If applying this to dietary salt intake, it would

be suggested that future studies employ multiple 24-hour urinary sodium measurements in addition to dietary methods, to determine food sources of sodium, which may inform a more tailored dietary intervention.

In summary, more research is needed to elucidate complex gene-diet interactions contributing to health outcomes such as hypertension and CVD, including the role of genetic variants in regulating/modifying disease mechanisms (i.e. their functional effects). If this is achieved then it is paramount to conduct more robust studies to determine whether providing nutrigenetic information elicits behaviour change both in short and long term. Such studies should focus on specific behaviour recommendation and, when salt intake is in question, using more accurate methods of measuring salt intake.

1.8. Thesis aims and selection of genetic variants

Increased BP response to a high salt intake (i.e. salt sensitivity) is an independent CVD and mortality risk factor prevalent in both normotensive and hypertensive individuals. If detected in healthy population it may lead to a more successful and effective hypertension and CVD prevention. The key biological mechanism predisposing to salt sensitivity is impaired sodium transport with an evident genetic background. Genetic association studies have identified genes associated with salt sensitivity of BP, however these studies require replication. Moreover, salt sensitivity is a clear example of gene-diet interactions where dietary salt intake plays a major role in development of hypertension. Dietary intake of salt may be influenced by salt taste sensitivity which may share similar genetic

background as salt sensitivity of BP. Since there is a need for a salt sensitivity biomarker that would replace a cumbersome dietary method, the potential of using genetic variants associated with salt sensitivity for salt sensitivity biomarker development will also be explored.

This thesis will comprehensively investigate all the above-described variables aiming to explore (Figure 1.8):

- Whether there is a genetic predisposition to salt sensitivity and, specifically, whether genetic variation in genes coding for sodium transport proteins affects salt-sensitive changes in BP (Chapter 2).
- If there is an association between genetic variations in genes coding for sodium transport proteins and salt taste perception and salt intake (Chapter 3 and 4).
- If there is an association between salt sensitivity of BP, salt taste perception and salt intake and whether these associations depend on genetics (Chapter 3 and 4).
- If genetic variations predisposing to salt sensitivity are affecting the phenotype (protein levels) and if there is a potential for salt sensitivity physiological biomarker discovery (Chapter 5).

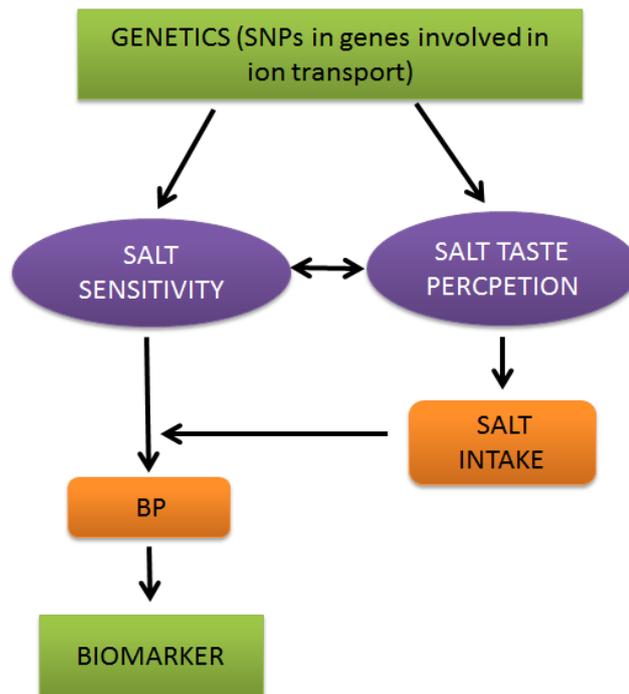


Figure 1.8 Schematic overview of the key variables and outcome measures to be investigated in the thesis. Blood pressure (BP), single nucleotide polymorphism (SNP).

Since the main outcome variables to be explored in this thesis are salt sensitivity of BP and taste, the choice of SNPs to be included in the analysis depended on their previously reported association with any of the two phenotypes and the genetic background of the study population. If these exact phenotypes were not investigated in the context of genetic variants of interest, then similar phenotypes were considered.

Following the extensive literature review, presented in this section and to be presented in introduction sections of the following chapters, four SNPs were selected for genotyping: rs7571842 (A/G) and rs10177833 (A/C) in the *SLC4A5* gene, rs239345 (T/A) in the *SCNN1B* and rs8065080 (T/C) in the *TRPV1* gene (Table 1.3).

The *SLC4A5* gene has been associated with various BP phenotypes to date. The C allele of rs8179526 was associated with higher SBP (Taylor et al., 2013). In Caucasians, rs6731545 and rs7571842 were significantly associated with resting and sub-maximal exercise PP ($0.0004 < p < 0.0007$ and $0.002 < p < 0.003$, respectively). Furthermore, rs6731545 was associated with sub-maximal-exercise SBP and rate pressure product (both $p = 0.002$) (Stütz et al., 2009). Regarding salt sensitivity as a distinct phenotype, Carey et al. (2012) noted that SNPs rs7571842 and rs10177833 had the most pronounced effects on salt sensitivity in a Caucasian population and were also associated with BP in black populations. Therefore, this gene, and specifically the latter two variants, were considered as excellent candidates to be explored in this thesis. In addition, it was suggested in an animal study that the *SLC4A5* may act jointly with ENaC (Wen et al., 2015). It was also reported that the *SCNN1B* (coding for ENaC β -subunit) rs239345 is associated with both hypertension and salt taste perception in humans and the *TRPV1* rs8065080 was strongly associated with salt taste perception (Hannila-Handelberg et al., 2005; Dias et al., 2013). This gene, in animals, has been linked to salt sensitivity of BP (Wang and Wang, 2006; Hao et al., 2011).

Table 1.3 Single nucleotide polymorphisms to be explored in the thesis.

Gene	SNP	MAF	Outcome	Reference
<i>SLC4A5</i> (electrogenic sodium bicarbonate cotransporter)	rs7571842	0.46	Blood pressure, pulse pressure, salt sensitivity (humans); <i>SLC4A5</i> may act jointly with ENaC (animals)	Wen et al. (2015)
	rs10177833	0.44		Carey et al. (2012)
<i>SCNNIB</i> (β -subunit of the ENaC)	rs239345	0.27	Salt taste suprathreshold sensitivity (humans), hypertension (humans)	Stütz et al. (2009)
				Hunt et al. (2006)
<i>TRPV1</i> (transient receptor potential cation channel, subfamily V, member 1)	rs8065080	0.36	Salt taste suprathreshold sensitivity (humans), <i>TRPV1</i> downregulation - salt sensitivity (animals)	Dias et al. (2013)
				Hao et al. (2011)
				Wang and Wang (2006)

Minor allele frequency (MAF), single nucleotide polymorphism (SNP).

Chapter 2 Genetic predisposition to salt sensitivity

2.1. Introduction

Salt sensitivity is defined as an increase in BP in response to a high dietary salt intake (Sullivan, 1991). Considering that some individuals do not exhibit such increase, the distinction is made between salt-sensitive and salt-resistant population (Weinberger, 1996). Salt sensitivity displays a strong heritable component and the genes involved in sodium transport across the cell membrane have shown a strong effect on salt-sensitive changes in BP (Hunt et al., 2006; Yang et al., 2014). As described previously, SNPs rs7571842 and rs10177833 in the *SLC4A5* gene coding for electrogenic sodium bicarbonate cotransporter 2 have been associated with salt sensitivity in Caucasian hypertensive and normotensive population (Carey et al., 2012). Fifty five hypertensive and 130 normotensive Caucasian participants from the UVA discovery cohort and 211 Caucasian hypertensive participants from the replication cohort (HyperPATH) participated in the study and were diagnosed for salt sensitivity based on the dietary method. The participants from the UVA cohort received 10 mmol of sodium/day for seven days on a low-salt diet and 300 mmol of sodium/day on a high-salt diet, for an additional seven days. HyperPATH study participants received 100 mmol of sodium less during a high-salt period and the duration of diets was five to seven days. Twenty-four hour urine samples were obtained on the last day of each diet period and when spot BP measurements were also performed. The two SNPs, rs7571842 and rs10177833 were significant predictors of salt sensitivity. These two SNPs had p values of 1.04×10^{-4} and 3.1×10^{-4} and OR of 0.221 and 0.221, respectively. After adjusting for BMI and age, the associations remained significant ($p = 8.9 \times 10^{-5}$ and 2.55×10^{-4} and OR 0.210 and 0.286, respectively). In a replication, HyperPATH, cohort significant associations

were found for rs7571842 ($p = 0.02$). SNP rs1017783 manifested trends for salt sensitivity that did not reach statistical significance ($p = 0.06$). In addition, the authors conducted a meta-analysis of the two cohorts for salt-sensitivity. As expected, both SNPs demonstrated highly significant associations with increased salt sensitivity (rs7571842 ($p = 1.2 \times 10^{-5}$), rs1017783 ($p = 1.1 \times 10^{-4}$) (Carey et al., 2012).

Moreover, a minor allele of the rs239345 in the *SCNN1B* gene coding for the β sub-unit of ENaC, a salt taste receptor, has been found to be more prevalent in Finish patients with hypertension compared to their normotensive counterparts (Hannila-Handelberg et al., 2005). The *TRPV1* gene coding for an amiloride insensitive part of the salt taste receptor, has been shown to be involved, in addition to salt taste sensitivity (Dias et al., 2013), in salt sensitivity of BP in animal experiments. Wang and Wang (2006) have reported that in Dahl S rats on a high-salt diet, *TRPV1* expression and function is impaired. Male Dahl S and Dahl salt-resistant (Dahl R) rats were fed a low-salt diet or high-salt diet for three weeks. As expected, high-salt diet significantly increased SBP only in salt-sensitive rats. *TRPV1* expression in renal cortex and medulla significantly decreased in the same group, whereas the opposite was observed in the salt-resistant rats fed a high-salt diet. Capsaicin, a *TRPV1* agonist, caused a decrease in BP in all animals, however, the magnitude of decreases induced by capsaicin (10 and 30 $\mu\text{g}/\text{kg}$) was significantly greater in salt-resistant rats on a high-salt diet (14 ± 3 and 27 ± 5 mmHg; $p < 0.05$) compared with salt-resistant rats on a low-salt diet (6 ± 1 and 11 ± 2 mmHg), salt-sensitive rats on a low-salt diet (5 ± 1 and 13 ± 2 mmHg) and salt-sensitive rats on a high-salt diet (8 ± 1 and 16 ± 3 mmHg). The results indicate that *TRPV1* function or expression is enhanced in salt-resistant rats on a high-salt diet but not in salt-sensitive rats fed a high-salt diet. Moreover, the decreases in BP induced by

capsaicin were blocked by capsazepine (3 mg/kg), a selective *TRPV1* antagonist, in all of the rats, indicating the specificity of capsaicin action. The authors concluded that the *TRPV1* receptor is activated and its expression upregulated during high-salt intake in salt-resistant rats, which acts to prevent salt-induced increases in BP. In contrast, *TRPV1* expression and function are impaired in salt-sensitive rats, rendering Dahl S rats sensitive to salt load in terms of BP regulation. In addition, *TRPV1* expression in mesenteric resistant arteries is also decreased in Dahl S rats with high-salt intake (Wang and Wang 2006). Hao et al. (2011) add to these findings reporting that chronic administration of capsaicin reduced the high-salt diet-induced endothelial dysfunction and nocturnal hypertension in male C57BL/6J wild-type mice by preventing the generation of superoxide anions and NO reduction of mesenteric arteries through vascular *TRPV1* activation.

Together with the observed single gene effects, a joint action on BP of the above-described genes has also been reported. Wen et al. (2015) hypothesised that a primary defect in sodium reabsorption in NBCe2-knockout mice could result from enhanced sodium reabsorption by the ENaC, NCC, or the recently described sodium dependent chloride bicarbonate exchanger (NDCBE). In their experiments, wild type (C57B/6) and NBCe2 knockout mice were fed a normal diet until acid loading for seven days. Urinary sodium excretion was significantly decreased and MAP increased in NBCe2 knockout mice compared to wild type. This difference in MAP was eliminated by treatment with amiloride, ENaC antagonist. Contrary to these results, treatment with hydrochlorothiazide, NCC antagonist, decreased the MAP in wild type but not the knockout mice, indicating that the increased activity of ENaC, but not NCC or NDCBE, contributes to the hypertension caused by NBCe2 deficiency. Western blot analysis showed that the quantity of plasmalemmal full-

length ENaC- α was significantly higher in knockout than in wild type mice. Based on the observed results, the authors emphasised the importance of identifying hypertensive patients with *SLC4A5* SNPs together with testing the ENaC function/amiloride effect on the BP of these patients.

The literature clearly indicates the association of genes coding for ion/sodium transport proteins with BP phenotypes. While some of them, such as *SLC4A5*, have been shown to affect salt-sensitive changes in BP in Caucasians, the ENaC and *TRPV1* warrant further research in the context of salt sensitivity as a distinct phenomenon, and the findings of the animal studies should be supported with research in humans. Furthermore, the *TRPV1* rs8065080 is a missense SNP resulting in amino acid change at position 585, from isoleucine to valine, potentially affecting protein function (Ng and Henikoff, 2006). Cantero-Recasens et al. (2010) have tested its functional effect by expressing it in HeLa cells and showed a decreased channel activity in response to two typical TRPV1 stimuli, heat and capsaicin, in TRPV1-Val-585 cells compared to TRPV1-Ile-585. The loss of function effect of the rs8065080, together with reduced expression and activity of the TRPV1 reported in salt-sensitive animals suggests this variant may also be involved in salt sensitivity in humans.

Considering that genetic association studies require replication and that all of the above-described genes have not been explored comprehensively in the context of salt sensitivity in humans, the aim of the present study was to determine if the SNPs in the genes involved in ion (sodium) transport affect BP response to a dietary salt intake manipulation in healthy participants. Specific objectives of this study are:

- To diagnose study participants for salt sensitivity using a “gold-

standard” dietary method.

- To determine the rs7571842, rs10177833, rs239345 and rs8065080 genotype.
- To explore the difference in BP response to a dietary salt intake manipulation between genotype groups of the selected SNPs.

2.2. Methods

2.2.1. Participants

The participants were predominantly young adult Caucasians, eight males and 12 females. Participants were excluded with current stage-2 hypertension (current or recent (less than one month prior to screening visit) use of anti-hypertensive medications or medications that affect BP. Further, those with secondary hypertension, history of CVD, chronic kidney failure, current diabetes were excluded. Also excluded were individuals with peptic ulcer disease or liver disease requiring treatment during the previous two years. In addition, pregnant women, underweight (BMI < 18.5 kg/m²) and obese (BMI > 30 kg/m²) individuals, individuals exceeding maximal recommended alcohol intake (4 units/day for men and 3 units/day for women), those currently adhering to a low sodium diet, or with an illness that permanently alters taste were also excluded from the study. Out of 20 participants, 19 completed the low- and high-salt dietary protocols, however, five participants were excluded due to incomplete 24-hour BP or urinary excretion data (Figure 2.1).

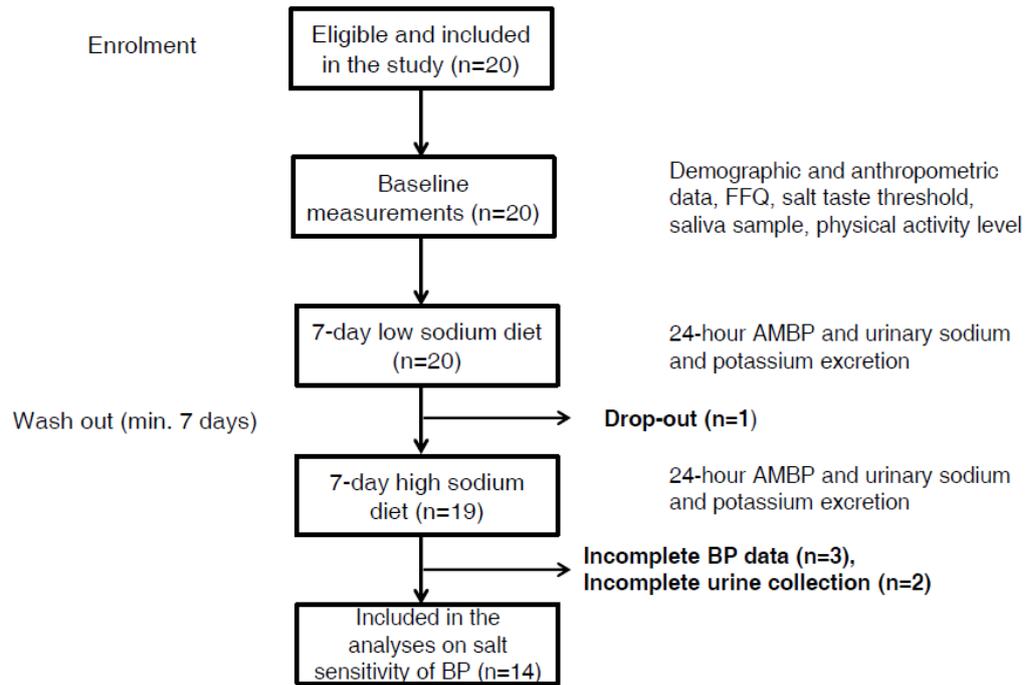


Figure 2.1 Overview of the study procedure. Automated blood pressure monitoring (AMBP), blood pressure (BP), food frequency questionnaire (FFQ).

This study was conducted according to the guidelines stated in the Declaration of Helsinki and all procedures involving human participants were approved by the Institutional Ethics Committee (Appendix A). Written informed consent was obtained from each participant before the baseline data collection informing they can withdraw from the study at any point (Research Registry unique identification number: researchregistry1652).

2.2.2. Baseline measurements

The baseline BP, height and weight were measured during the first examination. Body weight and height were measured with the participant in light indoor clothing without shoes. BMI was calculated according to the following

equation: $BMI = \text{body weight (kg)} / [\text{height (m)}]^2$. Participants were instructed to avoid alcohol, cigarette smoking, coffee/tea, and exercise for at least 30 minutes prior to their BP measurement. Seated BP was measured with an automated BP monitor (OMRON M24/7, Milton Keynes) using an appropriate size cuff after five minutes of rest. Two measurements were performed within five minute intervals and used for the analysis and calculation of the mean baseline SBP and DBP.

2.2.3. Demographic data, smoking habits, physical activity and health status

Demographic data (age, sex and race) was also collected and assessed together with smoking habits and health status information. Physical activity was assessed with the General Practice Physical Activity Questionnaire which is used in routine general practice to provide a simple, 4-level Physical Activity Index reflecting an individual's current physical activity (Department of Health, 2013). Participants were considered as: active, moderately active, moderately inactive or inactive according to the results of the questionnaire.

2.2.4. Dietary sodium intervention

Study participants received a low-sodium diet (3 grams of salt or 51 mmol of sodium/day) for seven days, followed by a high-sodium diet (18 grams of salt or 307 mmol of sodium/day) for an additional seven days. Minimal wash-out period between the diets was seven days. The low-sodium diet was designed by

investigators using nutritional analysis software (Nutritics, Nutritics LTD, Dublin, Ireland). The database used to calculate nutrient content was The Composition of Foods Integrated Dataset which included McCance and Widdowson “The Composition of Foods” (Finglas et al., 2015). Three meals and two snacks were designed to provide a total of 3 grams of salt per day and recommended macronutrient intake (Department of Health, 1991). Total energy intake was determined based on individual requirements of each participant. Participants were provided with written instructions and all food was consumed off site (Appendix B). They were also instructed to maintain their coffee, smoking and physical activity levels. During the high-salt diet, a low-salt diet was supplemented with additional 256.5 mmol of sodium per day (15g of salt/day) dispensed by research staff in small paper sachets each containing 1g salt (NaCl). To monitor participant compliance with the diets, at the last day of each period, 24-hour urine was collected for sodium, potassium and creatinine excretion measurements. During the same period, 24-hour BP measurements were performed with the 24-hour ambulatory BP monitoring device (ABPM). In addition, each participant filled in a three-day estimated food diary on the last three days of each diet to assess intake of nutrients other than sodium and potassium.

2.2.5. Twenty-four-hour automated BP monitoring

Twenty-four-hour ABPM was attached to the upper, non-dominant arm and BP was registered at 30-minute intervals during daytime and 60-minute intervals at night time. Data from the ABPM was downloaded using BP Tracker Software and mean SBP and DBP were calculated. Participant data with less than 30 successful

measurements on each occasion were excluded from the analysis for salt sensitivity (O'Brien et al., 2013). PP was calculated according to the formula: $PP = SBP - DBP$ and MAP as: $MAP = DBP + 1/3 PP$. Salt sensitivity was defined as an increase of ≥ 4 mmHg in MAP between the low- and high-salt diet (de la Sierra et al., 2002; Kurtz et al., 2017). The change in BP between the high-salt and low-salt diet (ΔBP) was calculated as: $\Delta BP = \text{high-salt diet BP} - \text{low-salt diet BP}$.

2.2.6. Biochemical measurements

The 24-hour urinary sodium and potassium were analysed using an automated clinical chemistry analyser (Rx Daytona, Randox laboratories, UK). Estimated salt intake was calculated using the equation $17.1 \text{ mmol of sodium} = 1 \text{ g of salt}$. Assessment of the completeness of the collection was assessed by measuring creatinine levels from the same urine samples. The following criteria were used: 1) incomplete urine = < 0.7 of $(\text{mmol urinary creatinine} \times 113) / (21 \times \text{kilograms of body weight})$ (Murakami et al., 2008), 2) urinary creatinine $< 4 \text{ mmol/day}$ for women, or $< 6 \text{ mmol/day}$ for men, or a 24 h urine collection of $< 500 \text{ ml}$ for either sex and extreme outliers for urinary creatinine (ie, $> 3 \text{ SD}$ from the mean) considered as unacceptable (Land et al., 2014). Participants with incomplete urine collection from any of the dietary intervention periods, based on any of the two criteria, were excluded from the analysis.

2.2.7. Food diaries

To monitor the compliance with the diet and calculate the intake of other nutrients that may affect BP response to dietary salt, participants were asked to keep a 3-day estimated food diary on two occasions. More precisely, on the last three days of both low and high salt dietary intervention periods. Participants were asked to record details of foods and beverages consumed at the time of consumption. Brand names, cooking and preparation methods were provided by participants. Portion sizes were estimated and household measures or natural unit sizes (e.g. slices of bread) were used to determine intake of nutrients other than sodium and potassium, which was calculated using nutritional analysis software (Nutritics, Nutritics LTD, Dublin, Ireland). As stated, the database used to calculate nutrient intake was The Composition of Foods Integrated Dataset which included McCance and Widdowson “The Composition of Foods” (Finglas et al., 2015).

2.2.8. Single nucleotide polymorphism genotyping

At baseline examination a 2 ml saliva sample was collected into a collection vial (SalivaGene collection module II, STRATEC Molecular, Berlin). A stabiliser provided by the manufacturer was added to the saliva sample and it was stored at -20 °C until DNA was extracted. Genomic DNA was extracted using a commercial kit PSP® SalivaGene 17 DNA Kit 1011 (STRATEC Molecular, Berlin, Germany) in accordance with the manufacturer protocol. Quality and quantity were assessed using Nanodrop (ThermoFisher, Waltham, MA, USA). Genotyping was performed using a pre-designed TaqMan® SNP genotyping assays for the SNPs: rs7571842,

rs10177833, rs239345, rs8065080 and the StepOnePlus thermocycler (Applied Biosystems, CA, USA) with two technical replicates for each sample. The primers and the probes were pre-designed by Applied Biosystems with the following codes (C____197439_10, C___1137534_10, C___2387896_30, C__11679656_10). The polymerase chain reaction (PCR) amplification was performed under the conditions specified by the manufacturer. All SNPs had minor allele frequencies higher than or equal to 30% and these reflect the frequencies reported in similar populations (National Institutes of Health, 2017).

2.2.9. Statistical analysis

Sample size calculation was based on the 4 mmHg difference in MAP when changing from low- to high-salt diet, to distinguish between salt-sensitive and salt-resistant individuals in the population. This difference in BP was observed in other studies investigating salt sensitivity in normotensive population and with a 24-hour ABPM (Sharma et al., 1994; de la Sierra et al., 2002). A sample size of 25 was calculated using an alpha of 0.05, confidence intervals of 95% and a standard deviation of 5 mmHg. This standard deviation was chosen due to lower variability of BP reported in younger and healthy individuals (Sharma et al., 1994; Mancia, 2012).

All continuous variables are presented as mean \pm standard error of the mean (SEM) or median (interquartile range). Categorical variables are presented as absolute (relative) frequencies. Before further statistical analysis, continuous variables were tested for normality with the Shapiro-Wilk test. Differences in baseline characteristics by salt sensitivity status and the confounding variables (sex,

age and BMI) according to genotype of interest were assessed using an independent samples t-test (with Levene's test for equality of variance) or Fischer's exact test. The difference between clinical characteristics and food diary data of participants between the low- and high-salt diets was assessed using paired samples t-test. An independent samples t-test (with Levene's test for equality of variance) or Mann-Whitney U test, as appropriate, was used to test for the difference in salt-sensitive changes in BP by genotypes of interest. The model used for the analysis was: major allele homozygous versus heterozygous plus minor allele homozygous. Analyses were performed using the SPSS software package (version 22.0, Chicago, IL, USA). All tests were two-tailed, with $p < 0.05$ considered statistically significant.

2.3. Results

2.3.1. Participant characteristics

Twenty participants completed the baseline examination. Of these, 14 participants provided a complete 24-hour ABPM and 24-hour urine excretion data and were included in the analysis on salt sensitivity of BP. Five participants were diagnosed as salt-sensitive using the criteria of ≥ 4 mmHg increase in MAP from low- to high-salt diet, with prevalence of salt sensitivity being 36%. The study population was normotensive, predominantly white, physically active and non-smoking with a median age of 28 years. There was no difference in any of the baseline variables between salt-sensitive and salt-resistant participants (Table 2.1).

Table 2.1 Baseline characteristics of study participants, total sample (n = 20) and according to salt sensitivity status (n = 14). Data presented as mean ± SEM or absolute (relative) frequencies. P value for difference between salt-sensitive and salt-resistant participants (Independent samples t-test, Fischer's exact test).

	Total (n = 20)	Salt-sensitive (n = 5)	Salt-resistant (n = 9)	P
Age (years)	28.0 (10.5) ^{a)}	35.8 ± 4.6	33.2 ± 2.7	0.612
Sex				
Male	8 (40)	2 (40)	2 (22)	0.580
Female	12 (60)	3 (60)	7 (78)	
Race				
White	16 (80)	4 (80)	6 (67)	0.999
Other	4 (20)	1 (20)	3 (33)	
BMI (kg/m²)	23.9 ± 0.7	24.7 ± 1.9	23.7 ± 0.7	0.633
SBP (mmHg)	121.3 ± 3.0	125.8 ± 9.2	118.2 ± 4.4	0.413
DBP (mmHg)	70.4 ± 2.1	71.9 ± 6.3	71.2 ± 2.9	0.913
Smoking status				
Yes	1 (5)	1 (20)	0	0.357
No	19 (95)	4 (80)	9 (100)	
Physical activity level				
Active	15 (75)	2 (40)	7 (78)	0.413
Moderately active	1 (5)	1 (20)	0	
Moderately inactive	2 (10)	1 (20)	1 (11)	
Inactive	2 (10)	1 (20)	1 (11)	

a), median (interquartile range); body mass index (BMI), diastolic blood pressure (DBP), systolic blood pressure (SBP)

2.3.2. Compliance with the diet

There was no difference in BP between the low-salt and high-salt dietary periods (Table 2.2). Urinary sodium excretion results demonstrated good compliance with the diet ($p = 3.3 \times 10^{-7}$). Potassium intake remained similar on both diets ($p = 0.243$).

Table 2.2 Clinical characteristics of study participants (n = 14) on low- and high-salt diet (mean \pm SEM, Paired samples t-test).

	Low-salt diet	High-salt diet	p
SBP (mmHg)	113.6 \pm 2.7	115.8 \pm 3.0	0.107
DBP (mmHg)	66.9 \pm 1.4	68.6 \pm 2.2	0.261
MAP (mmHg)	82.5 \pm 1.6	84.4 \pm 2.4	0.170
PP (mmHg)	46.7 \pm 2.2	47.2 \pm 1.8	0.656
Urine sodium excretion (mmol/24 hour)	66.1 \pm 8.9	281.5 \pm 24.4	3.3 x 10 ⁻⁷
Urine potassium excretion (mmol/24 hour)	75.8 \pm 5.5	81.8 \pm 5.8	0.243

Diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP), systolic blood pressure (SBP)

Considering the role that, besides sodium and potassium, other dietary factors play in salt sensitivity, the difference in intakes between low- and high-salt diets was tested. According to the results shown in Table 2.3, magnesium, alcohol, and caffeine intake remained unchanged during both dietary periods. Energy (2225 \pm 185 vs. 1781 \pm 130 kcal, p = 0.021), saturated fat (14 \pm 1 vs. 11 \pm 1 %TEI, p = 0.004), protein (96 \pm 7 vs. 74 \pm 6 g, p = 0.023) and calcium intake (988 \pm 85 vs. 649 \pm 67 mg, p = 0.004) were higher on the high-salt diet. However, when the intakes of protein, calcium, magnesium and fibre were energy adjusted the difference between the diets remained significant only for calcium (p = 0.018, Table 2.4).

Table 2.3 Difference in mean \pm SEM dietary intake of selected nutrients, caffeine and alcohol (assessed with a 3-day food diary), between low and high salt diet (n = 14, Paired samples t-test, Wilcoxon signed rank test).

	Low-salt diet	High-salt diet	p
Energy (kcal)	1781 \pm 130	2225 \pm 185	0.021
Total fat (%TEI)	34 \pm 2	38 \pm 2	0.066
SFA (%TEI)	11 \pm 1	14 \pm 1	0.004
MUFA (%TEI)	12 \pm 1	14 \pm 1	0.097
PUFA (%TEI)	6 \pm 1	6 \pm 1	0.572
Protein (g)	74 \pm 6	96 \pm 7	0.023
Carbohydrate (%TEI)	40 \pm 2	38 \pm 2	0.345
Free sugar (%TEI)	8 \pm 1	6 \pm 1	0.245
Fibre (g)	20 \pm 2	22 \pm 2	0.219
Calcium (mg)	649 \pm 67	988 \pm	0.004
Magnesium (mg)	323 \pm 30	362 \pm 23	0.139
Alcohol (g)	16 \pm 3	13 \pm 4	0.267
Caffeine (mg)^a	47 (96)	76 (100)	0.638

a, median (interquartile range); monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), saturated fatty acids (SFA), total energy intake (TEI).

Table 2.4 Difference in mean \pm SEM dietary intake of selected nutrients (energy adjusted), caffeine and alcohol (assessed with a 3-day food diary), between low and high salt diet (n = 14, Paired samples t-test)

	Low-salt diet	High-salt diet	p
Protein (g/1000 kcal)	42 \pm 3	44 \pm 2	0.450
Calcium (mg/1000 kcal)	360 \pm 25	461 \pm 38	0.018
Magnesium (mg/1000 kcal)	182 \pm 11	172 \pm 14	0.348
Fibre (g/1000 kcal)	11 \pm 1	10 \pm 1	0.298

2.3.3. SNP genotyping results

Observed genotype and allele frequencies with respective allelic discrimination plots are presented in Figure 2.2 and Table 2.5. From the table, it can be observed that the genotype and allele frequencies reflect the ones expected in similar populations (National Institutes of Health (NIH), 2017).

Table 2.5 Observed genotype and allele frequencies for the SNPs in the *SLC4A5* (rs7571842, rs10177833), *SCNN1B* (rs239345) and *TRPV1* (rs8065080) genes in the study population (n=20).

	Genotype	Observed Number (%)	Allele frequency	
rs7571842	A/A	7 (35)	A	G
	A/G	8 (40)	0.55	0.45
	G/G	5 (25)		
rs10177833	A/A	8 (40)	A	C
	A/C	8 (40)	0.60	0.40
	C/C	4 (20)		
rs239345	T/T	11 (55)	T	A
	T/A	6 (30)	0.70	0.30
	A/A	3 (15)		
rs8065080	T/T	10 (50)	T	C
	T/C	7 (35)	0.68	0.33
	C/C	3 (15)		

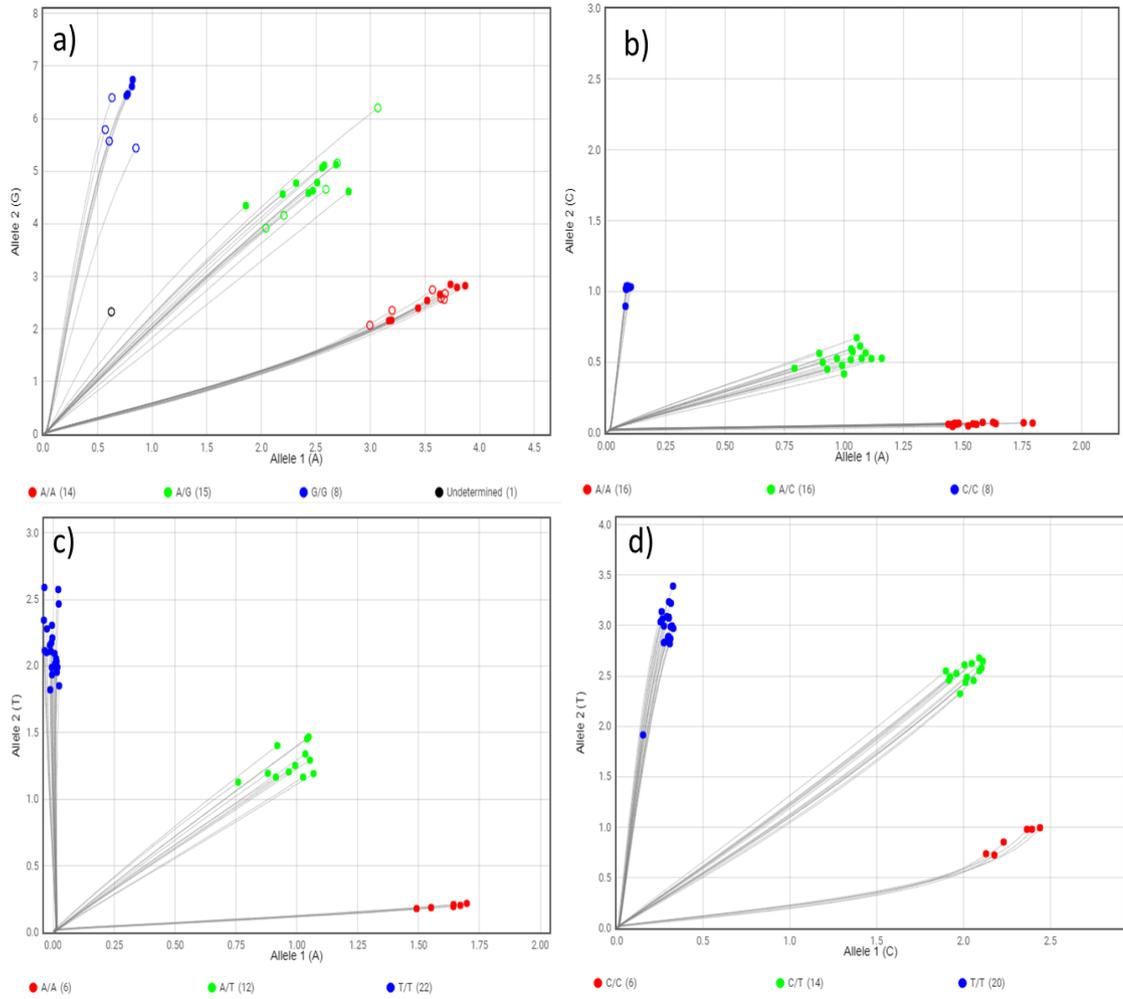


Figure 2.2 Allelic discrimination plots for the SNPs in the *SLC4A5* a) rs7571842, b) rs10177833, *SCNN1B* c) rs239345 and *TRPV1* d) rs8065080 genes. Plots are demonstrating the separation of the three genotypes, based on the intensity of the reporter dye (VIC, FAM) fluorescence.

2.3.4. The effect of genetics on salt-sensitive changes in BP

The mean change in BP between the low- to high-salt diets differed according to rs7571842 genotype groups. The participants with AA genotype had the highest increase in BP (Δ SBP = 7.75 mmHg, $p = 0.002$; Δ DBP = 6.25 mmHg, $p = 0.044$; Δ MAP = 6.5 mmHg, $p = 0.014$). SNPs rs10177833, rs239345 and rs8065080 did not have a statistically significant effect on the BP response to dietary sodium

manipulation (Figures 2.3 – 2.6). There was no difference in Δ PP between the genotype groups for any of the SNPs (Table 2.6).

Table 2.6. Mean pulse pressure change (mmHg) \pm SEM following dietary sodium intervention according to genotypes of interest (n= 14, Independent samples t-test)

	Major allele homozygous	Heterozygous + minor allele homozygous	p
rs7571842	1.50 \pm 2.40 (n = 4)	0.10 \pm 1.27 (n = 10)	0.585
rs10177833	0.33 \pm 1.97 (n = 6)	0.63 \pm 1.35 (n = 8)	0.902
rs239345	-0.43 \pm 1.27 (n = 7)	1.43 \pm 1.82 (n = 7)	0.420
rs8065080	1.00 \pm 1.53 (n = 8)	-0.17 \pm 1.67 (n = 6)	0.619

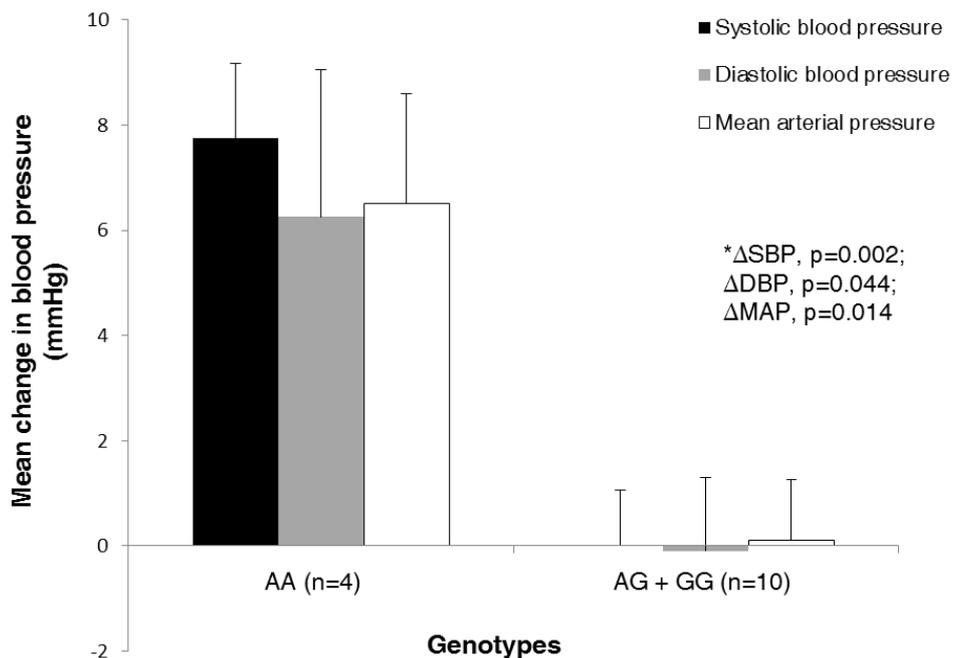


Figure 2.3 Systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) change from low- to high-salt diet according to *SLC4A5* rs7571842 genotype (n = 14). Error bars represent + SEM. (Independent samples t-test, *Mann-Whitney U test).

Considering a low number of participants in each genotype group and that the *a priori* calculated sample size was not achieved, *post-hoc* power calculation for the tests that revealed significant differences in BP was conducted. It showed that, with the two-tailed 0.05 significance level, this test had a power of 92% to detect a difference in SBP between the two *SLC4A5* rs7571842 genotype groups (mean values for Δ SBP 7.75 mmHg vs. 0.00 mmHg and standard deviations 2.87 mmHg vs. 1.06 mmHg). The test of difference for the DBP and MAP did not reach sufficient power.

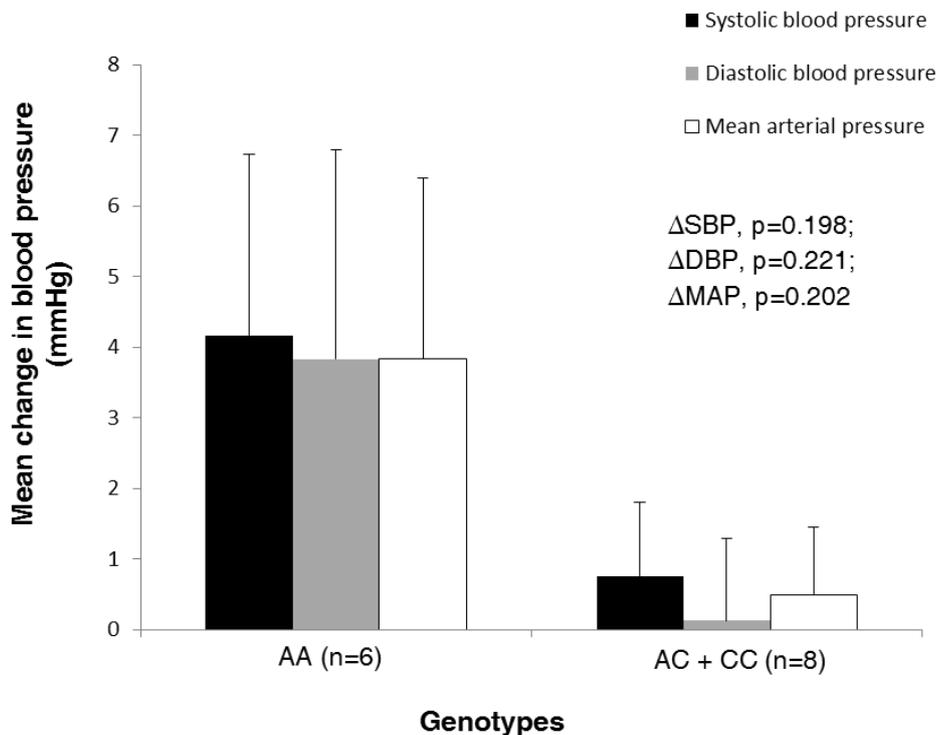


Figure 2.4 Systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) change from low- to high-salt diet according to *SLC4A5* rs10177833 genotype ($n = 14$). Error bars represent + SEM. (Independent samples t-test).

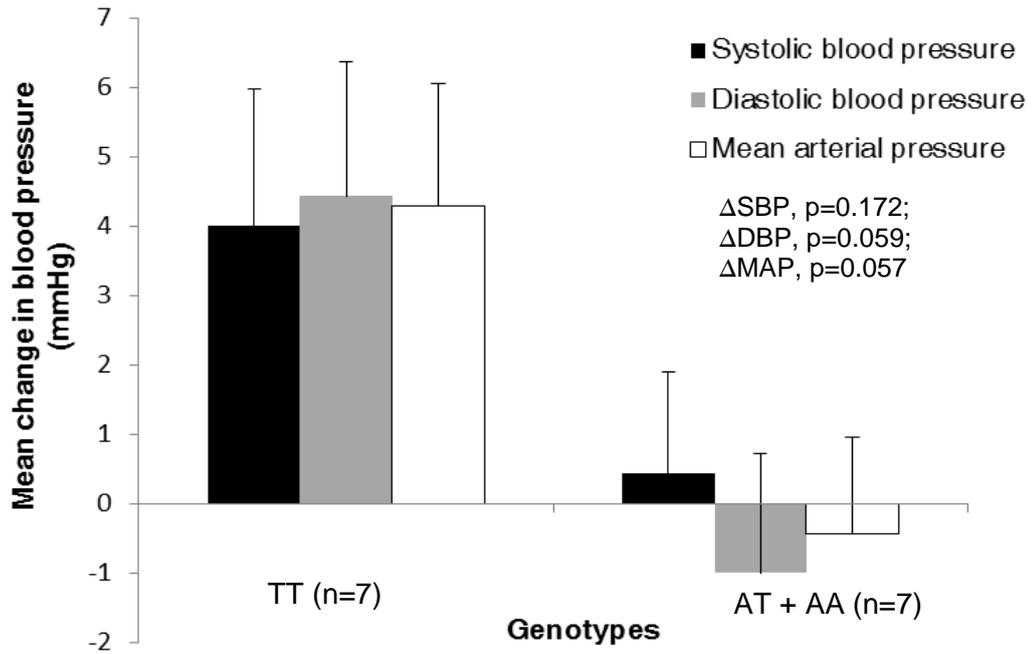


Figure 2.5 Systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) change from low- to high-salt diet according to *SCNN1B* rs239345 genotype (n = 14). Error bars represent + SEM. (Independent samples t-test).

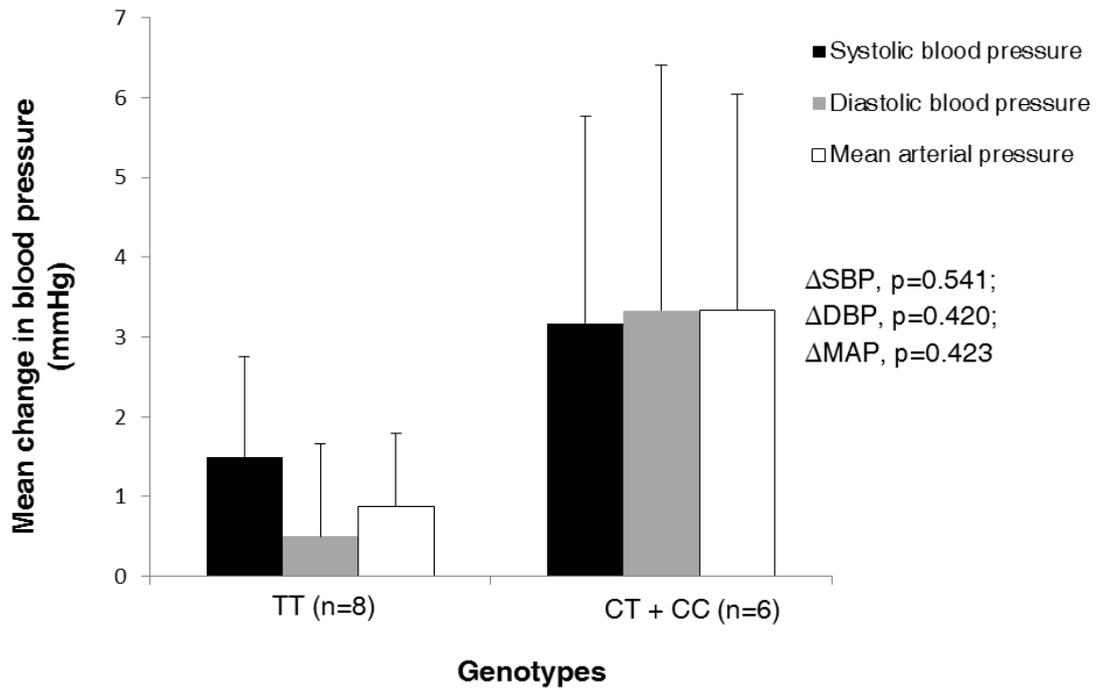


Figure 2.6 Systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) change from low- to high-salt diet according to *TRPV1* rs8065080 genotype (n = 14). Error bars represent + SEM. (Independent samples t-test).

2.4. Discussion

The aim of this study was to investigate the genetic predisposition to salt sensitivity of BP in healthy, adult participants. The study focused on genes involved in sodium transport. The results suggest a genetic predisposition to salt sensitivity in the study population with the *SLC4A5* rs7571842 affecting SBP response to a dietary salt intake manipulation. Other genes explored in this study do not appear to play a role in salt sensitivity of BP. These results confirm the results of a study by Carey et al. (2012) and are the first to report a genetic predisposition to salt sensitivity in healthy UK participants. Considering the small sample size, they should be considered as preliminary and should, therefore, be confirmed in a larger sample size study.

2.4.1. Adherence to dietary intervention

The dietary protocol used in this study is suggested as the method of choice by the AHA. The AHA suggests the period of the low-salt diet to be 50 mmol of sodium/day and the high-salt diet approximately 250 mmol of sodium/day (Elijovich et al., 2016). The present study was designed to achieve intakes of 51 mmol sodium/day on a low-salt diet and 308 mmol of sodium/day on a high-salt diet. Compliance with the diet was assessed by measuring urinary sodium and potassium excretion. In a study by Gu et al. (2013), where participants were tested for salt sensitivity using the same dietary method and sodium concentrations as in the present study, the mean urinary excretion of sodium on a low-salt diet was 54.1 mmol/24 hour and on a high-salt diet 248.6 mmol/24 hour respectively, making the

difference in sodium excretion between the two dietary periods approximately 200 mmol/24 hour. In the present study the mean urinary excretion of sodium on a low-salt diet was 66.1 mmol/24 hour and on a high-salt diet 281.5 mmol/24 hour making the difference in this study even higher which may indicate good compliance with the diets when sodium and potassium intakes are considered. Nevertheless, the results of food diaries administered on the last three days of each intervention period revealed that the energy intake on a high-salt diet was higher compared to the low-salt diet. Participants may have increased their overall energy intake to achieve required sodium intake on a high-salt diet (since sodium intake is associated with energy intake) instead of merely adding the provided amount of salt to the same food they consumed during the low-salt period. Results indicate that this may be due to increased fat, specifically saturated fat, and protein intake which may have affected BP during dietary intervention. This further indicates participants did not fully comply with the written instructions provided throughout each of the two periods.

As stated in the introduction, arginine supplementation may lower salt-sensitive rise in BP (Campese et al., 1997), however research on this specific topic is scarce and is therefore difficult to draw any definitive conclusions about possible effects of protein intake in this study population. If indeed, intake of arginine rich foods affects salt-sensitive changes in BP, higher protein intake on the high-salt diet would have resulted in a lower BP during this dietary period, which may explain the lack of significant difference between BP on a low- and high-salt diet in the total study population. Similar can be concluded for calcium intake, which was also higher during the high- compared to the low-salt period. Weinberger et al. (1993) reported that 600 mg of calcium supplementation attenuated a high-salt induced rise in BP in salt-sensitive individuals. If this was the case in this study population, then

similar to protein intake, it may have resulted in a lower than expected BP on a high-salt diet.

Regarding increased fat intake on the high-salt diet, which may partly explain the difference in energy intake, no research in humans has been conducted that would suggest its effects on salt-sensitive increase in BP. Animal studies show that the combination of high-fat and high-salt diet in rats genetically predisposed to hypertension increases their BP to a further extent than a high-salt diet alone (Nagae et al., 2009). However, the relevance of these findings in humans is questionable and is therefore difficult to draw any conclusions about the possible effects of increased fat intake during a high-salt period.

Overall, satisfactory intakes of sodium and potassium but lack of adherence to suggested intakes of macronutrients such as fat and protein indicate that providing only instructions on diets may compromise the study results. Even though allowing participants to source and prepare their own food may present lower burden, future studies should aim at providing the main meals which would be consumed at the research centre.

2.4.2. Salt sensitivity prevalence

The prevalence of salt sensitivity in this study was 36%. Considering the discrepancies in methods used in previous studies, it is difficult to draw any conclusion whether this prevalence could be expected in other populations with similar characteristics. Nevertheless, the rs7571842 allele frequencies, SNP with the effect on salt sensitivity in this and a previous study (Carey et al., 2012), reflect the

frequencies reported in European and specifically British populations (NIH, 2017). According to the 1000 Genomes project data, the prevalence of the AA genotype is 34%, similar to salt sensitivity prevalence observed in this study. Furthermore, salt sensitivity prevalence of 26% in normotensives was established using an intravenous protocol for diagnosis of salt sensitivity (Weinberger et al., 1986). However, more recent work suggests that this method can lead to misclassification and incorrect diagnosis (Sharma et al., 1994; de la Sierra et al., 2002). Conversely, in this study, salt sensitivity was determined with the “gold standard” protocol, which is reproducible, unlike other methods (Gu et al., 2013).

Another potential issue in comparison of different study results is the BP measurement. While most studies still use the conventional measurements, from the studies that employ 24-hour BP measurements together with the dietary method, only a limited number, is investigating salt sensitivity solely in healthy, normotensive population (Damasceno et al., 2000; de la Sierra et al., 2002; Brian et al., 2017). The criterion used to define salt sensitivity was an increase in MAP on the high-salt diet of at least 4 mmHg compared to the MAP on the low-salt diet. This cut-off was used by other researchers employing a 24-hour BP measurement in their studies (de la Sierra et al., 2002). Moreover, this is in line with the recommendations summarised in a recent review by Kurtz et al. (2017). The author suggests that when testing normotensive participants with the proposed dietary protocol and the physiological levels of salt intake already described, the cut-off for classifying someone with salt sensitivity should be considered as a change in MAP of at least 3 to 5 mmHg in response to the change in salt intake. Regarding the potential effects of this type of BP measurement, there is a decreasing trend in BP from the first to the second measurement period when ambulatory measurement techniques are applied.

This may also partly explain non-significant difference in BP (2.2/1.7 mmHg SBP/DBP) when transitioning from low- to high-salt diet in the total study population. To account for this effect, multiple 24-hour BP measurements may be employed throughout the intervention period together with randomisation of order of the diets.

When discussing salt sensitivity prevalence, the results show no difference in prevalence between Caucasian participants and participants of other race. Among four participants in the category “other”, three were of African origin. Considering the literature presented in Chapter 1, it may be expected that the prevalence of salt sensitivity would be higher in this group. Nevertheless, this study population was healthy and had normal BP whereas the majority of studies suggesting higher salt sensitivity prevalence in black populations were conducted in individuals with high BP. Considering low number of participants, stratification according to race and genotype could not be performed. If this was the case, then we may have observed higher BP response to sodium loading in individuals of African origin, a finding that was more commonly reported than the actual higher salt sensitivity prevalence. In this sense, there indeed may not be difference in prevalence of salt sensitivity between black and white populations when considering healthy, normotensive people. Conversely, a larger sample size compared to the one of this study may be needed to detect this difference. Similar can be concluded for sex differences reported previously. Considering low participant number and the age of the population, a larger sample may be needed to detect any difference.

Finally, the choice of the method employed in this study may not be the most appropriate to determine salt sensitivity prevalence. It should be noted that this study

primarily investigated the effects of sodium loading on BP and as such, the above-described salt sensitivity prevalence should be regarded with caution. When identifying participants as salt-sensitive or salt-resistant it is recommended that the low- and high-salt diet should be administered in a random order to achieve maximal reproducibility (Kurtz et al., 2017). When a low sodium period precedes high sodium period, RAAS may not be uniformly suppressed (Elijovich et al., 2016). This may result in an increased BP response on a low-salt diet and would require larger sample size compared to the one in this study to detect the true effect of dietary sodium manipulation on BP and estimate the salt sensitivity prevalence. Therefore, if the order of the diets was randomised and high-salt diet preceded the low-salt diet in a proportion of the study population, the RAAS may have been suppressed to an extent where more uniformity in the BP response to dietary intervention may have been observed. This in turn, may have resulted in a statistically significant difference in BP when transitioning from the low- to the high-salt diet in the total study population.

2.4.3. Genetic predisposition to salt sensitivity

The results suggest a genetic predisposition to salt sensitivity in the study population. The genetic variants explored in this study were the variants in genes involved in sodium transport since impaired sodium transport appears to be one of the most prominent mechanisms associated with salt sensitivity of BP (Ando and Fujita, 2012).

In this study, the *SLC4A5* gene, coding for a sodium hydrogen bicarbonate

transporter, was confirmed as the gene affecting salt-sensitive changes in BP. Carey et al. (2012) noted that SNPs rs7571842 and rs10177833 had the most pronounced effects on salt sensitivity in Caucasian population. In the present study individuals with AA genotype of the rs7571842 exhibited SBP increase of 7.75 mmHg after receiving a high-salt diet for seven days. In comparison, there was no change in SBP after high-salt diet in participants with AG + GG genotype. This confirms the findings of Carey et al. (2012) and the potential BP increasing effect of the A allele. Considering the statistical tests used to test for the difference in BP between the two diets according to genotypes of interest, the control for covariates could not be performed. However, the analysis was conducted to test for the possible difference in the prevalence of males and females, BMI and age, between the genotype groups. There was no difference in any of the variables between the AA and AG + GG group ($p = 1.000$, $p = 0.846$ and $p = 0.584$ for sex, BMI and age respectively).

The importance of this result lies in the allele and genotype frequencies for this SNP. The A allele is according to the HapMap, and, more recent, 1000 Genomes Project data present in approximately half of the European descent population with approximately a third of the population having the risky AA genotype (NIH, 2017). Additionally, salt-sensitive rise in BP, following a high-salt diet, was expressed as a continuous variable in this study. As discussed, the risk of CVD increases continuously and with each 2 mmHg increase in SBP there is a 7% increase in risk of mortality from IHD and a 10% increase in the risk of mortality from stroke (NICE, 2011). The increase in SBP in healthy participants with the rs7571842 AA genotype was 7.75 mmHg, which emphasises the clinical relevance of these results. Moreover, it has been estimated that approximately a third of deaths attributed to BP occur in individuals with BP lower than the hypertensive range (Appel, 2017). They

may represent a salt-sensitive part of the population.

The rs7571842 is in strong linkage disequilibrium (LD) with another SNP explored in this study, rs10177833 (Broad Institute, 2015) also reported by Carey et al. (2012). In the latter study, LD was greater in salt-resistant than salt-sensitive participants ($r^2=0.93$ and 0.61 respectively). In the present study, r^2 was 0.68 in the total study population. Due to the low number of participants diagnosed as salt-sensitive, LD was not calculated according to salt sensitivity status, however it reflects the results of Carey et al. (2012).

Other *SLC4A5* SNP, rs10177833, had no effect on salt-sensitive changes in BP in the present study. These results align with Carey et al. (2012) where the effect of rs10177833 on salt sensitivity observed in the UVA discovery cohort was not replicated in a HyperPATH study population. The HyperPATH study protocol differed from the one in the UVA cohort regarding the diagnosis of salt sensitivity and this may have also affected the results. The dietary protocol in a HyperPATH study was outpatient with participants receiving 10 mmol sodium/day during a low-salt dietary period and 200 mmol of sodium on a high-salt diet. Duration of diets was five to seven days and the authors reported how all participants had a urine sodium excretion ≥ 150 mmol/24 hours on the high-salt and ≤ 30 mmol/24 hours on the low-salt diet. The cut-off used for salt sensitivity diagnosis was an increase in MAP of at least 7 mmHg on a high- compared to the low-salt diet. In comparison with this protocol, the UVA cohort received 10 mmol of sodium on a low-salt and 300 mmol of sodium on a high-salt diet, with duration of both dietary protocols being seven days. Each meal was prepared and consumed in the Clinical Research Centre. The BP cut-off to diagnose for salt sensitivity was the same as in the former population.

Considering the differences in dietary protocols between the two cohorts, our inability to confirm the effect of rs10177833 may also be explained by the differences in dietary protocols used in this thesis and by Carey et al. (2102). Where the protocol is less demanding, smaller SNP effects may not be observed.

Besides the *SLC4A5*, the *SCNN1B* gene, coding for the β subunit of the ENaC has been associated with salt sensitivity in Chinese population (Zhao et al., 2011) and hypertension in Finnish patients (Hannila-Handelberg et al., 2005). The present study aimed at exploring these associations in the predominantly white population and in the context of salt sensitivity as a distinct phenomenon. The SNP rs239345 did not have an effect on salt-sensitive changes in study participants. The Δ SBP was 4 mmHg in the participants with TT genotype and 0.43 mmHg in participants with AT or AA genotype. Similar was observed for DBP and MAP. These findings, however, cannot be compared to the findings of Zhao et al. (2011) since the study population in the GenSalt study was Chinese suggesting a distinct genetic background. Regarding the effects of the rs239345 in Finnish participants, the prevalence of hypertension was higher in participants carrying the minor allele of this SNP (Hannila-Handelberg et al., 2005). The population in this study, however, was healthy and normotensive and the final outcome measure was salt sensitivity of BP, not hypertension *per se* making it, again, difficult to draw any comparisons between the studies.

The final SNP investigated in the current study was rs8065080 in the *TRPV1* gene. This gene codes for the receptor that may be involved in salt taste perception (Dias et al., 2013). Impaired *TRPV1* expression and activity has also been associated with salt sensitivity of BP in animal studies (Wang and Wang, 2006; Hao et al.,

2011), however there are no specific genetic variants in this gene reported to be associated with this phenotype. Nevertheless, the rs8065080 appears to be functional and is associated with lower channel activity, a trait observed in salt-sensitive rats (Wang and Wang 2006; Cantero-Recasens et al., 2010). In this population, it did not have an effect on salt-sensitive changes in BP, suggesting that other variants in this gene may have more pronounced effects on BP. Nevertheless Δ BP in the minor allele carriers was ~ 3 mmHg whereas it was ~ 1 mmHg in those homozygous for the major allele.

2.4.4. Potential for further exploration of the rs7571842

The *SLC4A5*, however, stands out as the gene with potentially detrimental effects on salt sensitivity. As described previously, it codes for the electrogenic sodium bicarbonate cotransporter which has, in humans, first been characterized in the heart (Pushkin et al., 2000). Regarding its role in the kidney, Carey et al. (2012) have been first to suggest its involvement in salt sensitivity of BP, most likely through increased sodium reabsorption in the nephron. The authors also state that even if *SLC4A5* plays a minor role in sodium balance, a decrease of only 0.1% in sodium excretion can in the long run lead to hypertension. As an example they state how a human excretes 1% of filtered sodium (~250 mmol/day). A decrease in sodium excretion of only 0.1% leads to sodium retention of 25 mmol/day or 250 mmol in 10 days and this cumulative effect can be substantial. In addition, impaired action of this cotransporter may lead to increased sodium reabsorption by other sodium bicarbonate cotransporters as it has been reported that the deletion of *SLC4A5* initiates compensatory bicarbonate reabsorption via other sodium-

bicarbonate transporters at the expense of increased tubule sodium uptake (Gröger et al., 2012). From the above-described it is evident that the role *SLC4A5* has in salt sensitivity warrants further investigation and one of the first steps should be exploring if the genetic variants associated with salt sensitivity affect gene expression or activity of this protein. If this is the case, then these variants may hold potential for development of new salt sensitivity diagnostic tools.

In this context, the rs7571842 is a non-coding SNP which may affect protein expression by having an effect on the transcription factor-binding site of the gene which can affect the level, location, or timing of gene expression. In addition, a SNP at the 'splicing site' may break the consensus splicing site sequence resulting in a different form of a protein (Yuan et al., 2006). Based on these facts and the findings of this study, it is warranted that this SNP is further explored regarding its potential for salt sensitivity biomarker development. Literature on the *SLC4A5* SNP functional effects and its biomarker potential will be discussed in Chapter 5.

2.4.5. Strengths and limitations

This study has several limitations. Firstly, even though the method to diagnose salt sensitivity is considered as the method of choice by other researchers and the AHA, participants were not provided with all the food and it was not consumed in the research centre. Instead, participants were provided with written instructions and dietary intake of sodium and potassium was monitored by collecting a 24-hour urine sample at the end of each intervention period. Intake of other nutrients was monitored with the food diaries collected during the last three days of

each diet. While urinary markers showed good compliance with the diet in terms of sodium and potassium intake, participants did not adhere to the instructions and have increased their energy intake by increasing the amount of fat and protein consumed on a high-salt diet. Even though it appears that this would potentially result in a smaller effect of the dietary intervention on BP than expected, it also suggests that the method may not be appropriate and the food should be provided and consumed in the research centre to achieve maximal compliance. This, in turn, would, at least to an extent, eliminate the need to monitor dietary intake by the means of estimated food diaries which are prone to reporting errors. This is especially relevant for studies that monitor salt intake as it highly correlated with, frequently misreported, energy intake (McLean, 2014). A more accurate dietary method would be the use of weighed food diaries, however due to high participant burden these were not considered in the present study. Eljovich et al. (2016) support the above described by suggesting that components of the diet other than sodium must be controlled due their effects on BP. Considering the nature of dietary methods which makes them highly prone to error, the use of biomarkers to estimate intakes of nutrients associated with BP, such as urinary nitrogen to estimate protein intake, should be considered (Bingham, 2003).

Furthermore, except for the difference in SBP between high- and low-salt diet in the rs7571842 genotype group, the study was underpowered. Even though salt-sensitive increase in BP was detected and other studies with similar sample sizes, 14-16 participants respectively, have successfully investigated and detected this phenomenon in normotensive population (Luft et al., 1979; Scuteri et al., 2003; Nichols et al., 2012), the genetics aspect was not taken into account in sample size calculations. Consequently, the number of participants in each genotype group may

be low. This should have been accounted for in participant recruitment stage, by taking into consideration the frequency of the effect allele. In this case, a number of participants three times than the one determined in the methods section should have been screened for the SNPs in *SLC4A5*, *SCNN1B* and *TRPV1*, prior to their inclusion in the study, to be able to detect true effects of genetics on salt-sensitive increase in BP (Grimaldi et al., 2017; NIH, 2017).

A strength of the study is the use of 24-hour ABPM procedure to determine the difference in BP between the diets. This method provides many more measurements than conventional BP measurement reflecting usual BP more accurately. It also allows identification of individuals with a “white coat” response or masked hypertension, and is a stronger predictor of cardiovascular morbidity and mortality than conventional measurement (O’Brien et al., 2013). The AHA suggests it as the method of choice when researching salt sensitivity of BP (Elijovich et al., 2016).

2.5. Conclusion

This preliminary data suggests there is a genetic predisposition to salt sensitivity in the study population. The *SLC4A5* rs7571842 was identified as the variant that may exhibit effect on salt-sensitive changes in SBP. Other genetic variants explored in this study may have smaller effect sizes which may be detected in a larger sample size study, however in the present study they were not associated with this distinct phenotype. The role *SLC4A5* has in salt sensitivity should be further explored starting with the possible effects of the rs7571842 on its protein expression. This may provide potential for development of a personalised salt sensitivity diagnostic assay. Finally, as increased dietary salt intake is necessary for development of this phenotype, it should be explored together with its main determinant - taste perception. These relationships will be discussed in Chapters 3 and 4.

Considering a number of limitations to this study it suggested that future studies take genetics into account when calculating required sample size. Achieving the adequate number of participants in each genotype group may be facilitated by prospectively recruiting participants based on their genetic profile. This may also serve as a confirmation of the results of the present study, similar to the two studies that explored the response to dietary interventions based on the *APOE* and *MTHFR* genotypes, discussed in Chapter 1. The method to diagnose salt sensitivity should be a dietary method used in this study, however the order of diets should be randomised and all food prepared and consumed in the research centre. Multiple 24-hour urine collections and BP measurements should be performed to achieve the maximal accuracy of the results and to determine compliance with the diet. Besides sodium

and potassium, other biomarkers of dietary intake should be measured, such as urinary nitrogen.

Chapter 3 The associations between salt sensitivity, salt taste sensitivity and salt intake

3.1 Introduction

High dietary salt intake is a major risk factor for hypertension (He and MacGregor, 2004; He et al., 2013) estimated to be responsible for one in 10 deaths from CVD events (Mozaffarian et al., 2014). In 2010, the estimated mean global salt consumption was 9.9 g/day, with regional mean levels ranging from 5.45 g to 13.7 g/day, exceeding the WHO reference intake of 5 g salt/day (Mozaffarian et al., 2014). In the UK, salt intake varies widely across the population, ranging from 0.8 to 24.2 g/day (Department of Health, 2016).

One of the main determinants of food intake, and most likely salt, is taste. The ability to perceive a certain taste may be genetically determined (Reed et al., 2006) and it may explain the variability in sodium intakes within and across populations. To our knowledge, only one study reports on the genetic predisposition to salt taste in humans. SNPs in genes coding for ion channels, the ENaC and the TRPV1, were associated with modified salt taste perception in 95 white participants (Dias et al., 2013). In brief, this was a cross-sectional study conducted in 28 male and 67 female Caucasians, between ages 21 and 31 years. The participants were genotyped for SNPs in the *SCNNIA*, *SCNNIB*, *SCNNIG* and *SCNNID* genes coding for the subunits of the ENaC and the TRPV1. They were also tested for their detection thresholds using a 3-alternative forced-choice up-down method. Test solutions were 0.25 log cycles apart in concentration ranging from 9×10^{-6} to 0.5 mol/l of sodium chloride in distilled water. Suprathreshold taste sensitivity to salt was also tested using a general labelled magnitudes scales (Bartoshuk et al., 2004). The concentrations of the solutions, 0.5 log cycles apart, ranged from 0.01 to 1.0 mol/l sodium chloride. Recognition threshold was not tested in this study. The

results of the study showed no association between SNPs in the *SCNN1A* and *SCNN1G* genes and salt taste thresholds. Individuals homozygous for the A allele of rs239345 in the *SCNN1B* gene had lower suprathreshold salt taste sensitivity incremental area under the curve (iAUC) (mean \pm SE) (70.82 ± 12.21) than those with either AT (93.81 ± 6.10) or TT (98.26 ± 4.79) genotype ($p = 0.05$). When the carriers of the T allele were combined to form the AT + TT group, the difference between the suprathreshold iAUC (mean \pm SE) was 70.82 ± 12.16 vs. 96.95 ± 3.75 , $p = 0.02$. Furthermore, individuals homozygous for the T allele of rs3785368 in the same gene (C>T) had lower suprathreshold iAUC (57.43 ± 19.85) than those with either CT (88.60 ± 6.61) or CC (98.60 ± 4.37) genotype ($p = 0.04$). Again, when genotypes were combined the effect was stronger ($p = 0.03$). Finally, the rs8065080 in the *TRPV1* (C>T) SNP was significantly associated with suprathreshold iAUC (mean \pm SE), where individuals with the CC genotype (74.15 ± 8.38) had significantly lower iAUCs than those with either CT (100.46 ± 5.44) or TT (97.53 ± 5.40) genotype ($p = 0.02$). Following the same pattern, when individuals carrying T allele were grouped together, the results showed that individuals with the CC genotype (74.14 ± 8.34) had a significantly lower iAUC than carriers of the T allele (98.3 ± 3.8) ($p = 0.008$) (Dias et al., 2013). This is the only study to date, reporting on the direct association between genetics and salt taste sensitivity in humans. Nevertheless, the actual sodium consumption has not been investigated in this study and the results warrant further investigation.

In addition, a link between salt taste perception, salt intake and BP is suggested, however with inconclusive results. While some studies report on positive association, other suggest how such an association does not exist (Okoro et al., 1998; Isezuo et al., 2008; Fischer et al., 2012; Piovesana et al., 2013). Besides

methodological issues discussed in Chapter 1, one of the proposed mediating variables in this relationship is salt sensitivity. Rabin et al. (2009) argue that the part of the population with high salt taste thresholds, with potentially higher salt intake but not a raised BP may be a salt-resistant part of the population. However, the authors do not explore the actual salt intake or salt sensitivity in this context, providing an avenue for further research. Moreover, the studies exploring these associations do not take into account genetic variation in the study population which may impact the results.

Furthermore, the mechanisms behind the possible link between salt taste perception and salt intake are unclear and confounded by other metabolic and physiological aspects of salt metabolism. Salt sensitivity is hypothesised as the main confounder.

In this context, there is research suggesting the presence of sensory mechanisms in the kidney (Pluznick and Caplan, 2015). More importantly, the gastrointestinal tract, the organ first exposed to components of food, has taste receptors and sensors for electrolytes (e.g., sodium, potassium, phosphate). Therefore, according to Yang et al. (2017), in addition to the kidney, there is increasing realisation of the importance of the gastrointestinal tract in the regulation of sodium balance, and consequently on BP. This may imply, and should be explored further, that the SNPs in genes affecting salt-sensitive changes in BP, by affecting renal sodium homeostasis, may also affect salt taste perception and vice versa. Moreover, renal sodium transport proteins may also be expressed in taste cells (Vinnikova et al., 2004; Sakamoto et al., 2016). Recent research in animals suggests an association between salt taste sensitivity and salt-sensitive hypertension which is

mediated by RAAS dysfunction (Sakamoto et al., 2016). There are no studies in humans confirming this association and conducted with the aim of comprehensively investigating salt sensitivity, salt taste sensitivity and salt intake, as the main environmental variable.

Thus, the aim of this study is to explore if there is an association between salt sensitivity of BP, salt taste sensitivity and salt intake and whether genetic predisposition to salt sensitivity/salt taste perception affects these associations. The objectives of this study are:

- To determine salt taste perception by measuring salt taste detection and recognition thresholds, as well as salt intake (non-discretionary and discretionary) of study participants.
- To explore if the SNPs rs7571842, rs10177833, rs239345 and rs8065080 are associated with salt taste thresholds and salt intake.
- To explore the associations between salt sensitivity, expressed as the change in BP response to dietary salt intervention (Chapter 2), salt taste thresholds and salt intake and if these associations depend on the rs7571842, rs10177833, rs239345 and rs8065080 genotype.
- To explore if there is an association between salt intake and baseline BP and whether it depends on salt sensitivity expressed as the change in BP response to dietary salt intervention.

3.2 Methods

3.2.1. Participants

The study participants were described in the Methods section of Chapter 2. All 20 participants completed the taste threshold determination test to assess salt taste perception, FFQ and provided a saliva sample. Out of 20 participants, 19 completed the low- and high-salt dietary protocol, however, five participants were excluded due to incomplete 24-hour BP or urinary excretion data.

This study was conducted according to the guidelines presented in the Declaration of Helsinki and all procedures involving human participants were approved by the Institutional Ethics Committee (Appendix A). Written informed consent was obtained from each participant before the baseline data collection informing they can withdraw from the study at any point.

For detailed baseline measurements, salt sensitivity diagnosis with 24-hour urine and BP measurements and SNP Genotyping see Chapter 2.

3.2.2. Taste thresholds for salt

Identification of taste thresholds for salt (salt taste perception) was determined using the British Standard BS ISO 3972:2011 methodology. Salt taste detection and recognition thresholds were determined using eight graded sodium chloride solutions (4 mmol/l – 49 mmol/l). Solutions were prepared by dissolving food grade sodium chloride in spring water. All solutions were prepared on the day of the testing. Participants were presented, repeating the procedure three times, with

a sample of each dilution by order of increasing concentration starting with the lowest concentration. Three additional vessels containing dilutions of the same concentration as the preceding vessel were presented randomly within the sample series. The STDT was identified as the lowest concentration of the sample where the participant can consistently perceive an impression but not identify the taste. The STRT was determined as the sample concentration where the participant consistently identifies the taste as salt.

3.2.3. Habitual dietary salt intake

Baseline energy and dietary salt intake were assessed using a semi-structured validated FFQ. The questionnaires were analysed using an open source, cross-platform tool FETA (Mulligan et al., 2014) and information on 46 nutrients, including sodium, was obtained. This FFQ uses 290 foods from the UK food composition database, McCance and Widdowson's 'The Composition of Foods' (5th edition) and its associated supplements. Habitual dietary sodium intake was energy adjusted and expressed as mg of sodium per 1000 kcal. In addition, information on the discretionary salt use was obtained. To the question about the frequency of adding salt while cooking and at the table, the participants could answer the following: 1) never, 2) rarely, 3) sometimes, 4) usually and 5) always.

3.2.4. Statistical analysis

All continuous variables are presented as mean \pm SEM or median (interquartile range). Categorical variables are presented as absolute and relative frequencies. Before further statistical analysis, continuous variables were tested for normality with the Shapiro-Wilk test. Salt sensitivity was expressed as a dichotomous variable with participants showing an increase in MAP \geq 4 mmHg, after seven days of a high-salt diet, considered salt-sensitive and a continuous variable as the difference in BP from high to a low-salt diet. Differences in baseline characteristics by salt sensitivity status were assessed with an independent samples t-test (with Levene's test for equality of variance) or Fischer's exact test. Fischer's exact test was also used to determine the association between STDT and STRT (low vs. high) and discretionary salt intake expressed as a dichotomous variable (adding salt while cooking and at the table: yes/no). Following the Fischer's exact test, a binary logistic regression was performed with threshold as an independent and discretionary salt as the dependent variable. To explore the effect of genotype an interaction term (threshold x genotype) was introduced in the model. A Cochran Armitage test of trend was run to determine whether a linear trend exists in the proportion of participants with low and high STDT and STRT according to genotype of interest (homozygous major allele, heterozygous and homozygous minor allele). Since there is no universal cut-off point provided to distinguish between the participants with low and high salt taste thresholds, a median was used as a cut-off. Participants with STDT \leq 8 mmol/l and STRT \leq 12 mmol/l were considered to have low thresholds. In addition, a binary logistic regression was performed with genotype (major allele homozygous plus heterozygous and minor allele homozygous

combined) as an independent and threshold (STDT/STRT low vs. high) as the dependent variable. Multiple linear regression analyses were performed with genotype and salt taste thresholds as independent and energy adjusted sodium intake (mg/1000 kcal) as a dependent variable. To explore if the association between thresholds and sodium intake depends on genotype, an interaction term (threshold x genotype) was introduced in the regression model. Further multiple regression analyses were performed with energy adjusted salt intake as independent and baseline BP as a dependent variable. To explore if these relationships depend on salt sensitivity, an interaction term (salt intake x salt sensitivity expressed as the change in BP response to dietary salt intervention). Finally, multiple regression analyses were performed to explore the relationship between salt sensitivity (Δ BP) and salt taste thresholds and salt sensitivity (Δ BP) and energy adjusted salt intake. Interaction term between the independent variable (salt sensitivity (Δ BP)) and genotype was introduced in the regression model. Regression analyses were performed without adjustments for covariates and after adjusting for sex, age and BMI. The model used for the analysis was: major allele homozygous versus heterozygous plus minor allele homozygous.

Analyses were performed using the SPSS software package (version 22.0, Chicago, IL, USA). All tests were two-tailed, with $p < 0.05$ considered statistically significant.

3.3. Results

3.3.1. Baseline characteristics

Twenty participants completed the baseline examination. Of these, 14 participants provided a complete 24-hour ABPM and 24-hour urine excretion data and were included in the analysis on salt sensitivity of BP (for compliance with the diets see Chapter 2). There was no significant differences in distribution of sex, participants with low or high salt taste thresholds, mean age and absolute and energy adjusted sodium intake between salt-sensitive and salt-resistant participants. The mean sodium intake in the total population was 2745 mg or 6.9 g of salt (Table 3.1).

Table 3.1 Baseline characteristics of study participants, total sample (n =20) and according to salt sensitivity status (n = 14). Data presented as mean \pm SEM or absolute and relative frequencies. P value for difference between salt-sensitive and salt-resistant participants (Independent samples t-test, Mann-Whitney U test, Fischer's exact test).

	Total (n = 20)	%	Salt-sensitive (n = 5)	%	Salt-resistant (n = 9)	%	P
Age (years)	28.0 (10.5) ^{a)}		35.8 \pm 4.6		33.2 \pm 2.7		0.612
Sex (n)							
Male	8	40	2	40	2	22	0.580
Female	12	60	3	60	7	78	
STDT (n)							
Low	15	75	4	80	7	77.8	1.000
High	5	25	1	20	2	22.2	
STRT (n)							
Low	12	60	2	40	6	66.7	0.580
High	8	40	3	60	3	33.3	
Mean sodium intake (mg)	2745 \pm 197		2860 \pm 428		3146 \pm 293		0.582
Mean sodium intake (mg/1000 kcal)	1409 \pm 57		1264 (309.5) ^{a)}		1493 (452) ^{a)}		0.364

a) median (interquartile range), salt taste detection threshold (STDT), salt taste recognition threshold (STRT)

3.3.2. Genetic predisposition to altered salt taste perception and dietary salt intake

As shown in Figure 3.1, the proportion of study participants with low and high STRT was similar according to genotypes of interest. The results of a Cochran Armitage test of trend between the different genotype groups (homozygous major allele, heterozygous and homozygous minor allele) and the proportion of participants with low and high STDT were: rs7571842 (p = 0.313), rs10177833 (p = 0.490),

rs239345 ($p = 0.160$), rs8065080 ($p = 0.213$). Similar was observed for STRT: rs7571842 ($p = 0.905$), rs10177833 ($p = 0.714$), rs239345 ($p = 0.456$), rs8065080 ($p = 0.078$) (Figure 3.2). When performing binary logistic regression similar was observed (data not shown). Moreover, results of multiple linear regression showed no relationship between genetics and salt intake (Table 3.2).

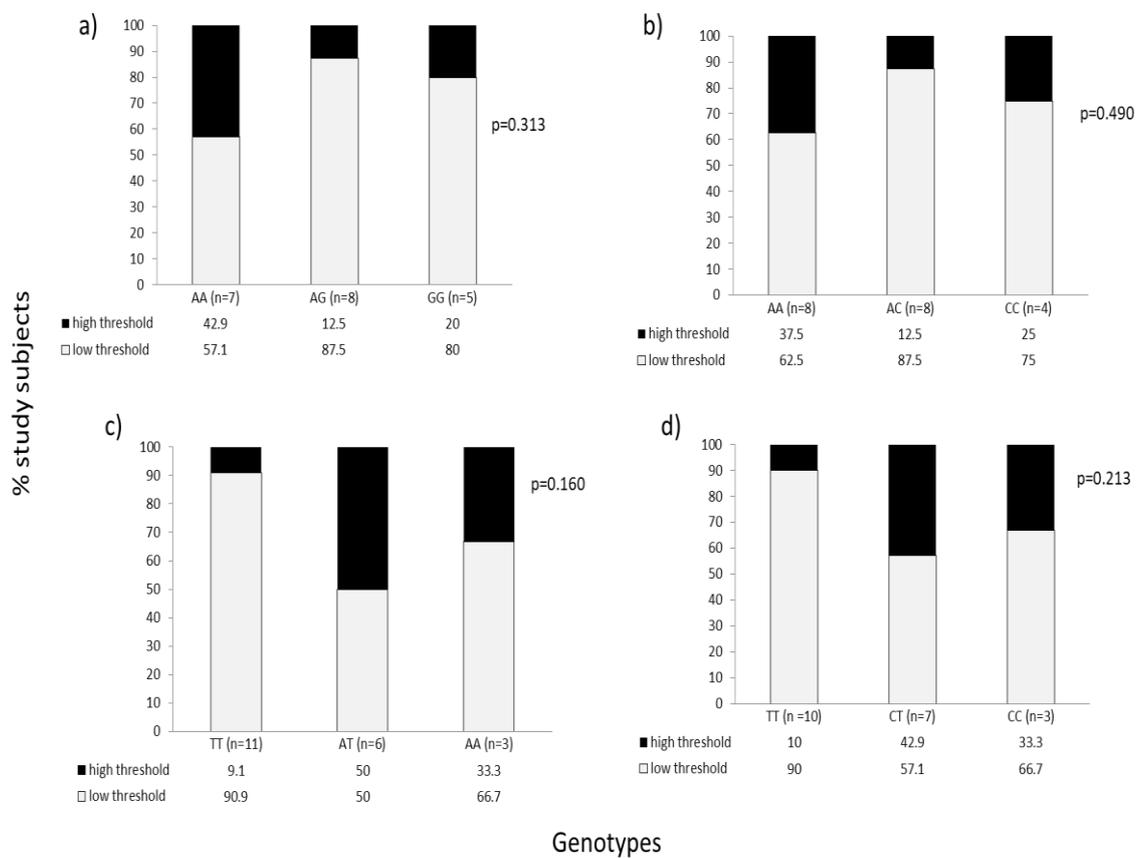


Figure 3.1 Proportion of participants ($n = 20$) with low and high salt taste detection thresholds according to *SLC4A5* rs7571842 (a) and rs10177833 (b), *SCNN1B* rs239345 (c) and *TRPV1* rs8065080 (d) genotype (Cochran Armitage test of trend).

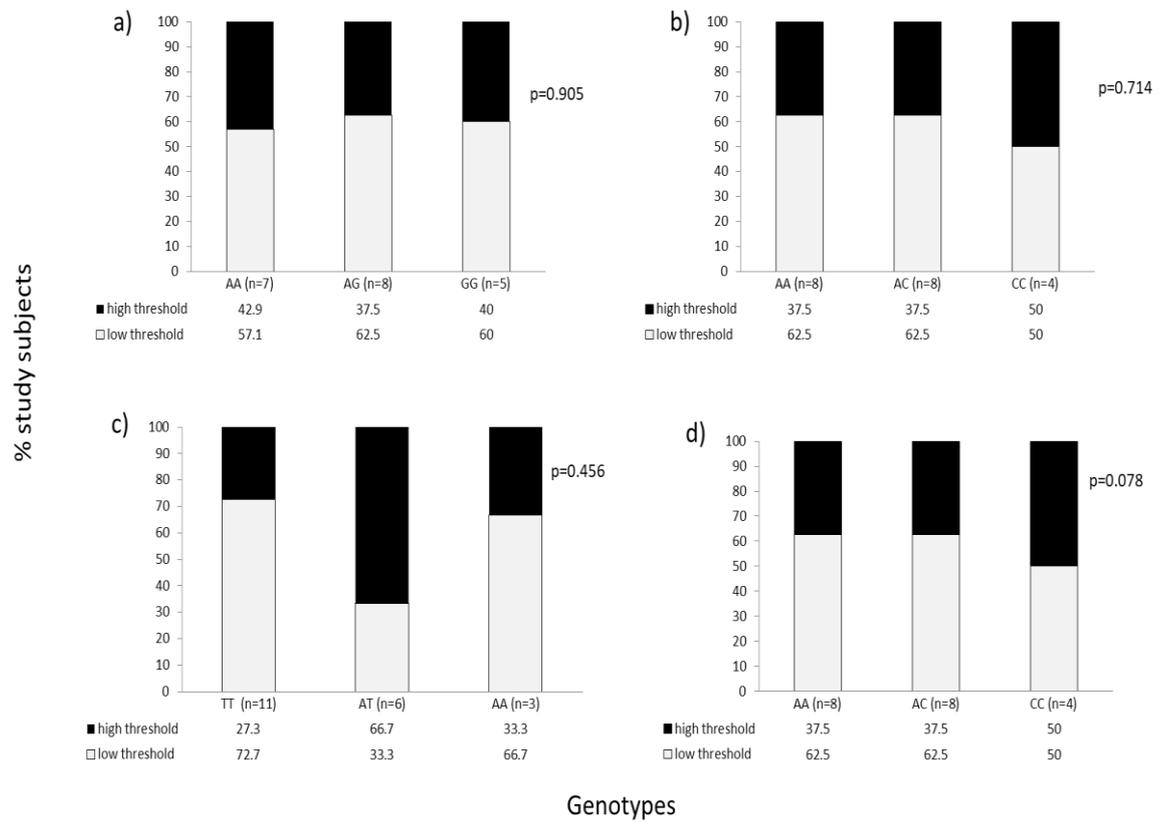


Figure 3.2 Proportion of participants (n = 20) with low and high salt taste recognition thresholds according to *SLC4A5* rs7571842 (a) and rs10177833 (b), *SCNN1B* rs239345 (c) and *TRPV1* rs8065080 (d) genotype (Cochran Armitage test of trend).

Table 3.2. Multiple regression analyses for sodium intake (mg/1000 kcal) (n = 20)

	Reference genotype	β	95% CI	p	p*
rs7571842	AA	0.037	-0.066, 0.087	0.774	0.988
rs10177833	AA	-0.004	-0.078, 0.071	0.921	0.501
rs239345	TT	0.034	-0.038, 0.106	0.331	0.330
rs8065080	TT	-0.001	-0.075, 0.072	0.967	0.323

* adjusted for age, sex and BMI; confidence interval (CI).

3.3.3. Association between salt sensitivity and salt taste thresholds and salt sensitivity and salt intake

Results of the regression analysis revealed that there was no relationship between salt sensitivity and salt taste thresholds (STDT and STRT, Table 3.3 and Table 3.4) after adjusting for sex, age and BMI. Introducing the interaction term (Δ BP x genotype) in the model revealed that genotype did not moderate the relationship between Δ BP and thresholds.

Table 3.3 Multiple regression analyses for salt taste detection threshold (mmol/l) (n = 14)

	β	95% CI	p	p*
Δ SBP (mmHg)	0.291	-0.104, 0.687	0.134	0.691
Δ DBP (mmHg)	-0.547	-8.964, 7.871	0.888	0.694
Δ MAP (mmHg)	0.802	-7.950, 9.553	0.842	0.701
Δ PP (mmHg)	0.294	-2.374, 2.961	0.811	0.496

* adjusted for age, sex and BMI; confidence interval (CI), diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP), systolic blood pressure (SBP).

Table 3.4 Multiple regression analyses for salt taste recognition threshold (mmol/l) (n = 14)

	β	95% CI	p	p*
Δ SBP (mmHg)	1.002	-9.267, 11.271	0.832	0.442
Δ DBP (mmHg)	0.311	-0.105, 0.728	0.129	0.141
Δ MAP (mmHg)	-0.558	-11.235, 10.119	0.910	0.482
Δ PP (mmHg)	-0.447	-7.545, 6.651	0.891	0.453

* adjusted for age, sex and BMI; confidence interval (CI), diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP), systolic blood pressure (SBP).

Moreover, there was no relationship between Δ BP and sodium intake after adjusting for age, sex and BMI (Table 3.5). Introducing interaction term (Δ BP x genotype) did not change the results (data not shown).

Table 3.5 Multiple regression analyses for sodium intake (mg/1000kcal) (n = 14)

	β	95% CI	p	p*
Δ SBP (mmHg)	-17	-49, 15	0.265	0.469
Δ DBP (mmHg)	-26	-51, 0.2	0.048	0.231
Δ MAP (mmHg)	-28	-58, 2	0.065	0.262
Δ PP (mmHg)	22	-16, 59	0.235	0.405

* adjusted for age, sex and BMI; confidence interval (CI), diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP), systolic blood pressure (SBP).

3.3.4. Associations between salt intake and baseline BP

Furthermore, there was no relationship between salt intake and baseline BP after adjusting for sex, age and BMI (Table 3.6). Salt sensitivity did not moderate the relationships after introducing the interaction term (sodium intake x Δ BP) to the model.

Table 3.6 Multiple regression analyses for baseline BP (mmHg) (n = 14)

	β	95% CI	p	p*
Systolic blood pressure				
Sodium intake (mg/1000 kcal)	-0.03	-0.065, -0.001	0.044	0.844
Diastolic blood pressure				
Sodium intake (mg/1000 kcal)	-0.01	-0.037, 0.11	0.265	0.367
Mean arterial pressure				
Sodium intake (mg/1000 kcal)	-0.02	-0.045, 0.006	0.116	0.528
Pulse pressure				
Sodium intake (mg/1000 kcal)	-0.02	-0.043, 0.002	0.073	0.477

* adjusted for age, sex and BMI; confidence interval (CI).

3.3.5. Associations between salt taste thresholds and dietary salt intake

As shown in Table 3.7 there was no relationship between salt taste thresholds and energy adjusted sodium intake. Introducing the interaction term (threshold x genotype) to the regression model did not change the results suggesting that genotype does not moderate the threshold- salt intake relationship in this study population. In addition, there was no association between salt taste thresholds and discretionary salt intake (adding salt while cooking/at the table) (Figure 3.3 and 3.4). Similar was observed when stratifying according to genotype and with binary logistic regression analysis (data not shown).

Table 3.7 Multiple regression analyses for sodium intake (mg/1000 kcal) (n = 20)

	β	95% CI	p	p*
STDT (mol/l)	-5	-16, 5	0.287	0.551
STRT (mol/l)	-1	-12, 9	0.782	0.949

* adjusted for age, sex and BMI; confidence interval (CI), salt taste detection threshold (STDT), salt taste recognition threshold (STRT).

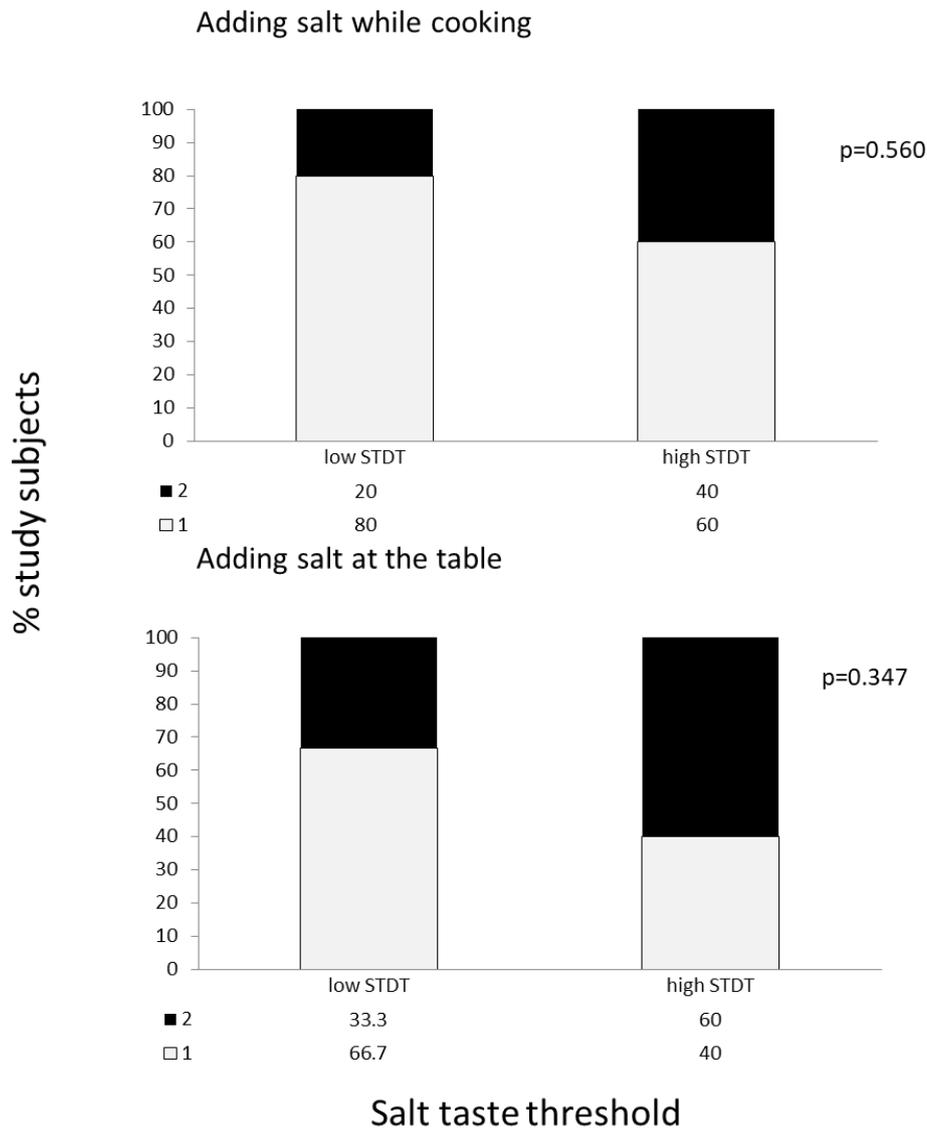


Figure 3.3 Discretionary salt intake according to salt taste detection threshold (low STDT n = 15, high STDT n = 5). 1: participants that add salt and 2: participants that do not add salt (Fisher's exact test). Salt taste detection threshold (STDT).

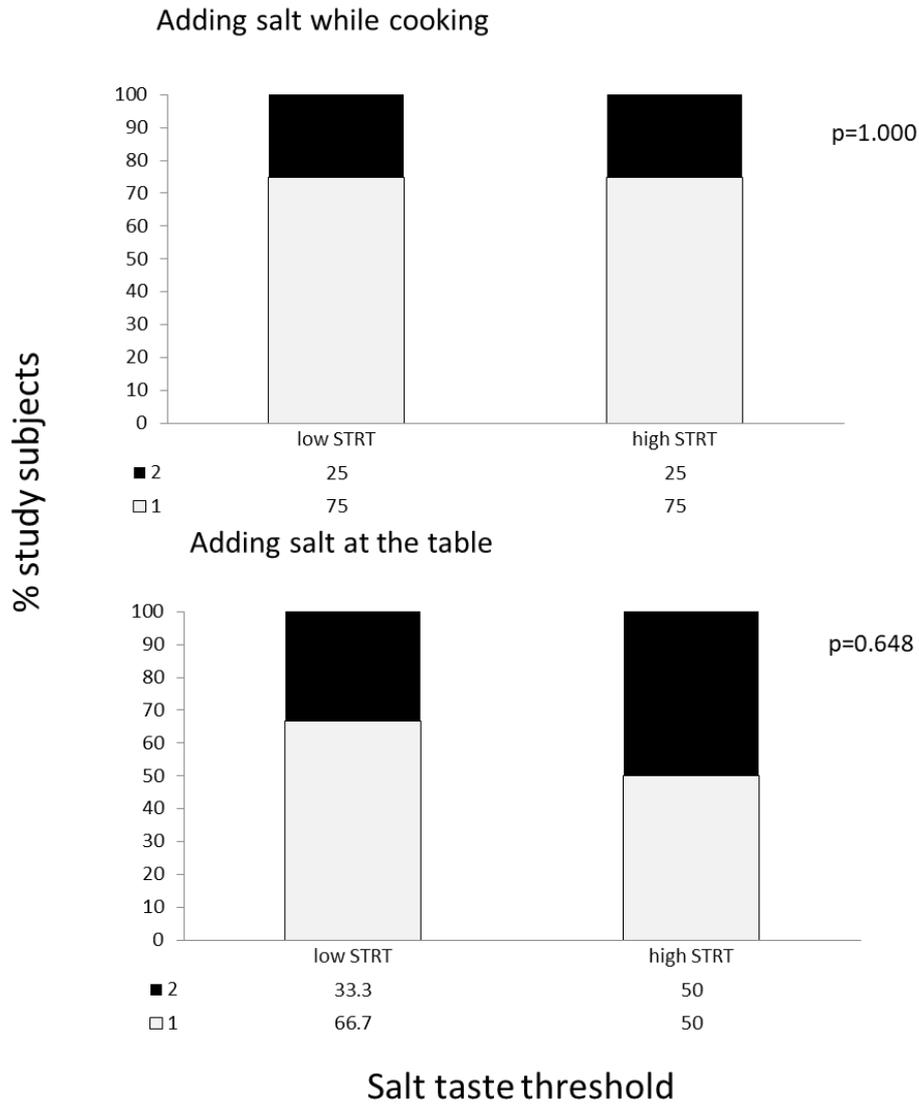


Figure 3.4 Discretionary salt intake according to salt taste recognition threshold (low STRT n = 12, high STRT n = 8). 1: participants that add salt and 2: participants that do not add salt (Fisher’s exact test). Salt taste recognition threshold (STRT).

3.4. Discussion

The results of the present study suggest that the *SLC4A5* rs7571842 and 10177833, *SCNN1B* rs239345 and *TRPV1* rs8065080 did not predict salt taste perception or salt intake in the study population. In addition, there was no relationship between salt sensitivity and salt intake as well as salt intake and baseline BP. These relationships did not depend on genotype or salt sensitivity. Similarly, there was no relationship between salt taste perception and salt intake. Finally, the association between salt sensitivity and salt taste perception may depend on the *SLC4A5* rs7571842 and *SCNN1B* rs239345 genotypes.

These results may be a consequence of several methodological issues which will be discussed in further sections. They may also suggest that complex gene-gene and gene-environment interactions, as well as other factors that contribute to eating behaviours, play a more pronounced role in explaining the outcomes such as salt taste perception and salt intake and should, as such, be explored in future studies. Each outcome will be discussed separately in sections that follow.

3.4.1. Genetic predisposition to altered salt taste perception and salt intake

One of the objectives of this study was to investigate if there is a genetic predisposition to altered salt taste perception, more precisely, whether the SNPs in the genes involved in sodium transport affect salt taste thresholds in the study population. Dias et al. (2013) showed there is an association between the SNPs in the *SCNN1B* and *TRPV1* genes and altered salt taste perception. Specifically, they have found that minor allele carriers of the rs239345 and rs8065080 have lower ability to

taste salt at a suprathreshold level. These results were not confirmed in the present study, potentially because of the difference in the level of salt stimulus. This will be discussed further in Chapter 4. Taste recognition threshold or the actual salt intake were not explored in the Dias et al. (2013) study. Nevertheless, the effect of rs8065080 on STRT may be detected in a larger sample size study. For such study to be clinically meaningful, in addition to salt taste perception, dietary salt intake should be measured. It has been shown that the reduction in salt intake results in important falls in BP, in both hypertensive and normotensive individuals (He et al., 2013) and a reduction in overall CVD risk (Lewington et al., 2002). This is supported by the authors who acknowledge the limitations of not measuring salt intake. The question they pose is whether the effect of the variation in *TRPV1* on the ability to taste salt at the higher intensity, potentially aversive level, means it would also affect the preference for salt or dietary salt intake.

Similar to salt taste perception, the genetic variants explored in this thesis did not predict salt intake in the study population. Considering that the power calculation was not based on the outcomes explored in this Chapter (salt taste perception and salt intake) but salt-sensitive change in BP after consuming high-salt diet, it is not to exclude the possibility of type 2 error and that the effect of genotype would be detected in a larger sample size study.

In this context, research suggests that genetic predisposition plays an important role in both sodium intake level and salt habits. Hundred and thirty three pairs of monozygotic twins, 29 pairs of dizygotic twins, and 880 singletons participated in a twin family Korean cohort. Salt intake was calculated from half-day urine samples. In addition, salt habit was investigated by asking questions such as

“How salty is your diet?” and “When you eat out, does your food taste salty?” The authors reported that moderate genetic influences existed ($h^2 = 0.31 - 0.34$) for sodium intake and sodium density (sodium mg/1000 kcal) (Kho et al., 2013). Considering a distinct genetic background and high salt intake (~12.4 g/day) in Korean population (Lee et al., 2015) as well as the limitations of estimating salt intake from a causal urine sample, these results warrant further investigation in other populations and with multiple 24-hour urine collections as a method of estimating salt intake.

Nevertheless, further supporting the notion that salt intake may be genetically determined, Smith et al. (2016) have reported how individuals with enhanced bitter taste perception genotype (GC and GG alleles for the bitter taste receptor gene *TAS2R38*) were significantly more likely than CC homozygous to have daily sodium intake higher than recommended. The authors pointed out how further research is needed to better understand genetic influences on sodium consumption and implications for CVD prevention. Furthermore, Kho et al. (2012) have conducted a GWAS in Korean population. They investigated 2209 healthy individuals from Healthy Twin Study cohort and estimated dietary 24-hour sodium excretion from half-day urine samples. Out of 537 158 markers tested for association with the 24-hour urinary sodium, several variants reached sufficient or probable genome-wide significance level and these were: locus p16.2 with genes regulating voltage-gated sodium channels and calcium/calmodulin kinase in cardiac muscle, p15.3 with genes related to sodium reabsorption in the nephron and q11.21 with potassium channel related genes. These results suggest that genes coding for sodium transport proteins may be associated with salt intake and contradict the findings of this thesis. Besides the small sample size and lack of *a priori* power calculation focusing on this specific

outcome, the method of measuring salt intake in the present study may have affected the results. Limitations of a FFQ to measure salt intake will be discussed in the Limitations section of this chapter. Due to these methodological issues, results should be replicated in a larger size study where salt intake would be estimated by the means of a 24-hour urinary sodium excretion measurement.

3.4.2. Associations between salt sensitivity and salt taste perception and salt sensitivity and salt intake

Analysis was conducted to explore the relationships between salt sensitivity of BP, salt taste thresholds and salt intake and whether these relationships depended on the *SLC4A5*, *SCNN1B* and *TRPV1* genotypes. To our knowledge, this is the only study to date exploring the association between salt sensitivity and salt taste sensitivity in humans. The mechanisms behind this association and the causality remain unknown. Bobowski and Mennella (2015) suggested that where there is no correlation between salt taste sensitivity and salt intake, high BP may have yet unresolved consequences on taste. In this study we hypothesised that genetics may play a role in this relationship, however the results revealed there was no moderator effect of the genotype. Sakamoto et al. (2016) reported how kidney ENaC is up-regulated and the ENaC expressed in taste receptor cells down-regulated in aldosterone/NaCl-induced hypertensive rats which would in turn lead to a higher sodium intake in hypertension prone animals. Moreover, this would imply a negative relationship between salt taste sensitivity and salt sensitivity of BP. The mechanism behind this association in the above-described study is RAAS dysregulation which in

turn affects sodium transport through changes in ENaC expression. The effect of RAAS was not investigated in the present study. Moreover, rs239345, an ENaC SNP was not associated with salt sensitivity or salt taste thresholds. In a larger sample size study potential effect of interactions between the *SLC4A5* and ENaC SNPs may be investigated when exploring this relationship. Small sample which may lead to a type 2 error and false negative findings is another reason for conducting these analyses in a larger size study. In such study, respective allele frequencies should be taken into account when calculating required sample size (Grimaldi et al., 2017).

Besides salt taste thresholds, this study aimed at exploring the relationship between salt sensitivity and salt intake. To the best of our knowledge no studies in humans explored this relationship. Conversely, a negative association was reported in animal studies. Bachmanov et al. (2002) observed how Dahl S rats have lower salt intake than Dahl R rats. The authors suggested that the difference in the intake and preference for ingestion of minerals, including sodium, may have a substantial genetic component which was hypothesised in this thesis. The results suggested that genotypes explored in this thesis did not moderate the relationship between salt sensitivity and salt intake. Other variants in genes involved in sodium transport, such as *SLC4A5*, and their interactions may play a role and should be explored in future studies. If salt-sensitive population indeed has lower salt intake then the efforts should be directed towards maintenance of such behaviour. Considering the lack of studies exploring both salt sensitivity and habitual salt intake in humans it is difficult to draw any definitive conclusions or make comparisons between studies. Nevertheless, the intake of salt in salt-sensitive participants in this study was 7.1 g, still above the recommendations, which supports the argument that the efforts to reduce salt intake still warrant attention.

Finally, another factor that should be taken into consideration when exploring salt intake as the outcome variable is BMI. Taken into account as a covariate in the analyses, it affected the observed relationships between salt sensitivity of BP and salt intake together with the relationship between salt intake and baseline BP. This is consistent with research showing a correlation between salt intake and measures of adiposity such as waist circumference and BMI in children, adolescents and adults and emphasises the importance of controlling for BMI in studies exploring salt intake (Campanozzi et al., 2015; Ma et al., 2015; Lee and Kim, 2016).

3.4.3. Associations between salt taste perception and salt intake and salt intake and baseline BP

The results of the present study suggest there is no association between salt taste thresholds and salt intake. Literature is conflicting regarding these associations. As discussed in Chapter 1, one of the main reasons may be discrepancies in methods and populations studied. In the present study it was hypothesised that genetics, a factor not taken into account in studies discussed in Chapter 1, may also play a role in this relationship. However, the results showed that genotype did not moderate the relationships between salt taste thresholds and salt intake (discretionary and non-discretionary).

This contradicts the findings of Fischer et al. (2012) who report the association of salt taste intensity, measured with a filter paper disk impregnated with 1.0 M sodium chloride, and the frequency of discretionary salt use in their study population. However, the population in the latter study was older than the population in the current study and it comprised of 33% hypertensives. The link between taste

and discretionary salt use has also been suggested by Shepherd and Farleigh (1986) who reported on the associations between beliefs about the taste of salt and discretionary salt usage. Sarmugam et al. (2013) support this with their results showing that beliefs about the importance of taste are a mediating variable in the relationship between age, gender, education and discretionary salt intake. Beliefs related to taste of salt were assessed using two items 1) “In general, low salt food tastes bad”, 2) “Salt should be used in cooking to enhance the taste of the food and measured on five-point Likert scales ranging from “certainly wrong” to “certainly true”. Besides being a mediating variable, beliefs about taste of salt were directly associated with discretionary salt use ($r = 0.51$; $p < 0.001$). Indeed, preference for salty taste may be one of the factors affecting salt intake. In a study conducted in 74 healthy participants with a median age of 35 years, the absolute salt taste threshold, measured by the participants’ ability to recognize the taste of salt in graded solutions of sodium chloride, was not associated with the estimated sodium intake. It was the participants’ self-reported salt-eating habit or a salt preference that was shown to predict salt intake. However, the study population was Korean with higher salt intakes than the UK (Lee et al., 2014). As suggested, taste preferences, in addition to physiological and genetic parameters, are influenced by a range of demographic and sociocultural variables (Drewnowski, 1997). Kho et al. (2013) state that health conscious people may intentionally lower their salt intake. This is supported by Luta et al. (2018) who state that higher salt awareness, encompassing (1) knowledge of the national salt intake recommendation, (2) the use of discretionary salt when eating at home and away from home, and (3) the impact of the salt content of a food or menu item on purchasing/choosing it, or not, when shopping for groceries or eating in a staff canteen, was associated with lower salt intake estimated with 24-hour

biomarker method in their healthy and physically active population. The above-described may be a reason for not observing the association with total salt intake in the total population that was healthy and comprised predominantly younger adults. Nevertheless, it was suggested that besides demographic and genetic factors, the actual methods used in different studies may explain the discrepancies in results on the association between salt taste perception and salt intake and this will be further discussed in Chapter 4.

In addition to the lack of research on the potential relationship between salt sensitivity and salt taste sensitivity there is a clear discrepancy in results of the studies exploring salt taste sensitivity, BP and salt intake, as discussed in Chapter 1. Rabin et al. (2009) suggest salt sensitivity as the mediating variable explaining the discrepancies in results, however the authors do not explore it themselves. Moreover, there are a number of studies exploring the association between salt taste thresholds and BP that do not take into account the actual salt intake (Isezuo et al., 2008; Michikawa et al., 2009; Rabin et al., 2009; Cho et al., 2016). This study thus, aimed to explore if salt sensitivity indeed, may moderate the relationship between salt intake and BP.

Regarding the salt intake – BP association, genetics may also be one of the explanatory variables. It has been suggested by Kim et al. (2012) that the characteristics of a population, such as genetic effects and the average sodium intake relative to the sodium threshold, all affect the relationship observed between sodium intake and BP. In addition, other dietary factors such as high intake of fruit and vegetables and dietary patterns similar to DASH diet may affect this association. In a large sample of healthy female Korean population a negative correlation was observed between salt intake and both SBP and DBP. They also had high intake of

vegetables which may have affected this relationship (Kim et al., 2012). The authors state how dietary patterns and salt sensitivity at the population level could explain the relationship between sodium intake and BP. In the current study, there was no relationship between salt intake and BP and it did not depend on the degree of salt sensitivity. Considering a small sample size, control for dietary factors affecting BP could not be performed which may have affected the results. Indeed, the lack of association may be due to a limited sample and the fact the study population was healthy with a normal BP which may require a larger sample to detect this well-established relationship, as suggested by Graudal and Jürgens (2018).

3.4.4. Strengths and limitations

This study is, to the best of our knowledge, the first study to date comprehensively investigating the associations between salt sensitivity of BP, salt taste sensitivity and salt intake in humans, also taking into account genetic variation in the study population. However, one of the limitations is the use of a FFQ to determine dietary salt intake. Even though FFQ represents dietary intake over a longer time-period and is often used in epidemiological studies due to low participant burden, it relies heavily on respondents' honesty and long-term memory. In addition, it is used predominantly to rank participants according to their intake of specific nutrients rather than analysing intakes as absolute values, conducted in this small sample size study (Dehghan et al., 2012). McLean et al. (2017) report on poor agreement between sodium intakes measured with a FFQ and 24-hour urinary biomarker method in 18 different studies. In the present study, sodium intake was energy adjusted, which may have resulted in improved accuracy. Freedman et al.

(2015) suggest that the attenuations and correlations with truth for the FFQs are improved when considering sodium densities, utilised in this study. Nevertheless, the results reporting on salt intake as the outcome measure should be interpreted with caution due to limitations of the dietary method. Future studies should, for this reason, validate the FFQ against multiple 24-hour urine sodium excretion measurements in the study population of interest to test its actual validity and reliability in measuring salt intake. In this thesis, FFQ was chosen due to generally high participant burden with the intention to compromise between accuracy and compliance to the study protocol.

Another limitation of this study is the small sample size which did not allow to draw definitive conclusions about the relationships explored in this Chapter. The results may therefore be considered as hypotheses generating at best. Small sample size may have resulted in type 2 errors and the results should be replicated in a larger sample size study.

3.5. Conclusion

The results of the present study suggest no associations between explored variables. As the sample was small, these results should be considered as preliminary and confirmed in a larger sample size study. In addition to the lack of statistical power, it is suggested that gene-gene interactions may play a role in these relationships and require further exploration. Regarding the relationship between salt taste thresholds and salt intake, preference for salty taste or awareness about health consequences of inadequate salt intake may have more pronounced effects and require further investigation. Considering the potential effect of BMI such studies should focus on ensuring sufficient sample which would allow for stratification according to BMI.

Finally, due to the limitations of a FFQ used in this study, the link between salt taste thresholds and total salt intake should be further explored using another validated method of measuring salt intake. This will be discussed in Chapter 4.

Chapter 4 The associations between salt taste perception and total salt intake - confirmation study

4.1. Introduction

Based on the results of the study reported in Chapter 3, it can be concluded how the variants in genes coding for the β subunit of the ENaC and the vanilloid receptor 1 are not associated with altered salt taste perception in healthy and younger participants. These results somewhat correlate with the findings of the only study to date exploring similar research question in humans. Dias et al. (2013) report on the associations between the rs239345 and the rs8065080 variants and salt taste suprathresholds, but not thresholds, in younger and healthy Caucasians. However, the authors do not explore the actual salt intake as the final, and arguably, the most relevant outcome variable. In the previous chapter, salt taste perception expressed as the detection and recognition threshold was not associated with salt intake.

Nevertheless, different measures of salt intake may result in different results obtained. Supporting this notion is the research on salt taste perception and intake by Piovesana et al. (2013). This is, to the best of our knowledge, the only study comprehensively employing different methods of measuring dietary intake with the aim of exploring its association to salt taste thresholds. The authors used the measures of discretionary salt use, sodium intake by 24-hour recall and the consumption of foods with high sodium content by a sodium-FFQ designed to capture the consumption of sodium rich foods. The authors argued that when considering different methods together it is possible to estimate the overall consumption, and when methods are analysed separately, the contribution of each source to the total consumption can be estimated. As expected and, similar to observed in previous chapter, results differed with the method employed. There was no correlation between salt taste thresholds and sodium intake measured with a FFQ

($r = 0.16$, $p = 0.099$ and $r = 0.1$, $p = 0.287$, for detection and recognition threshold respectively). A weak and positive correlation was observed when focusing on the discretionary salt use ($r = 0.20$, $p = 0.046$ and $r = 0.21$, $p = 0.030$, for detection and recognition threshold respectively), and the opposite was observed regarding the 24-hour recall for detection threshold, whereas no association was observed for recognition threshold ($r = -0.20$, $p = 0.040$ and $r = -0.15$, $p = 0.112$, respectively). Total intake, expressed as the combination of three methods was positively correlated with both thresholds, however the strength of the correlation was weak ($r = 0.23$, $p = 0.015$ and $r = 0.23$, $p = 0.017$, for detection and recognition threshold respectively). Contrary to these results, total sodium intake estimated from 24-hour urine samples was not correlated to either of the thresholds ($r = -0.04$, $p = 0.654$ and $r = -0.06$, $p = 0.578$, for detection and recognition threshold respectively) (Piovesana et al., 2013).

As stated in the introduction chapter, the non-discretionary salt use accounts for approximately 85% of the total intake in the UK population (SACN, 2003). Even though the FFQ used in this thesis was designed to reflect the consumption of foods most frequently consumed in the UK population and capture, in addition to discretionary salt intake, the non-discretionary fraction of sodium intake (Bingham et al., 1997), it may not accurately represent the total intake of salt.

Although 10% of urine samples in the Chapter 2, that were collected for salt intake measurement, were incomplete based on the urinary creatinine excretion, other studies suggest that the average percentage of incomplete collections is higher than 20% (Subar et al., 2003; Rhodes et al., 2013). The reason for incomplete collections is primarily high participant burden which may consequently result in

inaccurate salt intake estimations, especially in younger populations and presents one of the major limitations of this "gold-standard" method (McLean, 2014). Therefore, another method of measuring salt intake will be employed in the present study. As stated previously, in the USDA AMPM validation study, the mean sodium, calculated as the ratio of reported dietary sodium intake from 24-h recall to 24-h urinary sodium, was 0.93 for men and 0.90 for women, suggesting the USDA AMPM is a valid method for assessing dietary salt intake (Rhodes et al., 2013; McLean, 2014). This method has also been developed into an online system, Automated Self-Administered 24-h Recall which has been validated in a feeding study with 81 adults (Kirkpatrick et al., 2014).

With the potential impact of the method utilised to measure total dietary salt intake on its association with salt taste perception, the aim of this study is to conduct a Chapter 3 confirmation study in a population with similar demographic characteristics. The focus of the present study will primarily be an investigation of the taste perception- total salt intake relationship based on the 24-hour recall method. The objectives of the study are:

- To determine salt taste perception by measuring salt taste detection and recognition thresholds using a BS method used in Chapter 3.
- To determine salt intake using two 5-step multiple pass 24-hour dietary recalls.
- To explore if the SNPs rs7571842, rs10177833, rs239345 and rs8065080 are associated with salt taste thresholds and salt intake.

- To explore the associations between salt taste thresholds and dietary salt intake and if these associations differ according to rs7571842, rs10177833, rs239345 and rs8065080 genotype.

4.2. Methods

4.2.1. Study participants

The participants were predominantly young adult Caucasians, six males and 18 females. Participants were excluded with history of/current chronic disease or the use of any medications to treat chronic disease. In addition, pregnant women and participants with an illness that alters taste were also excluded from the study. All participants completed taste threshold determination for salt, two 24-hour recalls and provided a saliva sample for genotyping.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human participants were approved by the Institutional Ethics Committee. Written informed consent was obtained from each participant before the baseline data collection informing they can withdraw from the study at any point.

4.2.2. Baseline characteristics

Anthropometric (height and weight) and demographic data (age, sex and race) was collected and assessed together with smoking habits and health status information. Physical activity was assessed by the number of hours participants spent physically active (exercising) per week. The information was collected using a confidential medical history/physical activity readiness questionnaire (Appendix C).

4.2.3. Taste thresholds for salt

Identification of taste thresholds for salt (salt taste perception) was determined using the British Standard BS ISO3972:2011 methodology. Salt taste detection and recognition thresholds were determined using nine graded sodium chloride solutions (3 mmol/l – 49 mmol/l, geometrical ratio of 0.7). The lowest dilution (3 mmol/l) was used in the current study based on the results of the previous chapter and participants' ability to detect and recognise salty taste at low concentrations. Solutions were prepared by dissolving food grade sodium chloride in spring water. All solutions were prepared on the day of the testing. Participants were presented, repeating the procedure three times, with a sample of each dilution by order of increasing concentration starting with the lowest concentration. Three additional vessels containing dilutions of the same concentration as the preceding vessel were presented randomly within the sample series. The salt STDT was identified as the lowest concentration of the sample where the participant can consistently perceive an impression but not identify the taste. The STRT was identified as the sample concentration where the participant consistently perceives the taste as salt.

4.2.4. SNP genotyping

At baseline examination a 2 ml saliva sample was collected into a collection vial (SalivaGene collection module II, STRATEC Molecular, Berlin). A stabiliser provided by the manufacturer was added to the saliva sample and it was stored at -20 °C until DNA was extracted. Genomic DNA was extracted using a commercial kit

PSP® SalivaGene 17 DNA Kit 1011 (STRATEC Molecular, Berlin) in accordance with the manufacturer protocol. Quality and quantity were assessed using Nanodrop (ThermoFisher, Waltham, MA, USA). Genotyping was performed using a pre-designed TaqMan® SNP genotyping assays for the SNPs: rs7571842, rs10177833, rs239345, rs8065080 and the StepOnePlus thermocycler (Applied Biosystems, CA, USA) with two technical replicates for each sample. The primers and the probes were pre-designed by Applied Biosystems with the following codes (C___197439_10, C___1137534_10, C___2387896_30, C__11679656_10). The PCR amplification was performed under the conditions specified by the manufacturer.

4.2.5. Habitual dietary salt intake - 24-hour recall

Dietary salt intake was assessed using two 24-hour dietary recalls (one weekday and one day of the weekend). It was based on the USDA 5-step multiple pass method and administered via online platform (Bristol Online Survey, Appendix D). The 5-step multiple pass method is a more detailed approach in collecting 24-hour dietary recall data, whereby participants are asked detailed questions about the food and beverages consumed, such as quantities, occasion and the time of the food consumption. During the first step, the study participants were asked to list all the foods consumed in the last 24-hours (from midnight to midnight). This was followed by the administration of forgotten food list to elicit additional recall of foods by focusing participants' attention on different, often forgotten, categories of food. In addition to the typically forgotten foods such as tea, coffee, non-alcoholic and alcoholic beverages, sweets and snacks, this list also contained foods usually high in

sodium such as pickled vegetables, deli meats, smoked fish, cheese, bread and condiments. Participants were also asked to provide information about the quantity of the stock cubes or gravy granules if used while cooking. In the third step, participants were asked about the eating occasion to refresh the memory. Detailed description of each food reported, such as brand name, portion size (photographs provided) and method of preparation was also collected, in addition to reviewing each eating occasion to elicit additional recall. The final step included the review of all food listed with the option to add any food that may have been forgotten. Discretionary salt use was assessed asking questions on adding salt while cooking and at the table with participants also required to provide the quantities of added salt. The participants were classified into group that adds salt or the group that does not add salt. Energy and nutrient intake was calculated using nutritional analysis software (Nutritics, Nutritics LTD, Dublin, Ireland). The database used to calculate nutrient content was The Composition of Foods Integrated Dataset which included McCance and Widdowson The Composition of Foods (Finglas et al., 2015). Total sodium intake (non-discretionary and discretionary) was calculated as an average of sodium intake in both recalls and it was energy adjusted and expressed as mg of sodium per 1000 kcal.

4.2.6. Statistical analysis

All continuous variables are presented as mean \pm SEM or median (interquartile range). Categorical variables are presented as absolute (relative) frequencies. Before further statistical analysis, continuous variables were tested for normality with the Shapiro-Wilk test. Differences in baseline characteristics by sex

were assessed using an independent samples t-test (with Levene's test for equality of variance) or Fischer's exact test. Fischer's exact test was also used to determine the association between STDT and STRT (low vs. high) and discretionary salt intake expressed as a dichotomous variable (adding salt while cooking and at the table: yes/no). Following the Fischer's exact test, a binary logistic regression was performed with threshold as an independent and discretionary salt as the dependent variable. To explore the effect of genotype an interaction term (threshold x genotype) was introduced in the model. A Cochran Armitage test of trend was run to determine whether a linear trend exists in the proportion of participants with low and high STDT and STRT according to genotypes of interest. Participants with STDT \leq 6 mmol/l and/or STRT \leq 12 mmol/l were considered to have low thresholds. In addition, a binary logistic regression was performed with genotype as an independent and threshold (STDT/STRT) as the dependent variable. Multiple linear regression analyses were performed with genotype and salt taste thresholds as independent and energy adjusted sodium intake (mg/1000 kcal) as a dependent variable. To explore if the relationship between thresholds and sodium intake depends on genotype, an interaction term (threshold x genotype) was introduced in the regression model. Regression analyses were performed without adjustments for covariates and after adjusting for sex, age and BMI. The model used for the analysis was: major allele homozygous versus heterozygous plus minor allele homozygous. Analyses were performed using the SPSS software package (version 22.0, Chicago, IL, USA). All tests were two-tailed, with $p < 0.05$ considered statistically significant.

4.3.Results

4.3.1. Baseline characteristics

Baseline characteristics of study participants are presented in Table 4.1. Male participants spent more hours physically active per week compared to females ($p = 0.009$). There were no other differences between males and females. The mean number of physically active hours per week in total population was 4.7 and the mean sodium intake was 3305 mg (8.3 g of salt).

Table 4.1 Baseline characteristics of study participants (n = 24). Data presented as mean ± SEM or absolute and relative frequencies. P value for difference between males and females (Independent samples t-test, Mann-Whitney U test, Fischer's exact test).

	Male (n = 6)	%	Female (n = 18)	%	p
Age (years)	25.8 ± 1.6		26.9 ± 1.3		0.652
Race (n)					
White	5	83.3	11	61.1	0.621
Other	1	16.7	7	38.9	
BMI (kg/m²)	24.6 ± 1.6		24.2 ± 1.1		0.854
Smoking status (n)					
Yes	1	16.7	0		0.250
No	5	83.3	18	100	
Physical activity (hour per week)	8.3 (4.5) ^a		2.8 (6.5) ^a		0.009
STDT (n)					
Low	4	66.7	10	55.6	1.000
High	2	33.3	8	44.4	
STRT (n)					
Low	2	33.3	10	55.6	0.640
High	4	66.7	8	44.4	
Mean sodium intake (mg)	3775 ± 152		3148 ± 302		0.078
Mean sodium intake (mg/1000 kcal)	1308 ± 144		1644 ± 124		0.164

a, median (interquartile range), body mass index (BMI), salt taste detection threshold (STDT), salt taste recognition threshold (STRT).

4.3.2. SNP genotyping results

Observed genotype and allele frequencies, with an example allelic discrimination plot for the rs8065080, are presented in Figure 4.1 and Table 4.2. From the table, it can be observed that the genotype and allele frequencies for the rs7571842 and rs10177833 overestimate the prevalence of the A allele whereas allele frequencies of the rs239345 and rs8065080 reflect the ones expected in similar populations (NIH, 2017).

Table 4.2 Observed genotype and allele frequencies for the SNPs in the *SLC4A5* (rs7571842, rs10177833), *SCNN1B* (rs239345) and *TRPV1* (rs8065080) genes in the study population (n=24).

	Genotype	Observed Number (%)	Allele frequency	
rs7571842	A/A	11 (48)	A	G
	A/G	8 (35)	0.65	0.35
	G/G	4 (17)		
rs10177833	A/A	14 (58)	A	C
	A/C	6 (25)	0.71	0.29
	C/C	4 (17)		
rs239345	T/T	14 (64)	T	A
	T/A	6 (27)	0.77	0.23
	A/A	2 (9)		
rs8065080	T/T	12 (50)	T	C
	T/C	9 (37)	0.69	0.31
	C/C	3 (13)		

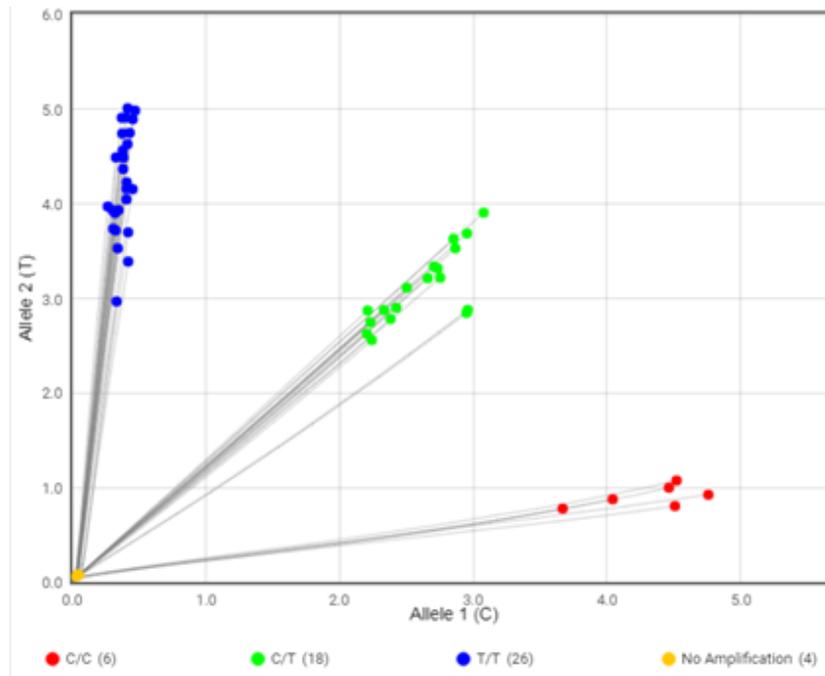


Figure 4.1 Allelic discrimination plot for the *TRPV1* SNPs rs8065080. Plot is demonstrating the separation of the three genotypes, based on the intensity of the reporter dye (VIC, FAM) fluorescence.

4.3.3. Genetic predisposition to altered salt taste perception and dietary salt intake

As shown in Figures 4.2 and 4.3, the proportion of study participants with low and high salt taste recognition thresholds was similar according to genotypes of interest. The results of a Cochran Armitage test of trend between the different genotype groups (homozygous major allele, heterozygous and homozygous minor allele) and the proportion of participants with low and high STDT were: rs7571842 ($p = 0.673$), rs10177833 ($p = 0.928$), rs239345 ($p = 0.471$), rs8065080 ($p = 0.181$). Similar was observed for STRT: rs7571842 ($p = 0.716$), rs10177833 ($p = 0.591$), rs239345 ($p = 0.515$), rs8065080 ($p = 0.379$). Logistic regression analyses yielded

similar results (data not shown). Similar to salt taste perception, genotype was not associated with energy adjusted salt intake (Table 4.3).

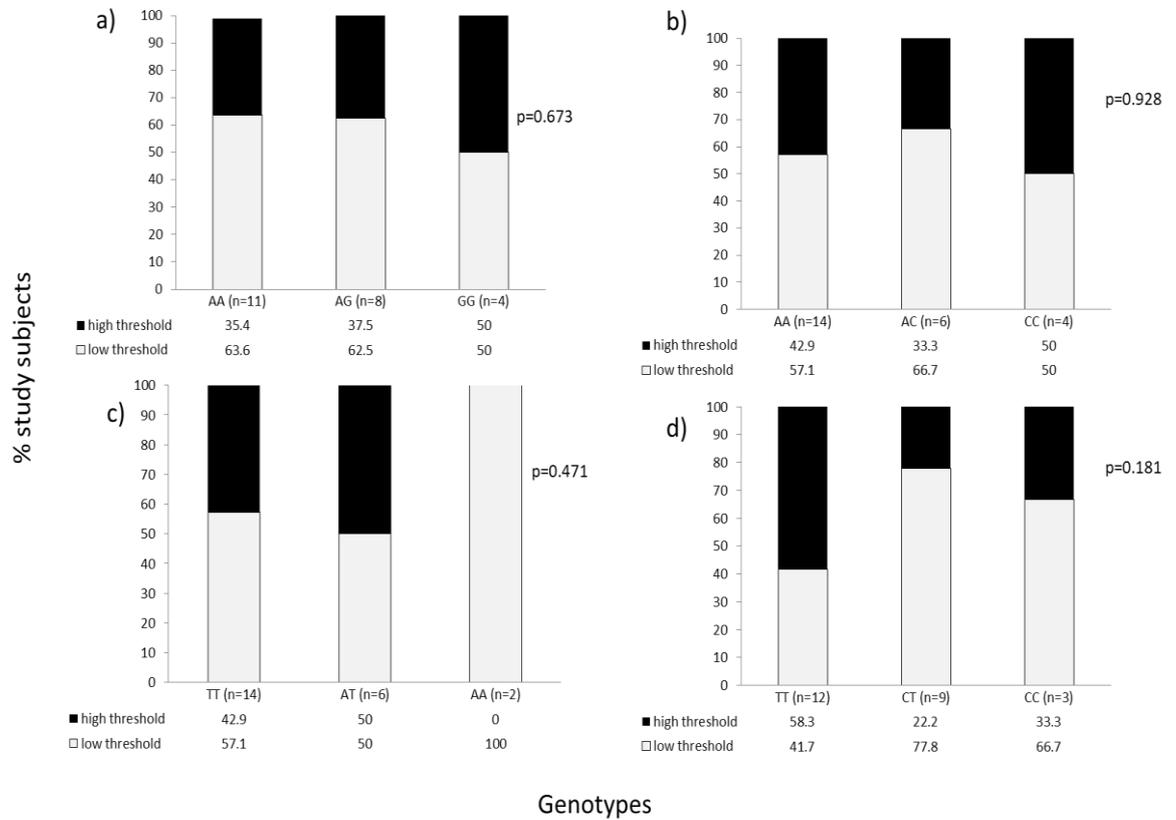


Figure 4.2 Proportion of participants (n = 22-24) with low and high salt taste detection thresholds according to *SLC4A5* rs7571842 (a) and rs10177833 (b), *SCNN1B* rs239345 (c) and *TRPV1* rs8065080 (d) genotype (Cochran Armitage test of trend).

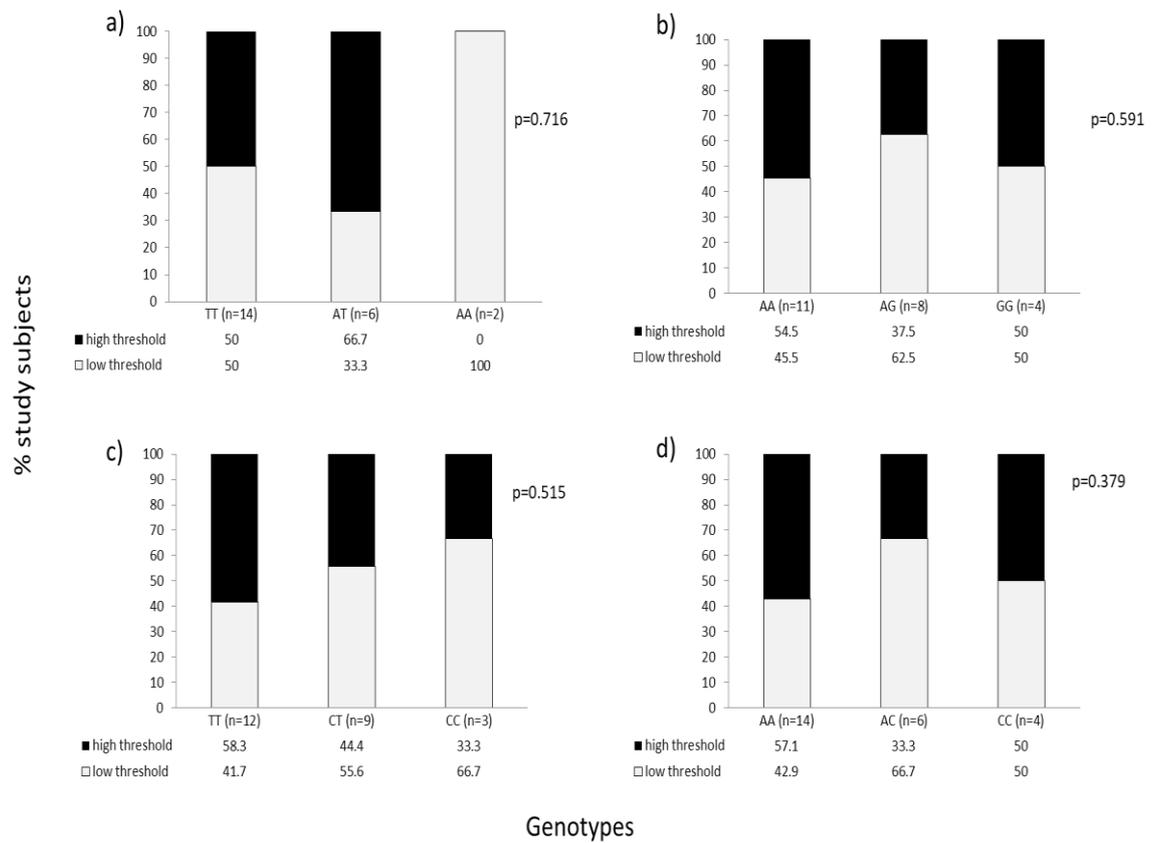


Figure 4.3 Proportion of participants (n = 22-24) with low and high salt taste recognition thresholds according to *SLC4A5* rs7571842 (a) and rs10177833 (b), *SCNN1B* rs239345 (c) and *TRPV1* rs8065080 (d) genotype (Cochran Armitage test of trend).

Table 4.3 Multiple regression analyses for sodium intake (mg/1000 kcal) (n = 22-24)

	Reference genotype	β	95% CI	p	p*
rs7571842	AA	169	-281, 622	0.442	0.600
rs10177833	AA	192	-242, 628	0.368	0.458
rs239345	TT	-125	-613, 363	0.598	0.515
rs8065080	TT	79	-357, 514	0.712	0.762

* adjusted for age, sex and BMI, confidence interval (CI).

4.3.4. Associations between salt taste perception and salt intake

As shown in Table 4.4 there was no relationship between salt taste perception and energy adjusted sodium intake. Introducing the interaction term (threshold x genotype) to the regression model did not change the results suggesting that genotype does not moderate the threshold- salt intake relationship in this study population. In addition, there was no association between salt taste thresholds and discretionary salt intake (adding salt while cooking/at the table) (Figure 4.4 and 4.5). Similar was observed when stratifying according to genotype and when logistic regression was performed (data not shown).

Table 4.4 Multiple regression analyses for sodium intake (mg/1000 kcal) (n = 24)

	β	95% CI	p	p*
STDT (mol/l)	312	-765, 1389	0.554	0.582
STRT (mol/l)	-126	-587, 334	0.575	0.588

* adjusted for age, sex and BMI; confidence interval (CI), salt taste detection threshold (STDT), salt taste recognition threshold (STRT).

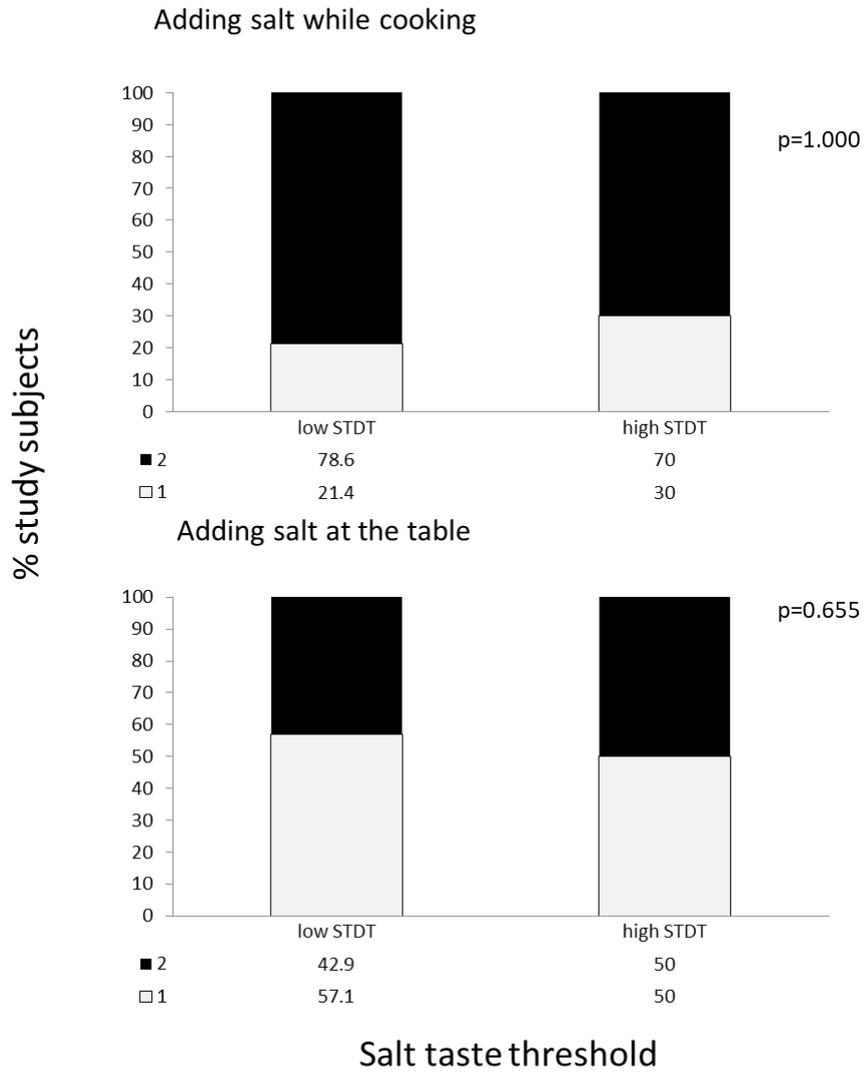


Figure 4.4 Discretionary salt intake according to salt taste detection threshold (low STDT n = 10, high STDT n = 14). 1: participants that add salt; 2: participants that do not add salt (Fisher's exact test). Salt taste detection threshold (STDT).

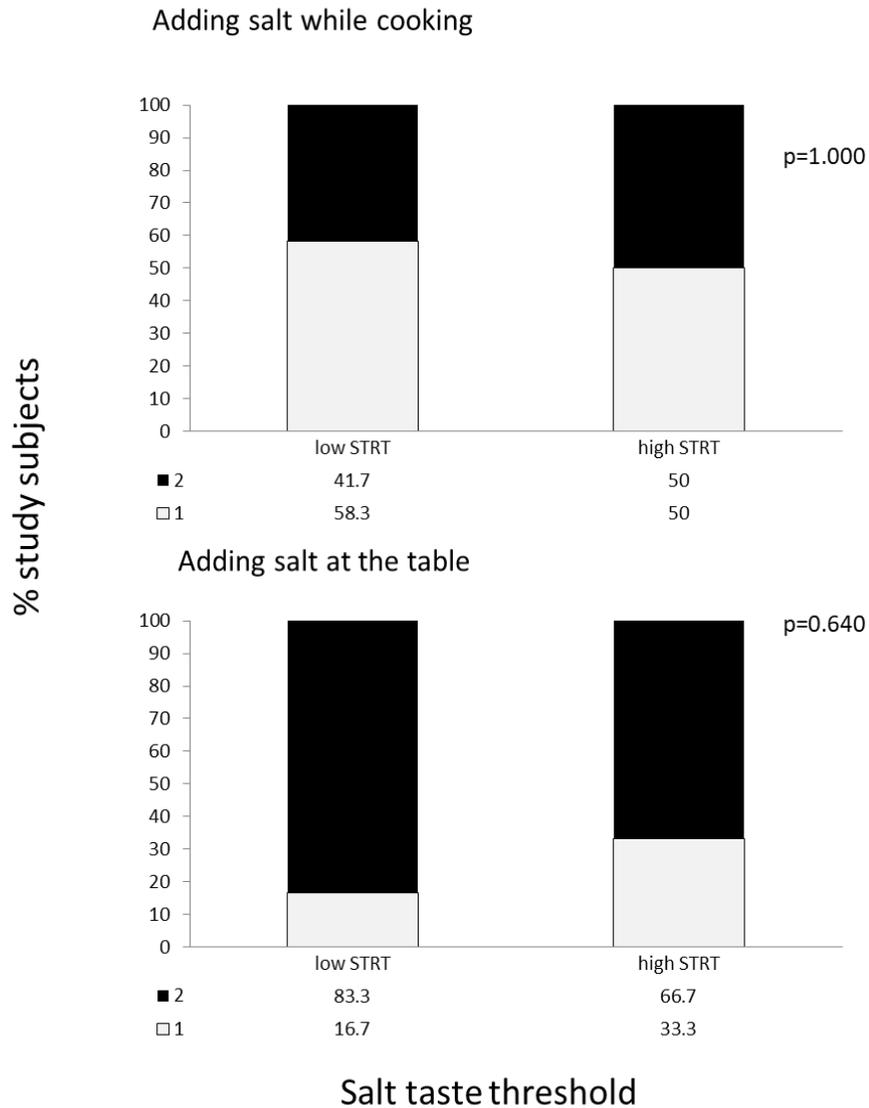


Figure 4.5 Discretionary salt intake according to salt taste recognition threshold (low STRT n = 12, high STRT n = 12). 1: participants that add salt and 2: participants that do not add salt (Fisher's exact test). Salt taste recognition threshold (STRT).

4.4. Discussion

The aim of the present study was to conduct a Chapter 3 confirmation study in a population with similar demographic characteristics. The focus of this study was salt taste perception - salt intake relationship measured with another validated method of assessing dietary salt intake, 24-hour recall. Similar to the findings reported in Chapter 3, there was no association between salt taste perception and salt intake in the total study population. Genotype did not moderate these associations. In addition, similar to Chapter 3, there was no association between genetics and salt taste perception and genetics and salt intake. These results may suggest that salt intake is determined by factors other than salt taste perception and that the association between the two variables does not depend on the method of measuring salt intake. Nevertheless, these results should be interpreted with caution as the study populations in Chapter 3 and 4 were different, albeit with similar demographic characteristics. These limitations, together with other limitations and alternative approaches, will be discussed in following sections.

4.4.1. Genetic predisposition to altered salt taste thresholds and salt intake

As in the previous chapter, there was no association between genetic variants in the *SLC4A5*, *SCNN1B* and *TRPV1* genes and salt taste thresholds. This thesis, for the first time, explored if the *SLC4A5* gene is associated with salt taste perception. No effect suggests that other genetic variants or their interactions may have more pronounced effects. Conversely, it was reported that minor allele carriers of the

rs239345 (*SCNN1B*) and the rs8065080 (*TRPV1*) had lower suprathreshold salt taste sensitivity (Dias et al., 2013). However, Dias et al. (2013) suggest how a large number of SNPs examined in each gene in their study increases the likelihood of a false positive association and that further studies aiming to replicate their findings with *TRPV1* and *SCNN1B* are needed.

The lack of association observed between detection thresholds and the above-described genetic variants in the current study aligns with the findings of Dias et al. (2013). In the current study also, there was no apparent genetic predisposition to altered recognition threshold. Wise et al. (2007) reported the lack of significant heritable component for the recognition thresholds for salty taste. However, the lack of heritability observed by the authors may be due to study design and imbalance between the sample size of monozygotic versus dizygotic twins which, if balanced, could provide the power needed to detect some influence of genetic factors for salt taste perception.

Recognition threshold was not explored in the study by Dias et al. (2013). In this thesis, it was included in the analysis as the heritability estimates to determine the effect of genetics on salt taste do not provide information on any specific genes. Furthermore, the proportion of population variation that is due to heritability is not a valid estimate of the proportion that is due to genetic factors (Vineis and Pearce, 2011). In addition, recognition thresholds have important applications as indexes to the magnitude of a compound's chemical activity towards a taste receptor (Schallenberger, 1993). Both *SCNN1B* and *TRPV1* are expressed in taste receptor cells (Dias et al., 2013).

With the above-described, the difference in the type of thresholds measured

between this thesis and Dias et al. (2013) may provide an explanation for the lack of effect observed in this study. It was shown that detection thresholds for sucrose do not correlate strongly with suprathreshold ratings of sweetness (Faurion et al., 1987). An explanation may be the underlying physiological mechanisms for these perceptual traits are distinct despite their activation by the same stimulus (Wise and Breslin, 2013). Wise et al. (2007) noted that some participants reported other taste qualities at lower concentrations (i.e., sweet or bitter for citric acid, or sweet for sodium chloride) before reaching concentrations that elicited the expected qualities of sour and salty. Moreover, when exploring the association between salt taste detection and recognition threshold, in 22 healthy adults, they found poor correlation between the two thresholds explaining it, in part, by their engagement in different underlying physiological mechanisms for this particular stimulus. However, they also state how individual differences in sensitivity seem to interact with the methodological differences, discussed in Chapter 1. Additionally, the taste quality of salt stimulus can be concentration dependent (Wise et al., 2007; Galindo-Cuspinera et al., 2009) which may explain the associations observed with suprathresholds in Dias et al. (2013) but not with lower concentrations used in this study. Finally, the effect of the *SCNN1B* and *TRPV1* SNPs may exist and be observed in a larger sample size study.

Together with salt taste perception, genotype did not predict salt intake in the present study which aligns with the findings reported in Chapter 3. Even though certain genes coding for sodium transport proteins appear to be associated with salt intake (Kho et al., 2012; Smith et al., 2016), we did not detect an effect of the *SLC4A5* rs7571842 and rs10177833 in the study populations of this thesis. It is not to exclude their potential effect in a different and larger study population. However,

this study was primarily intended as a confirmation study exploring the association between salt taste perception and salt intake and as such, no association between genetics and salt taste perception and salt intake may be a consequence of inadequate statistical power to detect an existing effect.

4.4.2. Association between salt taste thresholds and salt intake

Regarding the salt taste perception - intake relationship, as in the previous study, no association was found between either of the thresholds and salt intake. As discussed, 24-hour recall is another valid method of measuring total salt intake (McLean, 2014). When comparing the absolute salt intake between the two studies (Chapter 3 and 4), the intake in the first study was slightly lower and was 6.9 g compared to 8.3 g in the present study. The latter reflects current intakes, measured from 24-hour urine samples, in the UK population (Department of Health, 2016) implying the two dietary recalls may be more representative of total salt intake.

The fact that no association was observed between salt taste thresholds and salt intake may imply that factors other than taste thresholds affect salt intake. The study participants in this study were predominantly young adults, healthy, non-smoking and physically active. In a study by Drewnowski et al. (1996), salt taste perception was not related to sodium consumption, assessed with one 24-hour recall and 14 consecutive food records, in a sample of 24 young adults aged 20 to 30 years. The authors tested the participants' ability to taste the intensity of and acceptability of five aqueous salt solutions as well as their hedonic preference and the desire to consume chicken broths of four different sodium concentrations. A regression

analysis failed to show that individual hedonic response for salt in soup would predict the actual sodium consumption.

As already stated, taste preferences, in addition to physiological and genetic parameters, are influenced by a range of demographic and sociocultural variables (Drewnowski, 1997). In healthy and younger populations such as the one in this study, concern about nutrition and health may be the factor more important than the taste in food choices and food consumption. Kho et al. (2013) suggest that health conscious people may intentionally lower their salt intake. This may imply that higher levels of salt knowledge may result in lower intake of salt. Sarmugam and Worsley (2014), however, in their systematic review of 22 studies conducted across the world, including the UK, point out to limitations in methods of measuring salt knowledge. The authors state that the conclusions derived from these studies are unlikely to assess the true relationship of salt knowledge with behaviour and call for the development of validated comprehensive salt knowledge questionnaires. Salt knowledge may be associated with level of education and social status (Sarmugam et al., 2013), however this was beyond of the scope of this study.

Even though the results of this thesis suggest no association between salt taste thresholds and intake which was reported both in Chapter 3, where FFQ was used, and in Chapter 4, where two 24-hour recalls were employed, it is not to exclude the results would be different if the same population participated in both studies. While the populations were recruited from the similar pool of participants, were similar in terms of age, sex and race and both comprised of healthy individuals, other factors, such as social status or nutritional knowledge, associated with salt intake may differ between these two populations. This may have affected the results

and a preferred approach would be employing both FFQ and 24-hour recall in the same study population. This was the case in the study by Piovesana et al. (2013) described in the introduction of this chapter. The authors obtained different results depending on the method of measuring salt intake. However, the study population was Brazilian and the FFQ validated in low-income hypertensive Brazilian patients. In addition, only one 24-hour recall was employed and there was no control for completeness of urine collections which may have affected the accuracy of sodium intake estimation. In this sense, it is unlikely that similar results would be expected in this study population, comprising healthy UK adults. Therefore, it is suggested that future studies employ different methods of measuring salt intake in the same study population to explore the potential effect of methods to measure salt intake on taste-intake relationship.

4.4.3. Strengths and limitations

A strength of this study is the use of two 24-hour dietary recalls representing both dietary intake over the weekday and the weekend. By using more than one 24-hour recall, accuracy of total sodium intake measurement increases (Freedman et al., 2015). Furthermore, discretionary salt intake was quantified which was not the case with AMPM (Rhodes et al., 2013) suggesting this 24-hour recall may capture total salt intake more accurately. This is, nevertheless, only a speculation and future studies should validate the 24-hour recall used in this study against urine biomarker method to assess the actual accuracy.

A limitation of this study, as well as other studies using a dietary method to

measure salt intake, is the reliance on food composition databases to calculate intake of salt. Non-discretionary salt use accounts for the majority of salt intake in industrialised countries and it is supplied through commercially manufactured foods. However food composition tables do not usually reflect changes or differences in the salt content of processed foodstuffs by manufacturer, brand, or major restaurant chain (Conkle and van der Haar, 2016). Pennington et al. (2007) acknowledged that these databases are never complete because of the dynamic nature of the food supply. This may be the case in the UK, since the UK Salt Reduction Programme continuously publishes new salt reduction targets as guidance for food manufacturers and business, which require constant updating in the food composition databases (PHE, 2017). The food composition database used in this thesis was the latest edition of the The Composition of Foods Integrated Dataset which included McCance and Widdowson “The Composition of Foods” (Finglas et al., 2015). Even though it is commonly used by researchers, the above-described issues as well as the inability to accurately determine salt intake that comes from food preparation at home, restaurants, canteens, or catering operations, highlight the fact that the accuracy of the results may have been affected. As stated in Chapter 1, dietary methods tend to underestimate true intakes of sodium. Kelly et al. (2015) reported a weak correlation between 24-hour urinary sodium excretion and sodium intake obtained with a 24-hour dietary recall where calculations of sodium intake were based on McCance and Widdowson’s Food Composition Tables. From these results, it is, however, difficult to define the extent to which the validity of sodium intake data may be affected by limitations in food composition tables or the limitations attributed to participants’ reporting of dietary intake. Nevertheless, constant updating and expanding of food composition tables is necessary to accurately measure dietary intake of salt.

Additionally, the food composition database used in the AMPM validation study was USDA Food and Nutrient Database for Dietary Studies which is updated every two years to reflect changes in food supply. Sodium values in the database included the inherent sodium in foods as well as sodium added during processing as sodium chloride or other sodium-containing additives. Moreover, estimations of sodium intake included salt added in cooking or food preparation and the salt content of homemade dishes as well as some commercial products calculated by using recipes based on popular cookbooks or product nutrient profiles (Rhodes et al., 2013). The limitations of food composition databases to calculate salt intake acknowledged above may also apply to the USDA database.

Furthermore, the 24-hour recall used in this study was based on the USDA AMPM as the latter was validated against a 24-hour urine measurement and yielded comparable results. However, the study population in the AMPM validation study was aged 30–69 years and recruited from Washington metropolitan area. Considering the differences in demographic characteristics of different populations, the generalisability of these results to other populations, such as the one in this study, may be limited. A more valid approach would be to validate the 24-hour recall against 24-hour urine sodium measurement in this study population. If the results are satisfactory, the dietary method may be used instead of urine biomarker in a study exploring the association between salt taste perception and intake.

Finally, required sample size was not calculated *a priori* for the present study. *Post-hoc* sample size calculation, with the association between salt taste thresholds and salt intake as the main outcome, revealed that to conduct multiple linear regression with the medium effect size (Cohen's $f^2 = 0.15$), a power of 0.8 and

α of 0.05 and five predictors, required sample size would be 68. Taking into account respective allele frequencies may be facilitated by screening participants based on their genetic profile prior their inclusion in the study. If this was the indeed the case, then the sample required to screen would increase to 136 for the *SLC4A5* SNPs or 204 if the lowest MAF for the SNPs explored in this thesis is considered, which would be 0.3 for the *TRPV1* rs8065080 (NIH, 2017). This suggests that this study is underpowered and highlights the possibility of type 2 error considering no associations were found.

4.5. Conclusion

This study confirmed the results of the original study. There was no association between genetics and salt taste perception, genetics and salt intake as well as salt taste perception and salt intake in the study population. It is suggested that factors other than taste thresholds may influence total dietary intake of salt in healthy and younger participants such as salt knowledge, level of education or social status and warrant further research. Nevertheless, considering the limitations of the present study, future studies should explore the potential effect of different methods of measuring salt intake by employing the FFQ, 24-hour recall and 24-hour urine sodium measurement in the same study population. This is also suggested to validate the accuracy of the 24-hour recall used in this study against the “gold-standard” urine biomarker method.

Chapter 5 Protein expression in salt sensitivity – potential for biomarker discovery?

5.1. Introduction

This study follows up on the study presented in Chapter 2 and the observed effect of *SLC4A5* rs7571842 on salt-sensitive changes in BP. As discussed in Chapter 1, genetic association studies are a valuable tool in providing insight into the physiological role of specific genes by identifying links to particular phenotypes or diseases (Love-Gregory et al., 2011). However, the results are rarely replicated and functionally validated (Carlton et al., 2006; Love-Gregory et al., 2011).

Regarding salt sensitivity, Ray et al. (2016) have identified participants with salt-sensitive or salt-resistant BP, based on the described GenSalt dietary method, and sequenced their DNA for ENaC SNPs in *SCNNIA*, *SCNNIB*, and *SCNNIG* genes. The authors aimed to explore the functional effects of the ENaC variants by expressing them in *Xenopus laevis* oocytes and comparing amiloride-sensitive current amplitudes. In brief, cDNAs encoding human α , β and γ ENaC subunits were mutated to introduce selected variants. They were then transcribed in vitro and synthesised cRNAs were injected into oocytes. This was followed by incubation of oocytes and subsequent electrophysiological recordings. The changes in sodium self-inhibition or cell surface expression were explored for variants that altered channel activity. Finally, the authors examined whether gain-of-function variants were significantly more common in salt-sensitive study participants. The results revealed variants that increased or decreased channel activity. Several variants altered channel inhibition by extracellular sodium (sodium self-inhibition) and two variants had an opposite effect on cell surface expression, with one variant decreasing and one increasing the protein expression. Association between these variants and salt sensitivity, however, did not reach statistical significance.

Regarding the *SLC4A5*, one of the main foci of this thesis, Gildea et al. (2015) reported the localization and expression of its protein, NBCe2, in the apical membrane of the renal proximal tubule cell, where the majority of sodium is reabsorbed. It is also found in the cortical collecting duct, but not in the distal convoluted tubule. The only recently-reported localization of this protein in the human kidney can be explained by the lack of well-characterized antibodies against NBCe2. Newer NBCe2 antibodies have made it possible to determine the location of NBCe2 in the human kidney (Felder et al., 2016). NBCe2 protein is minimally expressed in the human and rodent renal proximal tubule under basal conditions and this may have limited the detection of its protein expression by immunohistochemistry and its functional activity in studies conducted to date (Felder et al., 2016). Nevertheless, Gildea et al. (2015) demonstrated vesicle-like structures at the apical membrane in polarised renal proximal tubule cells with internal reflection fluorescence microscopy, and confirmed the NBCe2 localization. This provides potential for measuring the difference in concentration of this protein between salt-sensitive and salt-resistant individuals, together with the functional effects of the *SLC4A5* SNPs. To date, the *SLC4A5* SNPs associated with distinct salt sensitivity phenotype are intronic rs7571842 and rs10177833.

As described, non-coding SNPs may affect the transcription factor-binding site of the gene which can affect the level, location, or timing of gene expression. In addition, a SNP at the 'splicing site' may break the consensus splicing site sequence resulting in a different form of a protein (Yuan et al., 2006). For example, CD36 is a gene associated with, among other, type 2 diabetes, metabolic syndrome and obesity (Febbraio and Silverstein, 2007). The rs3211909 lies within a CD36 intronic enhancer site and has been shown to reduce its protein expression in minor allele

carriers (Love-Gregory et al., 2011).

Regarding salt sensitivity, Felder et al. (2016), in their recent review, report that the *SLC4A5* rs10177833 may lead to an increase in expression and activity of NBCe2. HNF4A is a transcriptional regulator present in the renal proximal tubule and capable of inducing the mRNA expression of a number of genes important for proximal tubule identity and function, including solute carrier (SLC) and ATP-binding cassette (ABC) drug transporters (Martovetsky et al., 2016). The authors reported that *SLC4A5* rs10177833 caused an increase in HNF4A binding to the *SLC4A5* gene resulting in an increase in NBCe2 mRNA, NBCe2 protein expression, and increased NBCe2-mediated bicarbonate and sodium transport under conditions of elevated intracellular sodium (Felder et al., 2016).

Since the authors of the above-described studies focused on the NBCe2 expression in the renal proximal tubule, one of the key methods used in their research was “virtual renal biopsy”. This method entails isolation of renal proximal tubule cells from freshly voided urine of patients (Gildea et al., 2013a). A few research groups have successfully isolated these cells to date, with the aim of measuring expression and activity of various transport proteins or generation of pluripotent stem cells (Laube et al., 2005; Rahmoune et al., 2005; Zhou et al., 2012; Hauwaert et al., 2013; Gildea et al., 2013a). The method is based on isolation and immunomagnetic separation of proximal tubule cells using an adequate antibody and subsequent primary cell culture (Gildea et al., 2013a). The maintenance of cells in culture depends on the experiment to be conducted. With the aim of replicating this method and further exploring the potential of the “virtual renal biopsy” in terms of protein expression measurement, a primary cell culture of proximal tubule cells was

attempted as a part of this PhD project. The cells were isolated from freshly voided urine samples of participants diagnosed for salt sensitivity. The Appendix E comprises the methods used to isolate and culture renal proximal tubule cells. However, the maintenance of cells in culture was shown to be difficult, as confirmed by Gildea et al. (2012), putting their use in more applied aspects of salt sensitivity research, such as a rapid and easily detectable biomarker, into question. Therefore, another method was explored and will be the focus of this chapter.

The AHA urges for a salt sensitivity biomarker (genetic or other), ideally from an easily accessible tissue such as blood or urine. In their recent scientific statement they suggest such biomarker should be able to identify patients in whom reduced sodium intake would lead to a clinically important reduction in BP which would in turn help clinicians target therapy (Elijovich et al., 2016). From a personalised nutrition perspective such biomarker, together with other phenotypic traits associated with salt sensitivity such as age, race, and sex, could enable more targeted and effective dietary advice. If salt-sensitive population is identified while still healthy (i.e. normal BP) such dietary advice could result in more effective and successful CVD prevention. In addition, development of an easily obtained, inexpensive marker for salt sensitivity could allow the assessment of potential differential effects of reduced sodium intake on cardiovascular morbidity in salt-sensitive versus salt-resistant individuals (Elijovich et al., 2016). Finally, as a complex polygenic trait, salt sensitivity is associated with a number of genetic variants involved in different biological pathways which warrant further investigation. This is, among other reasons, due to the lack of GWAS examining salt sensitivity. The labour-intensive methods of diagnosing salt sensitivity constitute a major barrier in conducting large scale studies such as GWAS and would be

facilitated by the discovery of an easily measurable biomarker for this phenotype (Elijovich et al., 2016).

Urinary exosomes hold promise in discovery and development of such biomarker. Exosomes are small (40–100 nm) secreted membrane vesicles that are formed by inward budding of endosomal membranes which are released from the cell by fusion of the multivesicular body with the cell membrane. They contain plasma, proteins and RNA of the cells of origin (Figure 5.1). Exosomes can be isolated from different body fluids such as saliva, plasma and urine. Their function may be in cell-to-cell communication and intercellular protein and RNA exchange (Zhou et al., 2006).

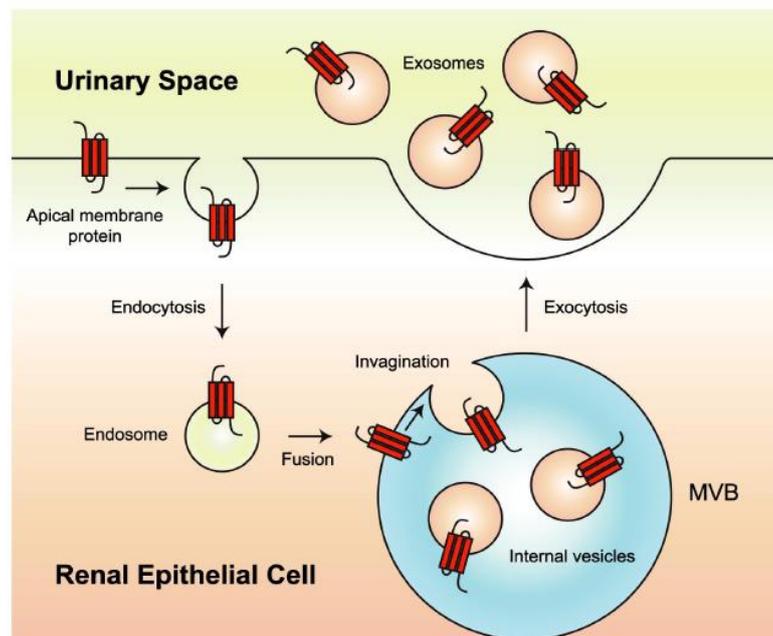


Figure 5.1 Formation and excretion of urinary exosomes (Pisitkun et al., 2006). Apical membrane proteins undergo endocytosis followed by targeting to the multivesicular body (MVB). The membrane proteins are segregated initially in the MVB outer membrane and then are internalised by membrane invagination, encapsulating cytosolic proteins in the process. After accumulation of numerous internal vesicles, the outer membrane of the MVB fuses with the apical plasma membrane releasing its internal vesicles, called exosomes, into the urinary space. Exosomes contain both membrane and cytosolic proteins (Pisitkun et al., 2006).

Exosomes are potentially a more targeted source of material for biomarker discovery than unfractionated urine (Street et al., 2017). They provide a unique view of renal metabolic activity (Gildea et al., 2013b) and the formation and excretion of microvesicles is theorised to take place in every segment of the renal tubulus (Rood et al., 2010). Alterations in the expression of different proteins present in the epithelial cells of the distal convoluted tubule, including NCC, are reflected in the composition of urinary exosomes (Tutakhel et al., 2017). Besides sodium transporters expressed in distal tubule, urinary exosomes contain proteins expressed in other nephron segments such as proximal tubule. These are, among others, NBCe1 (SLC4A4) and NHE3 (Gonzales et al., 2009). The predominantly used method for urinary exosomes isolation is differential ultracentrifugation which usually entails few ultracentrifugation steps with typical duration of ~2 hours. In addition, the number of samples that can be analysed largely depends on the number of slots in the rotor. Therefore, a rapid and simple method has been suggested (Cheruvanky et al., 2007).

In the context of the above-described, the *SLC4A5* rs7571842 has been associated with salt-sensitive increase in BP in this thesis, with the literature on its potential effects on protein expression in younger and healthy population being scarce. Moreover, with the need for discovery of an easily measurable salt sensitivity biomarker there are no studies to date reporting on an easily performed, rapid and a method readily used in a clinical setting for the measurement of the *SLC4A5* concentration from urinary exosomes. Pisitkun et al. (2006) suggest how the goal in biomarker research should be to identify proteins and peptides explicitly and this should be achieved with more cost-effective technologies such as antibody-based microarrays or enzyme-linked immunosorbent assay (ELISAs).

Thus, the aim of this study is to explore if the rs7571842 genotype is associated with phenotype (SLC4A5 protein level) using rapid and methods often used in a clinical setting. This will be a proof of principle study with its objectives being:

- To isolate urinary exosomes using a rapid and inexpensive method.
- To explore if the SLC4A5 protein concentration from urinary exosomes can be measured using commercially available ELISA kit.
- To measure the difference in SLC4A5 protein concentration between the participants previously diagnosed for salt sensitivity and homozygous for A or the G allele of the rs7571842.

5.2. Methods

5.2.1. Study participants

The study participants were four healthy females that have participated in the first study of this thesis (Chapter 2) and have been diagnosed for salt sensitivity with the dietary method. They were also genotyped for the *SLC4A5*, *SCNN1B* and *TRPV1* SNPs. The two participants were homozygous for the G allele and the other two for the "risk" A allele of the rs7571842. For the purpose of this study, the participants provided a spot urine sample. All other information was collected during previous examinations.

This study was conducted according to the guidelines stated in the Declaration of Helsinki and all procedures involving human participants were approved by the Institutional Ethics Committee (Appendix A). Written informed consent was obtained from each participant before the baseline data collection informing they can withdraw from the study at any point.

5.2.2. Urine sample collection, storage and transport

Fresh second morning urine samples were collected from study participants and stored at -80 °C for seven days until shipped on dry ice to the Molecular Biology laboratory, Department of Nutrition, Food Science and Physiology of the University of Navarra, Spain. Aliquots (1.5 ml) of each sample were kept at -80 °C at the Biochemistry laboratory at St Mary's University until analysed for creatinine excretion.

5.2.3. Urine sample preparation for exosome isolation

Upon arrival, urine samples were thawed and protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Basel, Switzerland) added immediately after thawing. Samples were aliquoted into 5 ml and 40 ml aliquots and vortexed extensively before treatment with NaOH, aimed at disrupting exosome membranes. In brief, 0.4 N NaOH was added to urine samples for 20 minutes under constant shaking and neutralised with HCl, as suggested by Sasaki et al. (2016).

5.2.4. Urinary exosome protein isolation

Urinary exosomes were prepared with a rapid method by Cheruvanky et al. (2007). Nanomembrane concentrators (Vivaspin 20, molecular weight cut-off 50 kDa, Sartorius Inc., Goettingen, Germany) were first washed to remove glycerol and other preservatives by adding approximately one volume of phosphate buffered saline (PBS) buffer then centrifuged at $3,000 \times g$ at room temperature according to manufacturer's instructions, before processing urine samples. Urine samples were centrifuged at $17,000 \times g$ at 4°C for 15 min after which 5 ml or 20 ml of urine supernatant was added to a Vivaspin nanomembrane concentrator and centrifuged at $3,000 \times g$ at 20°C for 27 min, as per manufacturer protocol. The urine proteins (~0.2 ml) were recovered from the bottom of the concentrate pocket with a pipette. Two 20 ml sample retentates (concentrated samples) from the same participant were pooled together to form a 40 ml original urine sample retentate. Recovered protein samples were stored at -20°C until further analysed.

5.2.5. SLC4A5 sandwich ELISA

To measure the SLC4A5 concentration in urinary exosome protein fraction previously isolated using a nanomembrane concentrator, a commercially available human SLC4A5 sandwich ELISA kit was used (Bioassay Technology Laboratory, Shanghai, China). The assay was performed according to manufacturer instruction. In brief, the standards were prepared prior to running an assay and 50 μ l was added to standard well. Sample (40 μ l), anti-SLC4A5 antibody (10 μ l) and streptavidin-HRP (50 μ l) were also added to all wells, besides blank control wells, and the plate was sealed and incubated for 60 minutes at 37 °C. The plate was then washed with the wash buffer five times when 50 μ l of substrate A and the equal volume of substrate B solution were added to each well. The plate was sealed and incubated for 10 min at 37 °C. The reaction was stopped by adding 50 μ l of stop solution and the optical density (OD) was measured at 450 nm using a Multiskan plate reader (Thermo Fisher Scientific, Waltham, MA, USA). After the blank well OD readings were subtracted from all standard and sample wells, the standard curve was generated based on the OD readings for standards of known concentration (Figure 5.2). Since urinary protein excretion rate differs throughout the day and creatinine excretion is fairly constant, the obtained SLC4A5 sample concentration was expressed as ng/mg creatinine (Warrell et al., 2003). The assay was performed at room temperature and each sample was tested in duplicate. The coefficient of variation (CV) was: % CV = 16.

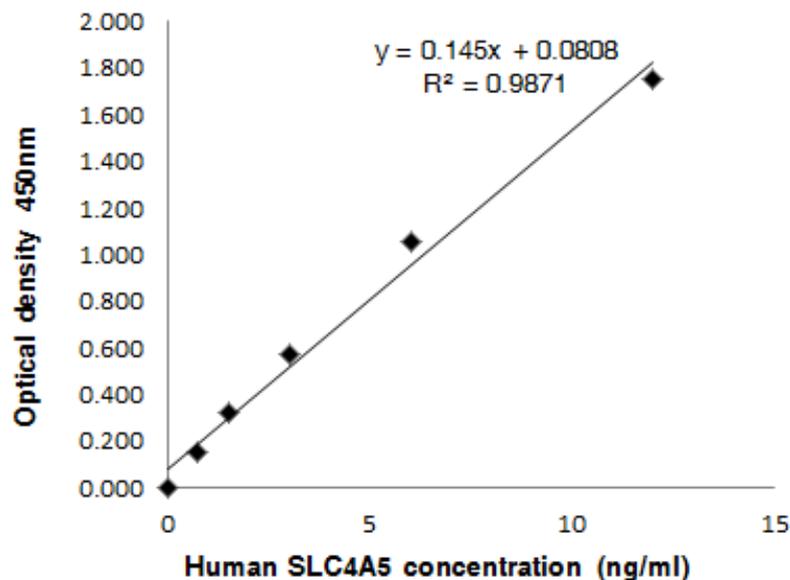


Figure 5.2 Standard curve for the human SLC4A5.

5.2.6. Urinary creatinine excretion

Urinary creatinine excretion was measured using a creatinine colorimetric assay kit (Cayman Chemical, MI, USA). This method is based on the Jaffe' reaction, wherein a yellow/orange colour forms when the metabolite is treated with alkaline picrate. The assay was performed in duplicate according to manufacturer instructions. In brief, urine samples were diluted 1:10 before assaying and standards prepared to create a standard curve (Appendix F). Equal volumes (15 μ l) of standard and sample were added to appropriate well, followed by the addition of alkaline picrate solution (150 μ l). The plate was covered and incubated for 10 minutes at room temperature under constant shaking. The initial absorbance was read at 492 nm using a Biochrom Assays Expert Plus plate reader (Holliston, MA, USA), after which 5 μ l of acid solution was added to each well. The plate was covered and

incubated on a shaker for 20 minutes at room temperature. Final absorbance was read at 492 nm. The corrected absorbance was obtained by subtracting the average final absorbance values from the average initial absorbance. Corrected absorbance of standard A was then subtracted from itself and all other standards and samples to obtain the final adjusted absorbance. The final sample concentration was calculated from the standard curve (Appendix F) and, after multiplying with the dilution factor, expressed as creatinine (mg/dl).

5.2.7. Statistical analysis

Baseline characteristics of study participants are presented as mean values. Differences in age and the SLC4A5 protein concentration between participants homozygous for A or G allele of the rs7571842 were tested with a Mann-Whitney U test. Analyses were performed using the SPSS software package (version 22.0, Chicago, IL, USA). All tests were two-tailed, with $p < 0.05$ considered statistically significant.

5.3. Results

5.3.1. Participant characteristics

As shown in Table 5.1, the participants were white females with the mean age of the participants in the AA group 37 and the GG group 41 years. The participants with the rs7571842 AA genotype were salt-sensitive and the GG genotype salt-resistant. The participants were genotyped and diagnosed for salt sensitivity during the first study of this thesis.

Table 5.1 Baseline characteristics of study participants (n = 4) according to the *SLC4A5* rs7571842 genotype. Data presented as mean (individual data points) or absolute (relative) frequencies.

	rs7571842 AA (n=2)	rs7571842 GG (n=2)
Age (years)	37 (27,47)	41 (30, 52)
Race		
White	2 (100)	2 (100)
Other	0	0
Salt sensitivity status		
Yes	2 (100)	0
No	0	2 (100)

5.3.2. Urinary exosome SLC4A5 concentration

SLC4A5 urinary exosome protein concentration was measured with a commercially available sandwich ELISA. During the exosome preparation with the nanomembrane concentrator, one of the 20 ml urine samples was not successfully concentrated, thus, for consistency in results, only 5 ml urine samples were analysed further. Figure 5.3 presents SLC4A5 concentration (ng/mg creatinine) which did not differ between the two rs7571842 genotype groups ($p = 0.667$). When absolute values for the concentrations (ng/ml) were analysed, no difference in expression was observed between the two genotype groups, $p = 1.000$, data not shown).

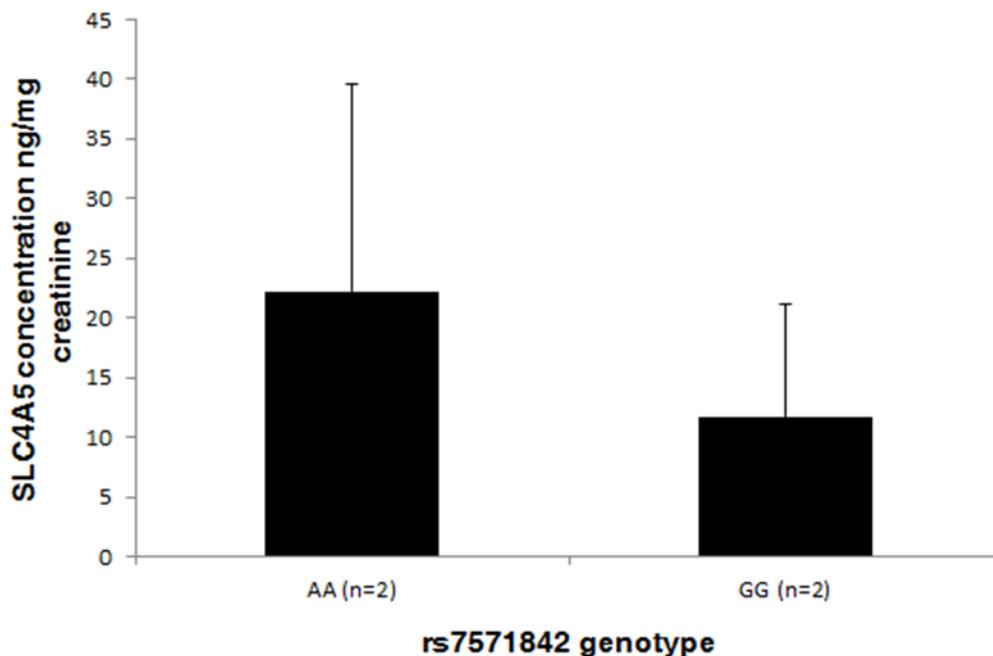


Figure 5.3 SLC4A5 urinary exosome concentration from 5 ml urine samples ($n = 4$) obtained with commercially available ELISA kit. Error bars represent + SEM. Protein concentration is normalised to creatinine (Mann-Whitney U test, $p = 0.667$).

5.4. Discussion

Considering the SBP increasing effect of the *SLC4A5* rs7571842 observed in the first study of this thesis, the aim of this proof of concept study was to explore the difference in the *SLC4A5* protein levels between salt-sensitive participants homozygous for the rs7571842 A allele and salt-resistant participants homozygous for the G allele. Since there is a need for an easily measurable biomarker for this phenotype, the methods used to measure the concentration of this protein were intended to be rapid methods readily used in a clinical setting. Even though results show there was no difference in *SLC4A5* concentration between salt-sensitive and salt-resistant participants, these should be taken into account with caution considering limitations in methods and a low number of participants. These will be discussed in the following sections. Nevertheless, this proof of concept study may serve as a starting point for future projects measuring *SLC4A5* concentration using methods other than Western Blot and with the aim of exploring its potential as a salt sensitivity biomarker. Recommendations for future research with certain critical points to consider will also be discussed in this chapter.

5.4.1. Twenty four hour vs. spot urine sample

Urine samples are often used as a source of protein biomarkers. As such they can be highly variable in terms of volume, protein concentration (particularly in the case of kidney damage or dysfunction), total protein excreted and pH (ranges from 4 to 8). The variability in urine components may be due to age, health status, diet, or other factors related to sample handling such as degradation upon storage. Sample

handling issues will be discussed in the following section (Thomas et al., 2010).

Similar to sodium excretion, there is a high between and within individual variability in urine protein excretion. Thomas et al. (2010) have reported high standard deviations for protein concentrations measured from urine samples of the same individual over the course of 10 days. Standard deviations were lowest for 24 hour collection (39%) and first morning collections (41%), while the second urine samples of the day (54%) and spot collection (61%) exhibited higher variability. Average concentrations were similar, ranging from 26 mg/ml for 24 hour, random spot, and second morning urine to 34 mg/ml for first morning urine. These results indicate there is high variability with each collection method and suggest multiple samples from the same individual may be needed to account for this issue.

With respect to urine collection procedures, 24-hour collections were initially advocated because of the circadian rhythm of urinary protein excretion. However, 24-hour urine collections present high participant burden and are prone to collection errors (i.e. under-collection or over-collection that were discussed in the Introduction). Moreover, long period of collection may lead to degradation and contamination of urine protein, particularly via lysis of suspended cells, as the samples are most commonly stored at 4 °C and transported under ambient conditions (Thomas et al., 2010).

For these reasons, a spot (random) urine sample is often used in clinical and research practice. Such collections present low participant burden and facilitate coordination between patients, clinicians, and researchers. As suggested by Thomas et al. (2010), first morning urine provides the least variability in protein concentration; second morning and random spot urine collection display somewhat

higher variability. However, first morning sample may be subjected to increased proteolysis due to the amount of time spent in bladder, highlighting limitations that can be attributed to each sample type.

Nevertheless, 24-hour samples are still considered as the “gold-standards” against which the results obtained from spot samples may be validated. For the latter, normalisation of the amount or concentration of protein is critical. This can be achieved by calculating ratios to other excreted small molecules or proteins. Creatinine normalisation has often been suggested, since the excretion of creatinine and protein is reasonably constant throughout the day when the glomerular filtration rate is stable (Ginsberg et al., 1983; Thomas et al., 2010). Newman et al. (2000) reported that the mean within individual variation in the protein:creatinine ratio was 38.6%, whereas that of the protein excretion was 96.5%. In a systematic review of 16 studies comparing protein:creatinine ratio in random urine samples with the respective 24-h protein excretion, Price et al. (2005) suggest there is a strong correlation between the protein:creatinine ratio in a random urine sample and 24-h protein excretion. However, primarily in patients with renal disease and preeclampsia. Similarly, Hogan et al. (2016) report on moderate correlation between the random urine protein creatinine ratio and 24-hour urine protein excretion in a group of 302 adult and paediatric patients with glomerular disease (r of 0.60 and 0.67, respectively). The \log_{10} transformation of values strengthened correlations in both groups (r of 0.85 and 0.82, respectively). Teo et al. (2015) reported similar in 232 patients with chronic kidney disease. The correlation coefficient between \log transformed 24-hour urine protein excretion and urine protein creatinine ratio was 0.79. In this sense, studies similar to this one, with the aim of measuring levels of exosomal sodium transport proteins in healthy participants, have used sport urine

samples (Cheruvanky et al., 2007; Isobe et al., 2016). Moreover, Gildea et al. (2013a) collected second morning urine samples from their cohort of healthy salt-sensitive and salt-resistant patients to measure differences in exosomal miRNA expression between the two groups.

Nevertheless, although previous studies have used spot urine samples and the literature suggests such type of sample may replace a cumbersome 24-hour urine collection, validation studies are predominantly conducted in patients with certain renal disease or another health condition, in whom urinary protein excretion may differ to the one in healthy individuals that participated in this study. Therefore, it is difficult to draw definitive conclusions whether similar would be observed in the latter group. For this reason, it would have been appropriate to collect both 24-hour and spot urine samples from participants in this study to explore if there are any differences in total protein and SLC4A5 concentrations between the two methods. This would serve as a validation step with the potential of informing future studies exploring urinary levels of SLC4A5 in healthy and younger population with characteristics similar to this one.

5.4.2. Urinary exosome preparation and NaOH urine pretreatment

Urinary exosome isolation can result in a very large enrichment of urinary proteins that are derived from renal tubule epithelial cells, especially membrane transporters and ion channels (Cheyron et al., 2003; Pisitkun et al, 2006). To date, five different approaches in exosome isolation have been reported, such as ultracentrifugation-based techniques, size-based techniques, exosome precipitation,

immunoaffinity capture-based and microfluidics-based techniques. All of these have both advantages and disadvantages and require further optimisation (Li et al., 2017). Nevertheless, ultracentrifugation method is considered as a “gold-standard” method against which other methods can be compared. This method requires high equipment cost, it is cumbersome, labour intensive and has low portability, thus other rapid and easy to use methods may be more appropriate in a clinical setting (Li et al., 2017). The nanomembrane method used in this study is a size-based technique developed by Cheruvanky et al. (2007). It can isolate urinary exosomes as effectively as a standard ultracentrifugation method, however quicker and with laboratories requiring only a benchtop centrifuge and a nanomembrane concentrator, making the cost of equipment lower. Other advantage of this method is the volume of the urine sample required to isolate exosomal proteins which can be as low as 0.5 ml to detect more abundant proteins such as aquaporin 2 (AQP2).

Considering that the sample handling, such as storage and shipping, was comprehensively explored in previous studies (Zhou et al., 2006; Cheruvanky et al., 2007), this was not considered necessary in the present study. For instance, Zhou et al. (2006) explored the effects of sample handling and storage conditions in the context of preservation of urinary exosomes. The authors used gel electrophoresis and immunoblotting to evaluate protein yield and integrity and suggested the use of protease inhibitors which prevented degradation of samples in a clinical setting. To prevent sample degradation, protease inhibitors were used in the present study.

The authors also confirmed the importance of freezing and mixing of thawed samples after retrieval from low temperature storage. Freezing at $-70\text{ }^{\circ}\text{C}$ resulted in a better recovery of exosomes than did freezing at $-20\text{ }^{\circ}\text{C}$. The samples in the

present study were stored at -80 °C and extensively vortexed after thawing.

Finally, the samples were collected in an institution different to the one where the analysis was conducted. Cheruvanky et al. (2007) have previously reported no difference in exosome protein concentration determined by a Western blot. The authors explored the levels of tumor susceptibility gene 101 protein (TSG101), AQP2, angiotensin-converting enzyme (ACE) and podocalyxin-like protein 1 (PODXL), in urine samples stored at -80°C, placed on wet ice for 24 hr for shipping to a remote laboratory and then stored at -80°C, compared to the fresh samples processed within one hour. The above-described suggests that the handling and storage conditions of the samples used in the present study was conducted according to suggestions provided previously in the literature and should therefore have minimal effects on the final results.

Regarding the methods of protein quantification after exosome preparation, the most commonly reported downstream method is a Western blot analysis (Zhou et al., 2006; Cheruvanky et al., 2007). However, a method such as ELISA may be the most suitable for clinical use because of its high sensitivity, ease of handling (no radioisotope compared to radioimmunoassay and high throughput for testing a large number of clinical samples (Sasaki et al., 2016). Above-described makes it a method of choice in this study which is also, to the best of our knowledge, the first study employing this method in the measurement of the SLC4A5 (NBCe2).

One of the potential issues arising from measuring exosome proteins with this method is the inability of detecting any signal, due to the localization of antibody recognition domains which are often inside exosomes, making lysis of exosome membranes necessary (Sasaki et al., 2016). This may be a potential reason

of the very limited use of ELISA in urinary exosome protein measurement.

However, the disruption of exosome membranes with NaOH has recently been reported as a simple method applicable to ELISA of membrane proteins (Nameta et al., 2016; Sasaki et al., 2016) and has thus been conducted in this study.

There are no studies reporting on the urinary exosome, or from any other source, SLC4A5 levels measured with ELISA. Therefore, urine volumes used in this study reflected a typical clinical sample (< 8 ml) (Cheruvanky et al., 2007) and a maximal sample volume that can be processed using two nanomembrane concentrators (40 ml).

5.4.3. SLC4A5 protein concentration according to the rs7571842 genotype

In the present study, the SLC4A5 protein concentration did not differ between salt-sensitive females homozygous for the rs7571842 risk allele (A) and salt-resistant ones homozygous for the G allele. This was observed when the protein concentration was expressed per mg of creatinine and when the absolute values were used. This is in line with Felder et al. (2016) who report increased in NBCe2 mRNA, NBCe2 protein expression, and increased NBCe2-mediated bicarbonate and sodium transport only under conditions of elevated sodium (Felder et al., 2016). The model used for the latter study was a primary culture of renal proximal tubule cells isolated from freshly voided urine of participants diagnosed for salt sensitivity (Gildea et al., 2013a; Gildea et al., 2015). These results thus reflect the changes in the SLC4A5 expression and activity specific for this nephron segment. Even though the majority of sodium is reabsorbed in proximal tubule, other nephron segments also play a role

in sodium homeostasis (Aviv et al., 2004; Capasso et al., 2005). *SLC4A5* expression has been reported in collecting duct where it also plays a role in sodium transport (Gröger et al., 2012). Since urinary exosomes are secreted from all nephron segments (Zhou et al., 2008), they may provide a more holistic view of the changes in *SLC4A5* protein expression in salt sensitivity than expression in proximal tubule only. Despite the fact urinary exosomes may also contain proteins from cells lining urinary drainage system, *SLC4A5* expression has not been reported in any of the segments of this system (UniProt, 2017).

The *SLC4A5* SNP reported in Felder et al. (2016) review is the rs10177833, suggested to increase the *SLC4A5* mRNA expression through increased HNF4 transcription factor binding to *SLC4A5* gene. As discussed, this SNP is in strong LD with the rs7571842 which was the focus of this study. Indeed, the participants homozygous for the rs7571842 “risk” allele A in the current study also had the rs10177833 risk allele reported by Carey et al. (2012). If this is the case, then rs10177833 may be the actual causal variant and the mechanism by which it increases *SLC4A5* expression, under conditions of high sodium concentration, already explained by Felder et al. (2016). However, together with the fact that urinary exosome *SLC4A5* may reflect its expression in all nephron segments and not only proximal tubule, a simple *in silico* analysis, revealed that the rs7571842 is in strong LD with another *SLC4A5* variant, rs7583544 (Broad Institute, 2015). This variant may be functional according to F-SNP database, which comprises the information on functional effects of SNPs obtained from 16 different bioinformatic tools and databases, and warrants further investigation (Queen’s University, 2017).

In a wider context, it can be argued how, specifically in healthy individuals,

increased *SLC4A5* expression may not lead to impaired sodium homeostasis and consequently salt sensitivity as the adverse effects of this cotransporter may be counteracted by the action of other sodium transport proteins. In addition, extrarenal regulatory mechanisms participate in the maintenance of sodium homeostasis and pressure natriuresis mechanism, together with the increased production and action of natriuretic hormones/factors and decreased production and action of anti-natriuretic hormones/factors that help to eliminate most of the ingested sodium (Felder et al., 2016). However, even if *SLC4A5* plays a minor role in sodium balance, a decrease of only 0.1% in sodium excretion can in the long run lead to hypertension. Humans excrete 1% of filtered sodium (~250 mmol/day). A decrease in sodium excretion of only 0.1% leads to sodium retention of 25 mmol/day or 250 mmols in 10 days and this cumulative effect can be substantial (Carey et al., 2012). As stated, Gildea et al. (2015) have demonstrated that *SLC4A5* mRNA, as well as its protein product, are increased with increased sodium intake in a model of human proximal tubule primary cell culture and this increase was apparent in salt-sensitive individuals (Felder et al., 2016). In addition, in some participants, the expression and activity of NBCe1 (*SLC4A4*) were only marginally attenuated. Finally, increased *SLC4A5* activity resulted in increased NHE3 activity resulting in a further increase in sodium reabsorption (Felder et al., 2016).

This thesis is, to the best of our knowledge, the first to explore habitual salt intake in relation to salt sensitivity in humans. Salt-sensitive participants had salt intake exceeding recommendations. If this is the case and *SLC4A5* protein expression increased in conditions of high sodium it may lead to above-described significant sodium retention and rise in BP. The results of this thesis and those of Felder et al. (2016), support the *SLC4A5* as a candidate for genetic and physiological

marker of salt sensitivity.

5.4.4. Strengths and limitations

A strength of this study is the use of rapid and methods more feasible in a clinical setting which may translate from salt sensitivity biomarker discovery to clinical practice. This is the first study to date aimed at measuring the SLC4A5 protein concentration from urinary exosomes using a sensitive, high throughput and cost-effective method such as ELISA. Urinary exosomes may provide a holistic view of changes in renal protein expression and activity and better represent changes in the kidney tissue than whole urine (Gudehithlu et al., 2015).

However, the present study was a proof of concept study exploring the difference in SLC4A5 concentration according to rs7571842 genotype only in a small sample of females. Furthermore, exosome proteins were not successfully isolated in one of the 20 ml urine samples even though the SLC4A5 ELISA signal was detected from a smaller and a urine volume more often used in clinical practice (5 ml). Besides potential issues with concentrating larger urine volumes, it was reported that some proteins may adhere to nanomembrane which may result in inaccurate protein quantification. However, Cheruvanky et al. (2007) report how this is not the case with all proteins and some are readily recovered from the nanomembrane. The method should, nevertheless, be optimised for maximal recovery of each exosomal protein, including SLC4A5. Considering the advantages of using a nanomembrane concentrator for urinary exosome protein concentration, in terms of speed and cost, the focus should be maximal recovery of exosomal proteins

that may adhere to nanomembrane. This could potentially be achieved by passivating the nanomembrane prior to exosome isolation. Since Cheruvanky et al. (2007) have optimised this method for subsequent Western blot measurement, protein concentrations obtained with ELISA can be compared to the ones obtained with Western blot to ensure correct protein quantification.

Another limitation of this study is the use of ELISA that has not been validated which may affect the accuracy of the results. Certain parameters such as sensitivity, specificity and spike and recovery were not explored. Even though the kit used was a commercial kit with reported inter-assay precision of < 8%, multiple assays should have been performed to ensure consistency of the results over time. In addition to inter-assay precision, intra-assay precision should be further confirmed including a larger number of technical replicates considering that only two were used in the present study. Five technical replicates for each sample have been suggested by Andreasson et al. (2015). Finally, positive control was not used for this experiment considering that the tissue samples that express the protein of interest were not available. Future experiments should include a positive control testing a tissue sample such as liver, where high levels of SLC4A5 were reported (Uniprot, 2018).

5.5. Conclusion

In this proof of concept study, we did not detect the difference in SLC4A5 protein concentration according to rs7571842 genotype and salt sensitivity status. These results should be regarded with caution considering a small sample size and inclusion of females only. In addition to limitations with the sample size, this study had several limitations regarding the methods. In this sense, future studies should validate SLC4A5 ELISA using standard procedures such as intra-assay validation using at least five technical replicates, together with inter-assay validation and measurement of sensitivity and specificity. Other critical point to be considered is the recovery of exosomal proteins. To ensure maximal recovery, nanomembrane passivation may be necessary and results obtained with ELISA should be validated with a Western blot. As for the biological variability in SLC4A5 levels, future studies should ensure a large sample size, including healthy males and females. Considering that age may affect urinary protein levels, the focus should be on healthy and younger population explored in this thesis. To account for intra- and inter-individual variability in protein excretion, multiple urine samples from the same individuals should be collected, again ensuring an adequate sample size. Moreover, SLC4A5: creatinine levels obtained from collected spot urine samples should be compared to SCL4A5 levels from 24-hour samples to explore if the use of a spot sample is appropriate. Finally, as sodium intake may affect SLC4A5 levels and activity, urinary sodium excretion should also be measured form 24-hour urine samples to further explore these effects, for the first time *in vivo*.

Despite the limitations, this study shows that the SLC4A5 expression in urinary exosomes, obtained with rapid ultrafiltration method, can be measured using

a commercially available ELISA kit. This may provide potential for measurement of other proteins associated with salt sensitivity.

Chapter 6 General discussion

6.1. Key findings and original contribution of the thesis

The aim of this thesis was to comprehensively explore the associations between salt sensitivity of BP, salt taste sensitivity and salt intake in humans, together with underlying genetic predisposition. The thesis comprised four research chapters with its main objectives being:

- To determine if selected genetic variants affect BP response to a dietary salt intake manipulation in healthy study participants (Chapter 2).
- To explore if there is a genetic predisposition to altered salt taste perception and/or salt intake (Chapter 3 and 4).
- To explore the associations between salt sensitivity, salt taste perception and salt intake and whether these associations depend on the genotype (Chapter 3 and 4).
- To explore if genetic variations predisposing to salt sensitivity are affecting the phenotype (protein concentration) and if there is a potential for salt sensitivity physiological biomarker discovery (Chapter 5).

Genetic variants explored in this thesis were the variants in genes coding for sodium/ion transport proteins associated with either salt sensitivity of BP, salt taste sensitivity, both or similar phenotypes, as discussed in Chapter 1.

Furthermore, salt sensitivity is present in both normotensive and hypertensive population and is an independent CVD risk factor. Research suggests that salt-sensitive individuals, irrespective of their hypertension status, exhibit similar mortality rate as individuals that suffer from hypertension (Weinberger et al., 2001). Moreover, the incidence of hypertension is higher in salt-sensitive than salt-resistant

individuals (Barba et al., 2007). From this, it is evident the research should be directed towards the, still, healthy population in whom early diagnosis and better understanding of factors associated with salt sensitivity may lead to more effective hypertension and CVD prevention. Thus, the study participants in this thesis were healthy, predominantly young adults with no prior history of hypertension.

With the above-described, the key findings of the thesis are presented in Figure 6.1 and below:

- There was a genetic predisposition to salt sensitivity in study participants with individuals homozygous for the A allele of the *SLC4A5* rs7571842 exhibiting the highest increase in BP after salt loading. This was, to the best of our knowledge, the only study replicating the findings of Carey et al. (2012) and the only study to date exploring this phenotype in healthy UK participants.
- The genetic variants in the *SLC4A5*, *TRPV1* and *SCNN1B* were not associated with salt intake (this is one of the few studies exploring the direct association between genetics and salt intake).
- The genetic variants in the *SLC4A5*, *TRPV1* and *SCNN1B* were not associated with salt taste perception measured as salt taste detection and recognition threshold in two separate cohorts with similar demographic characteristics (this is the first study to date exploring the *SLC4A5* in relation to salt taste perception).
- There was no association between salt sensitivity and salt taste perception and salt sensitivity and salt intake (this is the first study to date reporting on such association in humans).

- In two separate cohorts, salt intake was not associated with salt taste perception.
- We did not detect the difference in the *SLC4A5* urinary exosome protein concentration between different genotypes of the rs7571842 (this was the first study measuring the expression of this protein from urinary exosomes and with ELISA).

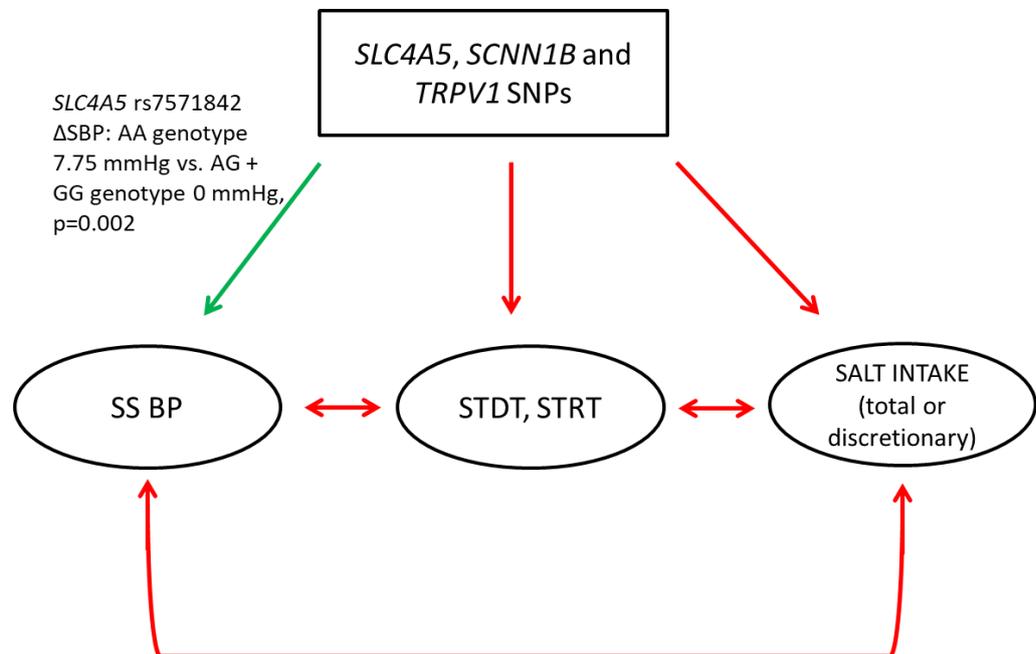


Figure 6.1 Overview of the key findings in the thesis. Red lines depict where no association was detected between the variables and green line represent the presence of a significant finding. More precisely, the green line represents a significant difference in SBP response (Δ SBP) to salt loading between individuals with AA and AG + GG genotype of the *SLC4A5* rs7571842. Salt sensitivity of blood pressure (SS BP), systolic blood pressure (SBP) salt taste detection threshold (STDT), salt taste recognition threshold (STRT).

6.1.1. *SLC4A5* as a genetic marker in prediction of hypertension and CVD

The results of this thesis suggest a genetic predisposition to salt sensitivity in study participants. Considering a small sample size the generalisability of the results is limited and is therefore difficult to draw conclusions whether similar would be observed in healthy and younger UK population. As stated in Chapter 1, hypertension is a major public health issue in the UK and worldwide (Mills et al., 2016; NICE, 2011). Despite the efforts to reduce its prevalence by reducing salt intake, as the major environmental contributor, the disease burden is still present which highlights the need for more effective prevention strategies that are focused on healthy populations. In this sense, nutrigenetics and genotype-based personalised nutrition may hold promise as alternative approaches in disease prevention. In this specific context, if the *SLC4A5* is the gene associated with salt-sensitive changes in BP in the UK population, then these individuals may be advised to reduce their salt intake and maintain it to the recommended level, while still asymptomatic, to reduce the risk of developing hypertension and/or CVD in the future. However, the limitations of this approach should be acknowledged. As the human genome contains more than 20000 genes with 10 million SNPs (NIH, 2010), it is unlikely that a single SNP may explain a large proportion of variation in a complex phenotype such as BP and salt sensitivity. Moreover, genetics can be viewed as a first layer in complex human physiology. Many posttranscriptional and posttranslational modifications occur on regular bases due to gene-environment interactions which may all affect the final phenotype. This is supported by the fact that single SNPs generally have poor predictive values for health outcomes and researchers are suggesting utilisation of risk scores based on multiple loci which may

serve as better predictors (Corella et al., 2017).

Nevertheless, *APOE* and *MTHFR* are examples of single genes/SNPs that were used in intervention studies to predict physiological responses (intermediary CVD risk factors such as BP and cholesterol levels) to dietary interventions (Caslake et al., 2008; Wilson et al., 2012). In this sense, it is not to exclude that the *SLC4A5* may serve as a predictor of BP response to dietary salt intake. The results of this thesis, due to study design limitations discussed in respective chapters, may be regarded only as preliminary and should be replicated in a larger sample size study. Such study should be a randomised crossover intervention where participants would be recruited prospectively based on their *SLC4A5* genotype to ensure adequate statistical power to detect the effect of genetics. Even if this is achieved and the results of this thesis confirmed, this could be considered as one of the first steps as it would not provide an indication on the actual disease development. Increase in BP due to salt loading is an intermediary risk factor, which may or may not lead to development of hypertension and CVD. Ideally, participants should be followed for a number of years to be able to determine incident cases of hypertension/CVD and if these are due to genetic predisposition and inadequate salt intake. In nutrition research this is often not feasible and randomised trials, as the highest level of scientific evidence, ethically not justified. Consequently, intermediary risk factors are most commonly used as indicators of potential disease development (Minihane et al., 2000; Caslake et al., 2008).

In the context of using SNPs as genetic markers for a specific health outcome, besides the association with the outcome, it is advised to explore their functional effects. Functional SNPs, affecting protein levels/activity, are more often

considered to be the causal variants. Finally, if feasible, it would be beneficial to explore if protein levels differ in individuals with or without a disease which would provide a more complete understanding of the phenotype. Regarding the *SLC4A5*, as stated, even if it plays a minor role in sodium balance, a decrease of only 0.1% in sodium excretion can in the long run lead to hypertension. Humans excrete 1% of filtered sodium (~250 mmol/day). A decrease in sodium excretion of only 0.1% leads to sodium retention of 25 mmol/day or 250 mmols in 10 days and this cumulative effect can be substantial (Carey et al., 2012). Furthermore, Gildea et al. (2015) have demonstrated that *SLC4A5* mRNA, as well as its protein product levels, are increased with increased sodium intake in a model of human proximal tubule primary cell culture and this increase was apparent in salt-sensitive individuals (Felder et al., 2016). In addition, in some participants, the expression and activity of NBCe1 (*SLC4A4*) were only marginally attenuated. Finally, increased *SLC4A5* activity resulted in increased NHE3 activity resulting in a further increase in sodium reabsorption (Felder et al., 2016).

Even though it appears that the *SLC4A5* SNPs are functional and that its protein product levels are increased only in salt-sensitive individuals, these findings should be replicated in a larger sample. In this sense, the *SLC4A5* ELISA measurement explored in this thesis should be optimised to allow for its measurement in a larger number of samples. If indeed, the *SLC4A5* protein levels differ between salt-sensitive and salt-resistant participants then it may be used as a salt sensitivity biomarker. The impact of such biomarker would be two-fold. Firstly, from a research perspective salt sensitivity biomarker would enable further GWAS studies exploring salt sensitivity as a distinct phenotype. Considering that salt sensitivity is difficult to diagnose and requires substantial time and resources, the

number of participants used in studies exploring genetic predisposition to salt sensitivity is too low to conduct a GWAS. Biomarker would enable recruitment and phenotyping of much larger number of participants which is required to further elucidate genetic variants associated with salt sensitivity. These newly identified variants may, in turn, serve to pinpoint specific physiological systems associated with this phenotype.

Secondly, from a clinical perspective, salt sensitivity biomarker would enable identification of individuals at risk of hypertension and CVD while still asymptomatic which would enable more targeted approaches in primary prevention of this disease and may therefore, have a substantial economic impact in reducing health care costs associated with hypertension and CVD treatment.

It is important to note that traditional risk factors for CVD are still considered as better predictors of disease development compared to genetic factors which is supported by the fact that the Framingham risk score is still used in predicting a 10-year CVD risk as opposed to genetic markers associated with this phenotype. Some argue that in certain cases, such as CVD, genetic predictive markers are redundant. Conversely, it can be argued that genetic markers may be especially useful in predicting disease in healthy individuals before traditional risk factors, such as increased BP or cholesterol have developed. Furthermore, such markers would be beneficial where traditional risk factors are more costly to investigate than a genetic marker or a protein biomarker. This is the case with salt sensitivity, especially in healthy and younger population where more traditional salt sensitivity risk factors such as age, sex and race are of limited predictive value (Müller et al., 2016).

6.1.2. Utilisation of nutrigenetics in providing genotype-based dietary advice

With the advancements in understanding the genetic basis of complex lifestyle-related diseases, there has been a rapid increase in direct to consumer (DTC) genetic testing services. Such services provide information on the risk of monogenic disorders, such as lactose intolerance, as well as macronutrient and energy metabolism, weight management and obesity. Moreover, consumers are often informed about their risk of hypertension due to high salt consumption (Guasch-Ferré et al., 2018). The DTC services are utilising an increase in the number of people proactively taking ownership about their health care. The increase in both the number of companies offering such services as well as the number of people interested in purchasing such services, is often not accompanied by the advancements in nutrigenetic and nutrigenomic research.

Despite the fact that better understanding of nutrient-gene interactions is needed before genotype-based personalised dietary advice can be applied, Grimaldi et al. (2017) state that DTC is a reality and as such it is important that it is based on valid and robust scientific evidence. With this notion, the experts in the field have developed a framework to evaluate scientific validity and evidence for genotype-based dietary advice. In brief, the framework takes into account the research on specific gene-diet interaction in a specific population. It emphasises the importance of study design quality, which can be assessed using strengthening the reporting of genetic association studies (STREGA) guidelines (Little et al., 2009), as well as biological plausibility of the interaction. Encompassed in a robust study design is the number of participants to be included in the study, which should take into account specific allele frequencies. For example, Grimaldi et al. (2017) suggest that, to be

able to consider an evidence for a specific gene-diet interaction convincing, two independent studies should show the relationship between the gene-diet interaction and the specific health outcome. Together those studies should include at least 100 participants carrying the effect allele (the presumed functional variant or in LD with the functional variant) for intervention studies and at least 500 participants for observational studies. The frequency of the effect allele should be taken into consideration (e.g. if the frequency of the effect allele is 10%, a total of at least 1000 and 5000 participants for intervention and observational studies would be required). The authors also emphasise the importance of strictly defining the population in whom the interaction is observed and may be valid (e.g. sex, ancestral background and other relevant subgroups such as age). Considering that a person's sex, age, race and other factors may modify the response to dietary intervention, personalised dietary advice should encompass all this information and not use genotype in isolation (Grimaldi et al., 2017).

Even though this is only the first draft of the guidelines, they currently present the state of the art for assessing the quality of nutrigenetic based dietary advice. In this sense, the interaction between the *SLC4A5* rs7571842 and dietary salt observed in this thesis in healthy and younger participants may only be considered as preliminary, and not as a basis for providing personalised nutrition advice. This is due to small sample size and other limitations discussed in Chapter 2. Even though the study by Carey et al. (2012), where the same interaction was observed, was a larger study and the findings replicated in two independent cohorts, study populations were heterogeneous, comprising of white individuals of both sexes with wide age range (18 - 70 years) and both healthy and individuals with hypertension, making it difficult to draw population specific conclusions about the scientific

validity of this gene-diet interaction. The fact that gene-diet interactions may be highly population specific is further supported by a recent meta-analysis by Jin et al. (2018) who explored under what covariate-defined conditions is an individual carrying the *MTHFR* rs1801133 at risk of elevated homocysteine. The authors analysed data from 256 studies and identified 36 homocysteine determinants that, besides the rs1801133, included age, sex, ethnicity, smoking and alcohol consumption. Given the covariates included in the analysis, the authors were able to predict that the risk of hyperhomocysteinemia occurred in only a small part of the covariate-defined conditions (e.g. rs1801133 TT genotype, young or middle aged female adult with low folate intake). These findings highlight the need for exploring gene-diet interactions in more homogenous populations and including information other than genotype when tailoring personalised nutrition advice. In this context, findings from this thesis should be replicated in a larger population of similar demographic characteristics. Considering a distinct genetic background, focus should be on specific ethnicity, or in case of mixed ethnic backgrounds, sufficient sample size should be ensured to allow for population stratification.

Furthermore, advances in personalised nutrition suggest integration of different omics technologies such as genomics, transcriptomics, proteomics and metabolomics into powerful tools for deeper and more robust phenotyping of individuals and understanding mechanisms behind diet-disease relationship (Wang and Hu, 2018). From this, it is evident that the findings of this thesis present only a small fraction of the complex biology underlying complex disease such as hypertension, and their interaction with diet and lifestyle. Taking CVD as an example, Ma and Ordovas (2017) suggest integration of genomics and epigenomics to further elucidate the mechanism behind gene-diet interaction in CVD. In this

sense, a recent study revealed that saturated fat induced methylation of the apolipoprotein A-2 (*APOA2*) cg04436964 was associated with changes in gene expression and changes in branched chain amino acid and tryptophan pathways that depended on the genotype of the *APOA2* functional variant rs5082 (Lai et al., 2018). In the context of salt sensitivity, it would be beneficial to explore potential epigenetic mechanisms of salt intake on the *SLC4A5* expression and its association with this phenotype as literature on the topic is limited.

Taken into account the current state of both nutrigenetic research and practice and the limitations acknowledged throughout the thesis, it may be concluded that further research is needed before translating these findings into practice. They may, nevertheless, serve to inform future studies on the topic which will be discussed in the following section.

6.2. Conclusion and recommendations for future research

In conclusion, the results of this thesis suggest a genetic predisposition to salt sensitivity in study participants with the *SLC4A5* rs7571842 confirmed as the variant with the effect on salt-sensitive changes in BP. There was no association between genetics and salt taste perception and genetics and salt intake as well as salt sensitivity, salt taste perception and salt intake. Considering issues with statistical power these results should be considered only preliminary and replicated in larger size studies. Nevertheless, preference for salty taste may be a driver of salt intake and warrants further investigation. The *SLC4A5* may be a candidate for genetic and physiological marker of salt sensitivity if the results of this thesis are replicated in larger size studies. In this regard, the ELISA measurement of its expression from urinary exosomes may serve as a method of choice in a clinical setting, if further optimised.

A number of methodological issues have been identified in this thesis and these, together with alternative approaches, have been discussed in respective chapters. Considering the key limitations, it is suggested that future studies take genetics into account when calculating required sample size. Achieving the adequate number of participants in each genotype group may be facilitated by prospectively recruiting participants based on their genetic profile. Regarding salt sensitivity diagnosis, even though a more participant friendly approach has been chosen in this thesis, poor adherence to dietary intervention regarding certain nutrients, such as protein and calcium, highlighted the need for a more strict control of dietary intake. This should be achieved together with randomisation of order of the low- and high-salt diets. Considering the limitations in methods of measuring dietary salt intake, it is suggested that future studies validate dietary methods (FFQ or 24-hour recall) against

a 24-hour urine biomarker method. Since dietary salt intake may depend on demographic and anthropometric characteristics such as age, sex, race as well as obesity related measures, it is important to rely on the dietary methods that have been validated against the 24-hour urine gold standard method in a respective population of interest.

In a wider context, it was hypothesised how genetic variants in the *SLC4A5*, *SCNN1B* and *TRPV1* may affect the key phenotypes explored in this thesis: salt sensitivity of BP, salt taste sensitivity and salt intake. However, no associations were detected. To further explore these associations, studies should ensure a sufficient sample size, taking into account genetic variation in study population. Further exploration of the effect of genetics on salt taste thresholds is also suggested which may be detected in a larger sample size study than this thesis. Taste is one of the main drivers of food intake in general and better understanding of its determinants may aid in development of novel personalised nutrition strategies. In this context, the UK Biobank, available for open access, may be used as a source of data. The Biobank comprises participants aged 40 years and older, it provides extensive phenotypic and genotypic detail about its participants, including data from questionnaires, such as 24-hour dietary recall, physical measures, sample assays, accelerometry, multimodal imaging, genome-wide genotyping and longitudinal follow-up for a wide range of health-related outcomes. In addition, it provides opportunities for re-contacting of study participants for further phenotyping (Sudlow et al., 2015).

Finally, the focus of this thesis was genetic predisposition to salt sensitivity, salt taste sensitivity and salt intake in predominantly white participants. Considering

the specific genetic background, these measurements should be replicated in other populations with different genetic and environmental backgrounds.

7.References

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8. Appendices

APPENDIX A: ETHICAL APPROVAL LETTERS



St Mary's
University
Twickenham
London

Cc Yiannis Mavrommatis

Leta Pilic (SHAS) GENETIC PREDISPOSITION TO SALT SENSITIVITY AND ITS EFFECTS ON DIETARY SALT INTAKE AND HYPERTENSION

11th May 2015

Dear Leta

University Ethics Sub-Committee

Thank you for submitting your ethics application for consideration.

I can confirm that that you have ethical approval to undertake your research.

Yours sincerely

A handwritten signature in black ink, appearing to read 'Conor Gissane'.

Dr Conor Gissane
Chair of the Ethics Sub-Committee



St Mary's
University
Twickenham
London

5 January 2017
17_036

Unique Ref: SMEC_2016-

Leta Pilic (SHAS): 'Discovery of salt sensitivity biomarkers based on protein measurement'.

Dear Leta

University Ethics Sub-Committee

Thank you for submitting your ethics application for the above research.

I can confirm that your application has been considered by the Ethics Sub-Committee and that ethical approval is granted.

Yours sincerely

A handwritten signature in black ink, appearing to read 'Conor Gissane'.

Prof Conor Gissane
Chair of the Ethics Sub-Committee

Cc Dr Yiannis Mavrommatis

**APPENDIX B: INSTRUCTIONS FOR PARTICIPANTS –
DIETARY SODIUM INTERVENTION**



St Mary's
University
Twickenham
London

***Genetic predisposition to salt sensitivity
and its effects on dietary salt intake and
hypertension***



***LOW SODIUM DIET PARTICIPANT
BOOKLET***

Thank you for deciding to participate in this study. This booklet will provide you with all the information and meal plans necessary for successful completion of the 7-day low-salt diet. If you are eating out or going grocery shopping, take this booklet with you. **Your daily salt intake should be 3 grams.** Please note that there are 3 different meal plans based on different energy needs of the study participant. The member of research staff will advise you which meal plan you should be following. On the pages 10 - 13 you can find food photographs that will assist you with the portion sizes. Please fill in the food diary at the end of this booklet.

Please note that this is a very important part of the study and will add greatly to the information we have already collected about you. If at any point during this 7-day period you feel nausea, dizziness, extreme fatigue or any other signs of discomfort, please stop it immediately and contact your GP. Research staff contacts can be found at the end of the booklet.

GENERAL TIPS:

- Stick to the meals and snacks advised by the research staff and avoid any other food
- Stick to your regular fluid (water), coffee/tea intake and physical/work activity and smoking habits
- Please avoid medications such as soluble paracetamol, aspirin, ibuprofen or Alka - or Bromo - Seltzer.
- Please limit your alcohol consumption to: 2-3 units per day for women and 3-4 units per day for men (glass of wine, pint of beer, double measure of spirit)
- Eat a varied selection of offered meals
- Do not add any salt at the table
- Use unsalted butter/margarine/spreads
- Do not use stock cubes while preparing your food
- If you feel hungry increase your portion of rice, pasta or noodles
- Certain drinks are high in salt. Avoid: soda drinks (club soda) or cocktails

FOODS HIGH IN SALT:

- bacon, ham and smoked meats
- cheese
- pickles, canned soups and vegetables
- salami, sausages
- salted and dry roasted nuts
- salt fish and smoked fish
- gravy granules, stock cubes and yeast extract
- bread, bread products like wraps, crumpets, scones and sandwiches
- pasta sauces, tomato ketchup, mayonnaise, soy sauce
- savoury snacks like crisps and salted nuts, crackers
- pizza, ready meals
- breakfast cereals (read the label carefully)
- salad dressings

FOODS LOW IN SALT:

- fresh fruits and pure fruit juices
- fresh vegetables (e.g. peppers, cauliflower, beans, peppers, mushrooms, broccoli, onions, lettuce, corn, radishes, aubergines, asparagus, tomatoes, avocados, cucumber)
- cooking oils, such as olive, canola, soybean, peanut and sunflower
- lentils, rice, oat bran, wheat flour
- pasta, noodles
- fresh meat and fish

1500 – 2000 kcal MEAL PLAN

Please use this table to plan your breakfast, lunch and dinner. For your lunch and dinner choose **one meal from the group Meal 1 and one meal from the group Meal 2 every day.**

	Portion Size	Note
BREAKFAST		
Yoghurt with grapes and granola	1 pot yoghurt + ½ cup granola	Use any type of yoghurt (low/full fat, fruit, Greek)
Porridge with milk	Medium	Use any type of milk
Muesli with milk	Medium	Use any type of milk
MEAL 1		
Vegetable risotto	Medium	No salt while cooking. Use homemade stock
Vegetable casserole	Medium	No salt while cooking, use homemade stock
Pasta Bolognese	Small	Pasta boiled without salt
Chicken curry	Small	Use canned curry sauce (Knorr or Uncle Ben's). Serve with rice, pasta, or noodles boiled without salt
Vegetable lasagne	Medium	Homemade
Meat lasagne	Medium	Homemade
Pasta with tomato sauce	Medium	Pasta boiled without salt. No grated cheese on top
Baked salmon with peas and jacket potatoes	Medium potato + average darn salmon	No added salt while cooking. Top the potato with sour cream and grated cheese (1 tablespoon of cream and ¼ cup cheese). Replace salmon with mackerel if preferred.
Jacket potatoes with baked beans	Medium potato	Baked beans reduced salt. Serve with salad with olive oil dressing and no added salt
Grilled steak (meat) with boiled broccoli or cauliflower	1 medium steak (130g)	(Poultry, beef, pork...). No added salt on meat. Vegetables can be boiled in salted water
Fish and chips	Medium fish fillet and average chips portion	Cod or haddock. No added salt or sauce
Roast dinner		Meat, broccoli, cauliflower, carrots, potatoes – no salt while cooking. 1 tablespoon of gravy per person

Chicken salad with yoghurt dressing	As wished	Limit dressing to 1 tablespoon
Chicken and sweetcorn sandwich	1 pack	
Salmon and cucumber sandwich	1 pack	
MEAL 2		
Pasta with meat and tomato sauce	Medium	Use Dolmio tomato stir-in sauce. No added salt while cooking
Chilli con carne -homemade	Medium	Serve with rice or pasta – no salt while boiling
Fried chicken breasts in crumbs with coleslaw	Average chicken breast portion and 2 tblsp coleslaw	Replace chicken with 2 fishcakes if preferred
Turkey stir fry	Medium	Reduced salt soy sauce. Serve with rice or noodles – no added salt while cooking
Potato and leek soup - homemade	Large - 1 bowl (2 cups)	No added salt while cooking, use homemade stock. Serve with 1 thick slice of toast with unsalted butter/spread
Tomato and red lentil soup- homemade	Large - 1 bowl (2 cups)	No added salt while cooking, use homemade stock. Serve with 1 thick slice of toast with unsalted butter/spread. Do not add bacon
Chicken salad sandwich	1 pack	
Egg and cress sandwich	1 pack	
Egg mayonnaise sandwich	1 pack	
Tuna and cucumber sandwich	1 pack	
Tuna and sweetcorn sandwich	1 pack	
Tuna salad with yoghurt dressing	1 can of tuna	Drained tuna in brine with 1 tablespoon of dressing. If you wish to increase the portion of tuna use tuna canned in spring water instead of brine

SNACKS: Unsalted mixed nuts and dried fruit, plain unsalted popcorn, plain chocolate bar (e.g. Dairy milk), yoghurt, fresh or baked fruit, fruit juices, smoothies or salads, vegetable salad (oil or vinegar dressing, no added salt). Limit your chocolate or yoghurt to 1 bar/pot per day.

2000 – 2500 kcal MEAL PLAN

Please use this table to plan your breakfast, lunch and dinner. For your lunch and dinner choose **one meal from the group Meal 1 and one meal from the group Meal 2 every day.**

	Portion Size	Note
BREAKFAST		
Yoghurt with grapes and granola	1 pot yoghurt + ½ cup granola	Use any type of yoghurt (low/full fat, fruit, Greek)
Porridge with milk	Medium	Use any type of milk
Muesli with milk	Medium	Use any type of milk
MEAL 1		
Vegetable risotto	Medium	No salt while cooking. Use homemade stock. Serve with avocado salad.
Vegetable casserole	Medium	No salt while cooking, use homemade stock. Serve with avocado salad
Chicken curry	Small	Use canned curry sauce (Knorr or Uncle Ben's). Serve with large portion of rice, pasta, noodles boiled without salt
Meat lasagne	Medium	Homemade
Baked salmon with peas and jacket potatoes	Medium potato + average darn salmon	No added salt while cooking. Top the potato with sour cream and grated cheese (1 tablespoon of cream and ¼ cup cheese). Replace salmon with mackerel if preferred.
Jacket potatoes with baked beans	Large potato + ¼ can of beans	Baked beans reduced salt. Serve with salad with olive oil dressing and no added salt
Grilled steak (meat) with boiled broccoli or cauliflower	2 medium fillets (2*130g)	(Poultry, beef, pork...). No added salt on meat. Vegetables can be boiled in salted water
Fish and chips	Medium fish fillet and average chips portion	Cod or haddock. No added salt or sauce
Roast dinner		Meat, broccoli, cauliflower, carrots, potatoes – no salt while cooking. 1 tablespoon of gravy per person
Chicken salad with yoghurt dressing	As wished	Limit dressing to 1 tablespoon

Chicken and sweetcorn sandwich + fruit smoothie	1 pack + 1 bottle	
Salmon and cucumber sandwich + fruit smoothie	1 pack + 1 bottle	
MEAL 2		
Pasta with meat and tomato sauce	Medium	No added salt while cooking
Chilli con carne - homemade	Medium	Serve with rice or pasta – no salt while boiling
Pasta Bolognese	Medium	No salt while boiling pasta
Fried chicken breasts in crumbs with coleslaw	Average chicken breast portion and 2 tablespoons coleslaw	Replace chicken with 2 fishcakes if preferred
Turkey stir fry	Medium	Reduced salt soy sauce. Serve with rice or noodles – no added salt while boiling
Pasta with tomato sauce and garlic bread	Medium pasta + 1 angled slice of bread	Pasta boiled without salt. No grated cheese on top
Potato and leek soup - homemade	Large -1 bowl (2 cups)	No added salt while cooking, use homemade stock. Serve with 1 thick slice of toast with unsalted butter/spread
Tomato and red lentil soup- homemade	Large - 1 bowl (2 cups)	No added salt while cooking, use homemade stock. Serve with 1 thick slice of toast with unsalted butter/spread. Do not add bacon
Chicken salad sandwich + fruit smoothie	1 pack + 1 bottle	
Egg and cress sandwich + fruit smoothie	1 pack + 1 bottle	
Egg mayonnaise sandwich + fruit smoothie	1 pack + 1 bottle	
Tuna and cucumber sandwich + fruit smoothie	1 pack + 1 bottle	
Tuna and sweetcorn sandwich + fruit smoothie	1 pack + 1 bottle	
Tuna salad with yoghurt dressing	1 can of tuna	Drained tuna in brine with 1 tablespoon of dressing. If you wish to increase the portion of tuna use tuna canned in spring water instead of brine

SNACKS: Unsalted mixed nuts and dried fruit, plain unsalted popcorn, plain chocolate bar (e.g. Dairy milk), yoghurt, fresh or baked fruit, fruit juices, smoothies or salads, vegetable salad (oil or vinegar dressing, no added salt). Limit your chocolate or yoghurt to 1 bar/pot per day.

2500 – 3000 kcal MEAL PLAN

Please use this table to plan your breakfast, lunch and dinner. For your lunch and dinner choose **one meal from the group Meal 1 and one meal from the group Meal 2 every day.**

	Portion Size	Note
BREAKFAST		
Yoghurt with grapes and granola	2 pots yoghurt + ½ cup granola	Use any type of yoghurt (low/full fat, fruit, Greek)
Porridge with milk	Large	Use any type of milk
Muesli with milk	½ cup muesli	Use any type of milk
MEAL 1		
Vegetable risotto	Large	No salt while cooking. Use homemade stock. Serve with avocado salad.
Vegetable casserole	Large	No salt while cooking, use homemade stock. Serve with avocado salad
Baked salmon with peas and jacket potatoes	Medium potato + 2 average salmon darns	No added salt while cooking. Top the potato with sour cream and grated cheese (1 tablespoon of cream and ¼ cup cheese). Replace salmon with mackerel if preferred.
Jacket potatoes with baked beans	Large potato + ¼ can of beans	Baked beans reduced salt. Serve with avocado salad with olive oil dressing and no added salt
Grilled pork loin with boiled broccoli or cauliflower	1 large pork loin chop (200g)	No added salt on meat. Vegetables can be boiled in salted water
Fish and chips	Medium fish fillet and average chips portion	Cod or haddock. No added salt or sauce
Roast dinner		Meat, broccoli, cauliflower, carrots, potatoes – no salt while cooking. 1 tablespoon of gravy per person
Chicken salad with yoghurt dressing	As wished	Limit dressing to 1 tablespoon

Chicken and sweetcorn sandwich + fruit smoothie	1 pack + 1 bottle	
Salmon and cucumber sandwich + fruit smoothie	1 pack + 1 bottle	
MEAL 2		
Meat lasagne	Large	Homemade
Pasta with meat and tomato sauce	Medium	No added salt while cooking
Chilli con carne -homemade	Medium	Serve with rice or pasta – no salt while boiling
Pasta Bolognese	Medium	No salt while boiling pasta
Turkey stir fry	Medium	Reduced salt soy sauce. Serve with rice or noodles – no added salt while boiling
Pasta with tomato sauce and garlic bread	Medium pasta + 1 angled slice of bread	Pasta boiled without salt. No grated cheese on top
Potato and leek soup - homemade	Large – 1 bowl (2 cups)	No added salt while cooking, use homemade stock. Serve with 1 thick slice of toast with unsalted butter/spread
Tomato and red lentil soup- homemade	Large - 1 bowl (2 cups)	No added salt while cooking, use homemade stock. Serve with 1 thick slice of toast with unsalted butter/spread. Do not add bacon
Chicken salad sandwich + fruit smoothie	1 pack + 1 bottle	
Egg and cress sandwich + fruit smoothie	1 pack + 1 bottle	
Egg mayonnaise sandwich + fruit smoothie	1 pack + 1 bottle	
Tuna and cucumber sandwich + fruit smoothie	1 pack + 1 bottle	
Tuna and sweetcorn sandwich + fruit smoothie	1 pack + 1 bottle	
Tuna salad with yoghurt dressing	1 can of tuna	Drained tuna in brine with 1 tablespoon of dressing. If you wish to increase the portion of tuna use tuna canned in spring water instead of brine

SNACKS: Unsalted mixed nuts and dried fruit, plain unsalted popcorn, plain chocolate bar (e.g. Dairy milk), yoghurt, fresh or baked fruit, fruit juices, smoothies or salads, vegetable salad (oil or vinegar dressing, no added salt). Limit your chocolate or yoghurt to 1 bar/pot per day.

CURRY – SMALL



CHILLI CON CARNE – MEDIUM



RISOTTO – MEDIUM



RISOTTO - LARGE



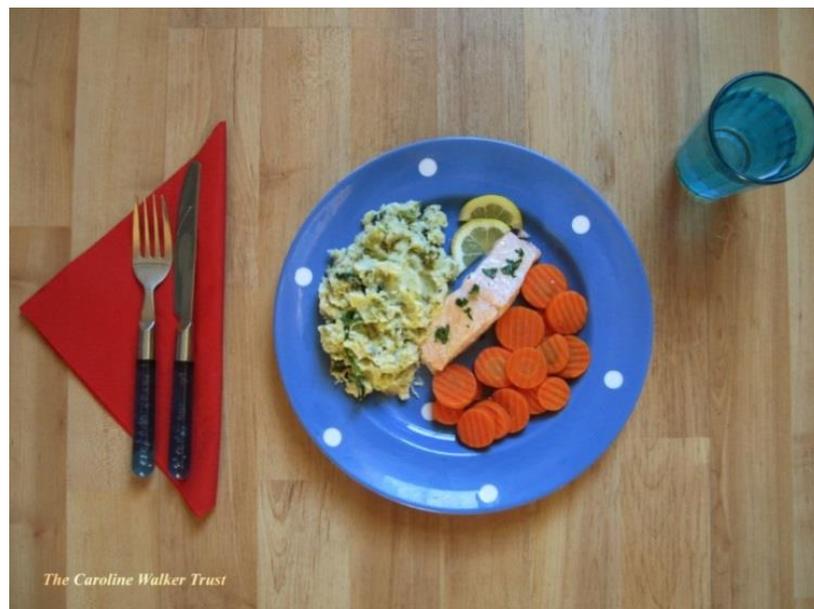
CHICKEN FILLET – 100g



PORK FILLET – 100g



SALMON STEAK – AVERAGE DARN



PASTA BOLOGNESE – SMALL



PASTA BOLOGNESE SAUCE – MEDIUM



LASAGNE – MEDIUM





St Mary's
University
Twickenham
London

***Genetic predisposition to salt sensitivity
and its effects on dietary salt intake and
hypertension***



***HIGH SODIUM DIET PARTICIPANT
BOOKLET***

Thank you for deciding to participate in this study. This booklet will provide you with all the information and meal plans necessary for successful completion of the 7-day high-salt diet. If you are eating out or going grocery shopping, take this booklet with you. **Your daily salt intake should be 18 grams (3 grams from food + 15 grams of added salt).** Please note that there are 3 different meal plans based on different energy needs of the study participant. The member of research staff will advise you which meal plan you should be following. On the pages 10 - 13 you can find food photographs that will assist you with the portion sizes. Please fill in the food diary at the end of this booklet.

Please note that this is a very important part of the study and will add greatly to the information we have already collected about you. If at any point during this 7-day period you feel nausea, dizziness, extreme fatigue or any other signs of discomfort, please stop it immediately and contact your GP. Research staff contacts can be found at the end of the booklet.

GENERAL TIPS:

- Stick to the meals and snacks advised by the research staff and avoid any other food
- Stick to your regular fluid (water), coffee/tea intake and physical/work activity and smoking habits
- Please avoid medications such as soluble paracetamol, aspirin, ibuprofen or Alka - or Bromo - Seltzer.
- Please limit your alcohol consumption to: 2-3 units per day for women and 3-4 units per day for men (glass of wine, pint of beer, double measure of spirit)
- Eat a varied selection of offered meals
- If you feel hungry increase your portion of rice, pasta or noodles.

FOODS HIGH IN SALT:

- **bacon, ham and smoked meats**
- **cheese**
- **pickles, canned soups and vegetables**
- **salami, sausages**
- **salted and dry roasted nuts**
- **salt fish and smoked fish**
- **gravy granules, stock cubes and yeast extract**
- **bread, bread products like wraps, crumpets, scones and sandwiches**
- **pasta sauces, tomato ketchup, mayonnaise, soy sauce**
- **savoury snacks like crisps and salted nuts, crackers**
- **pizza, ready meals**
- **breakfast cereals (read the label carefully)**
- **salad dressings**

FOODS LOW IN SALT:

- **fresh fruits and pure fruit juices**
- **fresh vegetables (e.g. peppers, cauliflower, beans, peppers, mushrooms, broccoli, onions, lettuce, corn, radishes, aubergines, asparagus, tomatoes, avocados, cucumber)**
- **cooking oils, such as olive, canola, soybean, peanut and sunflower**
- **lentils, rice, oat bran, wheat flour**
- **pasta, noodles**
- **fresh meat and fish**

For the successful completion of the high-salt diet please try to stick to the same meal plan and meals you have been following while on the low-salt diet. It is especially important for the foods rich in potassium listed below:

- **Winter squash**
- **Sweet potato**
- **Potato**
- **Beans, lentils**
- **Yogurt and milk**
- **Oranges, orange juice**
- **Broccoli**
- **Banana**
- **Pork tenderloin, chicken breasts**
- **Salmon, tuna**
- **Raisins**

In addition to following these meal plans please add salt sachets (containing 1g of salt) provided by the research staff to your meals. Each day you are supposed to add additional 15g/sachets to your meals. **Add it to already prepared food on the plate, not while cooking.** Try to distribute it evenly throughout the day. Drink as much water your body requires.

**APPENDIX C: CONFIDENTIAL MEDICAL
HISTORY/PHYSICAL ACTIVITY READINESS
QUESTIONNAIRE**

**CONFIDENTIAL MEDICAL HISTORY / PHYSICAL ACTIVITY READINESS QUESTIONNAIRE
(PAR-Q) FORM**

This screening form must be used in conjunction with an agreed Consent Form.

Full Name: Date of Birth:
Height (cm): Weight (kg):

Have you ever suffered from any of the following medical conditions? If yes please give details:

	Yes	No	Details
Heart Disease or attack	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/>
High or low blood pressure	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/>
Stroke	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/>
Cancer	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/>
Diabetes	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/>
Asthma	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/>
High cholesterol	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/>
Epilepsy	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/>
Allergies	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/>
Other, please give details	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text"/>

Do you suffer from any blood borne diseases? If yes please give details;

Please give details of any **medication** you are currently taking or have taken regularly within the last year:

Please give details of any **musculoskeletal injuries** you have had in the **past 6 months** which have affected your capacity to exercise or caused you to take time off work or seek medical advice:

Other Important Information

During a typical week approximately how many hours would you spend exercising?

If you **smoke** please indicate how many per day:

If you drink **alcohol** please indicate how many units per week:

Are you currently taking any **supplements or medication**? Please give details:

Is there any reason not prompted above that would prevent you from participating within the relevant activity?

By signing this document I agree to inform the relevant individual(s) of any change(s) to my circumstances that would prevent me from participating in specific activities.

APPENDIX D: 24-HOUR RECALL

24-hour dietary recall - salt 1

0% complete

Page 1: 24- hour dietary recall

1. Please enter your unique identification number (e.g. 101) * *Required*

Thank you for choosing to take part in this study!

We would like you to tell us everything you had to eat and drink yesterday. **Please include all meals, snacks and drinks (including alcohol).**

This survey has 6 sections (A - Descriptive information, B - Quick list, C - Forgotten foods list, D - Occasion , E - Details about food and drink, and F - Final step). **Please read and answer all of them.**

This survey will take approximately 20 minutes to complete.

For the purpose of this study you are required to take the questionnaire twice, on 2 different days, recalling **1 week-day and 1 weekend**.

A - Descriptive Information

2. What is your age?

3. What is your gender?

Male
 Female

4. What is your height? Please provide units (i.e. feet)

5. What is your weight? Please provide units (i.e. Kilograms)

6. Which of the below best describes your Ethnic Origin?

Please select at least 1 answer(s).

- White
- Gypsy or Traveller
- Black or Black British: Caribbean
- Black or Black British: African
- Other Black background
- Asian or Asian British: Indian
- Asian or Asian British: Pakistani
- Asian or Asian British: Bangladeshi
- Chinese
- Other Asian background
- Mixed – White and Black Caribbean
- Mixed – White and Black African
- Mixed – White and Asian
- Other Mixed background
- Arab
- Other Ethnic background
- Other

a. If you selected Other, please specify:

B - Quick list

7. Please list all the foods you have eaten in the past 24-hours (from midnight to midnight). Please also include all beverages, including alcohol. **We will also ask you to return to this question and add additional information throughout the survey.** * *Required*

C - Forgotten foods list

Please choose (tick) **all** foods from the list below that you have consumed in the same 24-hour period but you may have forgotten to list in the question 1. **Also, go back to question 1 and add them to the list, together with any other food or drink you have just remembered but have not included in the question 1.**

8. Non-alcoholic beverages

- Tea
- Coffee
- Hot chocolate
- Diet fizzy drink
- Fizzy drink
- Pure fruit juice (e.g. 100% orange or apple)
- Fruit squash or cordial
- Milk (e.g. with tea or coffee)

9. Alcoholic beverages

- Wine
- Beer
- Cider
- Liqueur (e.g. port, sherry, vermouth)
- Spirit (e.g. gin, brandy, whiskey, vodka)

10. Sweets and snacks

- Biscuits/cookies
- Cake
- Pastry
- Pie
- Ice cream
- Chocolate (single or squares)
- Chocolate bar
- Sweets
- Crisps or other packet snacks
- Peanuts or other nuts
- Crackers
- Sugar (e.g. with tea or coffee)

11. Fruit

- Apple
- Pear
- Orange
- Satsuma
- Mandarin
- Grapes
- Tinned fruit
- Dried fruit

12. Vegetables

- Carrots
- Celery
- Sweetcorn
- Avocado
- Baked beans
- Tinned vegetables
- Pickles

13. Dairy products and spreads

- Sour cream
- Double cream
- Yoghurt
- Dairy dessert
- Cheese (e.g. Cheddar, Brie, Edam)
- Cottage cheese or low fat soft cheese
- Halloumi cheese
- Feta cheese
- Salted butter
- Unsalted butter
- Margarine

14. Breads and rolls

- Bread and rolls (white, brown or wholemeal)
- Scones
- Crumpets
- Wraps

15. Deli meats

- Smoked bacon
- Unsmoked bacon
- Ham or other smoked meats
- Salami
- Sausages

16. Fish

- Salted fish (e.g. kippered herring, dried and salted cod)
- Smoked fish
- Tinned fish

17. Condiments

- Ketchup
- Mayonnaise
- Salad dressing
- Soy sauce
- Yeast extract
- Coleslaw

18. Did you add any salt while cooking? If yes, please state how much using household measures (e.g. teaspoon). * *Required*

19. Did you add any salt at the table? If yes, please state how much using household measures (e.g. teaspoon). * *Required*

20. Did you add any stock cubes while cooking? If yes, please state how many and indicate how many people this meal(s) was produced for and any leftovers? * *Required*

21. Did you add any gravy granules while cooking? If yes, please state how many and indicate how many people this meal(s) was produced for and any leftovers? * *Required*

D - Occasion

Now tell us the occasion at which you ate each food from the section A (e.g. breakfast at home, dinner at the restaurant, watching TV). Think about the activities you had throughout the day and list all the food you may have forgotten in the section A. Think about the activities such as watching TV, working on the computer, going to a cinema, restaurant or a bar. Also, try to remember the food you would not typically have or you had in between meals. **Return to question 1 and add it to the food list.**

E - Details about food and drink

Describe the food you have listed and chosen in the section A and B in as much detail as possible. It is important that you tell us the brand (if known) and any brand variation (e.g. reduced salt, sugar etc.), how was the food prepared (e.g. fried, roasted, boiled), was the food raw or canned. Also tell us how much you ate. Use exact quantities if known or household measures such as teaspoon, tablespoon, mug, cup, bowl (small, medium, large), plate (small, medium, large). **Return to question 1 and add it to the food list.**

If you need help with the portion sizes, scroll down to the end of the questionnaire where you will be able to find some photographs (question 16). If you do not need help, continue filling in the survey.

F - Final step

This is the final step. Look over all the food you have listed and described so far and add any food or drink that you may have forgotten, including the occasion and details of the food and drink. **Return to question 1 and add it to the food list.**

APPENDIX E: CELL CULTURE EXPERIMENT

Primary cell culture of human urine-derived renal proximal tubule cells

Urine sample collection and processing (Gildea et al., 2013a)

Morning urine samples were collected from seven participants already genotyped for the *SLC4A5*, *SCNN1B* and *TRPV1* SNPs and diagnosed for salt sensitivity in the study reported in Chapter 2.

Immediately after collection, the entire voided sample (~100 ml) was aliquoted in 50 ml tubes and centrifuged at 800 x g for 5 minutes at room temperature and allowed to decelerate without a brake, not to disturb the pellet. The pellet was aspirated with a 10 ml volume of urine and placed into a 15 ml tube. Pellet was washed three times in 10 ml of cold PBS (with Ca²⁺ and Mg²⁺) and centrifuged at 800 x g for 5 minutes to produce a new pellet each time. The final pellet was collected and placed in 1.5 ml tube followed by renal proximal tubule cell isolation.

Isolation of renal proximal tubule cells

Renal proximal tubule cells were isolated with the principle of immunomagnetic separation using a commercial kit (Anti-Biotin MicroBeads, Milteny Biotec, Bergisch Gladbach, Germany). The principle of this method is indirect magnetic labelling and separation of cells that are labelled with a biotinylated primary antibody. The biotinylated molecule is recognised by a monoclonal anti-biotin antibody coupled to MicroBeads. Anti-Biotin MicroBeads have the advantage of not binding to free biotin, which is often present in culture media.

Prior to cell isolation, MACS buffer (PBS, 0.5% bovine serum albumin

(BSA), 2mM ethylenediaminetetraacetic acid (EDTA), pH 7.2) was prepared by diluting MACS BSA stock solution 1:20 with autoMACS rinsing solution (Milteny Biotec, Bergisch Gladbach, Germany). Buffer was kept at 4 °C until further use.

For the cell isolation, the cell pellet obtained in the previous step was resuspended in MACS buffer for cell counting and subsequently centrifuged at 300 x g for 10 minutes. Supernatant was aspirated completely and cells were resuspended up to 10^7 nucleated cells per 100 μ L of buffer. The 1:11 dilution of the CD13-biotin antibody (human, clone: REA263, Milteny Biotec, Bergisch Gladbach, Germany) was added to buffer, according to manufacturer instructions, mixed well and incubated for 10 minutes in the dark at 4 °C. The cells were washed twice by adding 1 ml of buffer and centrifuged at 300 \times g for 10 minutes. Supernatant was aspirated completely, cells resuspended in 80 μ L of buffer followed by the addition of 20 μ L of Anti-Biotin MicroBeads (Milteny Biotec, Bergisch Gladbach, Germany) per 10^7 total cells. The suspensions was mixed well and incubated for 15 minutes at 4 °C. The cells were washed by adding 1 ml of buffer and centrifuged at 300 \times g for 10 minutes. Cell pellet was resuspended in 500 μ L of buffer per 10^8 cells.

Magnetic separation of cells

The cells were separated with MACS MS column and a suitable MACS separator (Milteny Biotec, Bergisch Gladbach, Germany). The column was rinsed with 500 μ L of MACS buffer. Cell suspension was applied onto the column and flow-through of unlabelled cells collected. The column was washed three times with the 500 μ L of buffer and flow-through combined with previously collected effluent. Column was then removed from the separator and placed on a 2 ml tube. After

pipetting 1 ml of buffer onto the column, magnetically labelled cells were flushed out by firmly pushing the plunger into the column. Isolated cells were centrifuged at 200 x g for 5 minutes.

Cell culture media

Primary medium contains DMEM/F12 (Invitrogen, Paisley, UK) supplemented with 10% (vol/vol) FBS (Invitrogen, Paisley, UK), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Paisley, UK), the REGM SingleQuot kit (Lonza, Basel, Switzerland) supplements and 2.5 µg/ml amphotericin B (Sigma Aldrich, MO, USA). To prepare 500 ml of medium, 50 ml of FBS was mixed together with 2.5 ml of penicillin/streptomycin, 1 ml of amphotericin B and each supplement vial of the REGM SingleQuot kit and filled up to 500 ml.

Proliferation medium was prepared by adding the entire amount of each supplement vial of the REGM BulletKit to the renal epithelial cell basal medium contained in the same kit (Lonza, Basel, Switzerland).

Primary renal proximal tubule cell culture (Zhou et al., 2012)

Isolated cells were resuspended in 1 ml of primary medium and then transferred to a 12-well plate. The cells were grown at 37°C in full humidity with 5% CO₂. For the first 72 hours, 1 ml of primary medium was added to the culture, without removing any medium. Approximately 96 hours after plating, 3 ml of medium was aspirated leaving ~ 1 ml, and 1 ml of proliferation medium was added. The cells were then fed every day, changing half of the proliferation medium and

leaving the other half intact. During the initial experiments and method optimisation, after approximately 10 days, colonies were seen (Figure 8.1). However, cell culture from study participants was not successful, suggesting this method may not be suitable for a rapid and easily measured salt sensitivity biomarker.



Figure 8.1 Example of a renal proximal tubule cell culture ~10 days after seeding.

Magnification x40

**APPENDIX F: CREATININE EXPERIMENT
STANDARD CURVE**

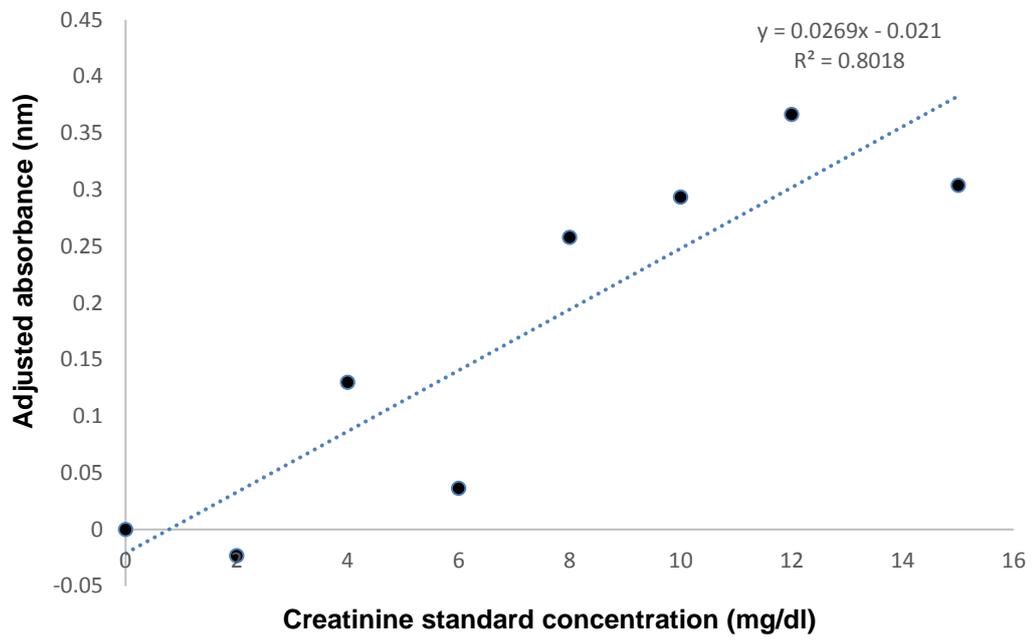


Figure 8.2 Standard curve for creatinine

The Author's Declaration

- I declare that this thesis is my own work and has not been previously submitted for an award of this university or any other institution;
- Content of the thesis is legally allowable under copyright.

Leta Pilic

06/10/2017

A handwritten signature in black ink, appearing to read "Conor Gissane".

Prof. Conor Gissane