The role of glycosaminoglycans in vascular stiffness and non-osmotic sodium storage

Thesis submitted for the degree of Doctor of Philosophy

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Summary

The role of glycosaminoglycans in vascular stiffness and non-osmotic sodium storage by Kathleen Marie Connolly

The goal of this thesis was to investigate the interplay between sodium, glycosaminoglycans, vascular stiffness, and hypertension. In contrast to the traditional view of salt-dependent hypertension, recent studies have found that sodium accumulation can occur without commensurate fluid retention. Researchers hypothesise that this sodium is stored non-osmotically via association with negatively charged glycosaminoglycans (GAGs) in the extracellular matrix. The interaction of sodium and GAGs, the influence of sodium on GAG production, and the ability of GAGs to affect vascular stiffness are of key interest.

This thesis first investigates the link between hypertension, vascular stiffness, and GAGs in ex vivo human aortae. Aortae from hypertensive donors were found to be stiffer than normotensive controls even after controlling for both pressure and age, a novel finding in humans. In these aortae, hypertension was associated with GAG remodelling, but not with changes in total GAG content.

Next, an interventional rat study is presented to examine the effects of dietary salt on vascular stiffness and GAGs, and to distinguish between salt-dependent and blood pressure-dependent effects. In vivo vascular stiffness was found to be salt-dependent but pressure-independent, with ex vivo stiffness unaffected by salt. Ex vivo stiffness was also independent of aortic GAG content, similar to the human aortae described previously. GAG content in the skin was both salt-dependent and pressure-dependent.

Finally, this thesis closes with an interventional study in humans. This study was designed to examine the effects of diuretic-induced salt loss on sodium storage, GAGs, and haemodynamics. An eight-day diuretic course corresponded to a ~10% reduction in skin sodium content, without associated water loss or cardiovascular changes. GAG mRNA expression was decreased in the skin, suggesting reduced GAG content. Pilot work from this study supports the use of $^{23}$Na MRI as a non-invasive measurement of skin sodium, but only for pre- vs post-treatment comparisons rather than absolute quantification.

In conclusion, this thesis demonstrates that both salt and blood pressure influence GAG accumulation and distribution, but that GAGs do not directly affect vascular stiffness. However, GAGs do play a direct role in osmotically inactive sodium storage, which may modulate development of hypertension.
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List of Abbreviations

ABPM ambulatory blood pressure monitoring
Aix augmentation index
ACE angiotensin-converting enzyme
Ang I angiotensin I
Ang II angiotensin II
ADH anti-diuretic hormone
BP blood pressure
BV blood volume
BW body weight
CO cardiac output
CNS central nervous system
CS chondroitin sulfate
DBP diastolic blood pressure
deoxycorticosterone acetate
DOCA deoxycorticosterone acetate
DS dermatan sulfate
EC endothelial cell
ECG electrocardiogram
ECM extracellular matrix
ECF extracellular fluid
ECFV extracellular fluid volume
ECG electrocardiogram
EH essential hypertension
FCD fixed charge density
GAG glycosaminoglycan
HA hyaluronan
HRV heart rate variability
HS heparan sulfate
Hep heparin
ICF intracellular fluid volume
ICP-OES inductively coupled plasma - optical emission spectroscopy
KS keratan sulfate
PP pulse pressure
PPA pulse pressure amplification
PBS phosphate buffered saline
PWA pulse wave analysis
PWV pulse wave velocity
SBP systolic blood pressure
SD Sprague Dawley (rat)
SH spontaneously hypertensive (rat)
TPR total peripheral resistance
TBS total body sodium
TBW total body water
UHP ultra-high purity
VSMC vascular smooth muscle cell
WKY Wistar-Kyoto (rat)
WS Wisconsin solution
Declaration

• This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

• It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

• It does not exceed the prescribed word limit for the relevant Degree Committee.
Main Thesis
Chapter 1

Introduction

Essential hypertension (EH, also called primary hypertension, or idiopathic hypertension) is defined as sustained blood pressure above 140/90 mmHg with no identifiable cause. Roughly 90-95% of hypertensive cases are classified as essential hypertension, with the remaining 5-10% denoted secondary hypertension - caused by diseases affecting the kidneys, arteries, heart, or endocrine system. Originally, all hypertension was thought to be of renal origin, and it was not until the twentieth century that EH became widely recognised as both distinctly extra-renal and responsible for the vast majority of hypertensive cases.

Despite its unknown origins, EH is a global health problem, affecting over six billion people worldwide. Epidemiological studies have shown a strong correlation between high blood pressure and cardiovascular risk, with EH accounting for 62% of strokes and 49% of coronary heart disease and contributing to nearly nine million deaths annually [122]. If EH is to be prevented or effectively targeted for treatment, it becomes crucial to identify the origins of EH as well as the mechanisms through which blood pressure is chronically elevated. While the causes of EH are by definition unknown, the present consensus is that physiological, genetic, and environmental factors all influence onset and progression.

1.1 Physiological mechanisms of blood pressure regulation

Hypertension results when the body fails to properly regulate changes in blood pressure (BP). It is therefore important to understand the mechanisms by which blood pressure is typically controlled before focusing on the causes of regulatory failure. Blood pressure is a composite property dependent on cardiac output (CO) - the volume of blood leaving the heart per minute - and total peripheral resistance (TPR) - the cumulative resistance to blood flow in the systemic vasculature:

\[ BP = CO \times TPR \]
This means that the body can regulate blood pressure by initiating physiological changes that affect either CO or TPR. Proper regulation of blood pressure is key to maintaining optimal tissue perfusion, and regulatory failure can result in tissue damage and organ failure. Blood pressure is regulated at three different levels: (1) short-term regulation (within seconds) via the nervous system, (2) intermediate-term regulation (within hours) via the endocrine system and changes in fluid balance, and (3) long-term regulation (within days) via the kidney.

1.1.1 Short-term regulation

The nervous system is responsible for near-instantaneous short-term corrections in blood pressure, relying on the activation of baroreceptors and chemoreceptors for stimulation. Fluctuations in blood pressure are detected by baroreceptors in the carotid artery and aortic arch. An increase in pressure stretches these baroreceptors, increasing their firing frequency. This activation decreases sympathetic activity and causes arteriolar dilation and venodilation, ultimately resulting in decreased TPR, decreased venous return, and decreased CO. Baroreceptor activation also increases parasympathetic activity, which decreases heart rate and vessel contractility, causing a further decrease in CO. Together, these changes in TPR and CO reduce arterial blood pressure. Because baroreceptors require deformation to activate, sensitivity decreases in hypertensive individuals as the vessel walls become stiffer and less deformable. The baroreceptor mechanism functions in the normal blood pressure range, responds within seconds, and remains active for 1-2 days.

Like baroreceptors, chemoreceptors are generally located in the carotid artery and aortic arch. Chemoreceptors respond to changes in blood pO$_2$, pCO$_2$, and pH, activating when pO$_2$ is low, pCO$_2$ is high, pH is low, or blood flow is very low. Activation of chemoreceptors increases TPR, heart rate, and stroke volume via increased sympathetic activity. These increases in heart rate and stroke volume increase CO. The chemoreceptor mechanism is only active when blood pressure is below 80mmHg. It responds within seconds, but is only active for 1-2 hours.

In addition to the chemoreceptors in the carotid artery and aortic arch, there are also pCO$_2$- and pH-sensitive chemoreceptors in the medulla. These chemoreceptors similarly respond to increased pCO$_2$ and reduced pH by increasing sympathetic activity to increase BP. This central nervous system (CNS) ischemic mechanism is incredibly powerful. Functioning as a “last-ditch” response when blood pressure falls below 40mmHg, it can raise pressures as high as 250mmHg. Like the carotid and aortic baroreceptors and chemoreceptors, this mechanism responds within seconds but only acts for a short duration.
1.1.2 Intermediate-term regulation

Intermediate mechanisms of blood pressure regulation include the renin-angiotensin-aldosterone system (RAAS), stress relaxation, capillary fluid shift, and antidiuretic hormone (ADH) regulation. These mechanisms activate within minutes or hours, and last up to several days. The RAAS is the most prominent of the intermediate mechanisms, and relies on modulation of salt and fluid balances. A drop in blood pressure induces vascular changes which decrease renal perfusion, triggering an increase in renin release and salt reabsorption. Renin catalyses the conversion of angiotensinogen from the liver to angiotensin I (Ang I), which is then converted to angiotensin II (Ang II) by angiotensin-converting enzyme (ACE). Ang II stimulates sympathetic nervous activity, arteriolar vasoconstriction, increased renal reabsorption of salt directly and via stimulated aldosterone secretion, and increased renal absorption of water via increased anti-diuretic hormone (ADH) production. Sympathetic nervous activity and vasoconstriction increase TPR, while reabsorption of water and salts effectively increases extracellular fluid volume, which increases CO.

Stress-relaxation and capillary fluid shift moderate small changes in blood pressure. Stress-relaxation is the mechanism by which increases in arterial blood pressure gently stretch the blood vessels, increasing the cross-sectional area and reducing overall BP. Capillary fluid shift occurs when increased arterial pressure increases the hydrostatic pressure of capillaries, inducing transduction of fluid out of the capillaries and into the surrounding tissues. This leads to a decrease in blood volume and subsequent decrease in CO, decreasing BP.

1.1.3 Long-term regulation

Long-term regulation of blood pressure is accomplished by the renal pressure-natriuresis mechanism, which controls salt and fluid intake and excretion. Long-term, intake and excretion must be balanced. A rise in blood pressure increases excretion of both salt and water. This mechanism is highly effective, with an increase from 100mm Hg to 200 mmHg resulting in a six- to eight-fold increase in urinary loss [101, 188]. This increased loss results in decreased blood volume and subsequent reduction in CO, leading to decreased arterial pressure. Similarly, as blood pressure drops, renal excretion is reduced, causing an accumulation of salt and fluid and the resultant increase in blood volume and CO. This mechanism can also be affected by changes in dietary salt and fluid intake, as well as renal impairments that affect excretory capacity.

1.2 Factors affecting blood pressure

The body regulates fluctuations in blood pressure by altering cardiac output and total peripheral resistance. However, certain genetic and environmental factors can also influence CO and TPR, manifesting in sustained pressure deregulation. These factors are
often cumulative, and contribute to the wide range of blood pressures observed in a given population. Extensive work by a Pickering et al in the 1950s and 1960s showed definitively that blood pressure is normally distributed, and that there is no obvious demarcation between normotensives and hypertensives [157, 158, 146, 57]. Indeed, there exists a near-linear relationship between BP and cardiovascular risk with no apparent floor [28, 114]. This means designating what constitutes “hypertensive,” and the threshold for clinical intervention, is somewhat arbitrary. However, it is important to understand the influence of these factors on blood pressure as they can be useful targets for antihypertensive treatment.

1.2.1 Age

Pickering et al showed that blood pressure was normally distributed across a given population, and found the this distribution shifts upwards with age [157, 158, 146, 57]. Age has long been the most identifiable predictor of increased blood pressure, influencing both CO and TPR. With respect to CO, a number of studies have documented the rarefaction of the renal vasculature with age, and subsequent decrease in glomerular filtration. This decreased filtration reduces the efficiency of sodium excretion, which can lead to increased extracellular fluid volume and elevated CO.

Similarly, as individuals age their blood vessels stiffen and vascular compliance decreases, causing an increase in TPR. While numerous studies have shown that both systolic blood pressure (SBP) and diastolic blood pressure (DBP) increase with age, DBP seems to plateau around 60 years while SBP continues to rise, with the rate often increasing after 60-70 years [57, 102, 182]. This suggests that the two are controlled by different mechanisms, with SBP in particular influenced by structural changes in the vessel walls which decrease distensibility [146, 129, 127, 128]. This is particularly important because isolated increases in systolic pressure lead to a wider pulse pressure, which is an independent predictor of cardiovascular risk [14]. Age can also result in rarification of the renal vasculature and impairment of sodium excretion, leading to increased sodium retention and subsequent increases in CO [88]. In general, chronic hypertension mimics these effects and induces accelerated vascular aging.

1.2.2 Gender

The effects of age on blood pressure and cardiovascular risk are to some extent influenced by sex hormones, producing gender-specific differences. Women generally have lower blood pressure and cardiovascular risk than men until menopause, after which both increase substantially and women surpass men [221, 222, 118]. Women over 60 years were found to have a higher incidence of hypertension than men regardless of ethnicity [20], and this BP increase was independent of age or BMI [226]. Interestingly, the effectiveness of hormone replacement therapy on mitigating these post-menopausal changes seems to be
age-dependent, reducing incidence of ischemic stroke in women with premature menopause (occurring before 50 years), but potentially increasing risk in post-menopausal women over 50 [168]. Such gendered effects on blood pressure are also seen in animals, with ovariectomy increasing blood pressure in salt-sensitive Dahl rats [68].

The exact mechanisms by which sex hormones influence blood pressure are still widely debated. Estradiol has been shown to have antihypertensive properties, increasing endothelial nitric oxide production through calcium modulation [217] with resultant vasodilation and inhibiting ACE production [80]. In contrast, androgens have been shown to promote sodium reabsorption, with testosterone in particular increasing Ang I and II synthesis. The effects of sex hormones on the nervous system are still largely unclear.

1.2.3 Familial genetics

Studies have shown that people with a family history of hypertension are more likely to develop hypertensive symptoms themselves [183, 25], with adoption and twin studies in particular able to quantify of the genetic component of hypertension. For systolic and diastolic blood pressures, successive adoption studies found that the regression coefficients between parents and their natural children, and between natural siblings, were double those between parents and adopted children and between adopted siblings [213]. Interestingly, the coefficients between children (both natural and adopted) were double those between parents and children (both natural and adopted), suggesting that a shared home environment also has a significant effect on adolescent blood pressure.

Similarly, twin studies have found that the regression coefficients between blood pressures of monozygotic twins were more than double than the relationship between dizygotic twins, and that genetics play an important role in modulation of blood pressure-controlling factors such as catecholamine, aldosterone, renin, and obesity [110, 52, 73]. Analysing several studies together, it has been estimated that genetics contribute 30% of blood pressure variance, shared environment contributes 15%, and non-familial environment contributes 55%.

1.2.4 Ethnicity

A variety of studies have shown ethnic trends in blood pressure. In particular, hypertension is more common and more severe in black people than white [108, 29], with stroke mortality two-fold greater in African Americans than in their Caucasian counterparts. These issues extend to clinical outcomes, with African Americans showing poorer blood pressure control for a variety of treatments. The reasons for such racial disparities are not well understood [92], and incorporation of ethnicity into clinical practice is complicated by reliance on self-defined ethnicity.

Self-identified ethnicity features prominently in both the UK National Institute for Health and Care Excellence (NICE) and North American hypertension guidelines, but
there is scant evidence supporting the usefulness of such self-defined stratification in hypertensive treatment. This is likely because the phenotypic and cultural components generally used to determine ethnicity are not necessarily indicative of relevant genetic polymorphisms and conceal the increased genetic mixing between historically isolated populations. The currently ongoing AIM-HY (Ancestral and biological Informative Markers for stratification of Hypertension) study is investigating whether stratification using ancestry informative makers (AIMs) – genetic polymorphisms whose frequency differs substantially between distinct geographic regions – improves treatment outcomes.

1.2.5 Stress

A number of studies have demonstrated that psychological stress elevates blood pressure, and indeed one explanation for the higher incidence of hypertension in African Americans is increased stress due to racial and socioeconomic hardship [29]. But even within Caucasian groups, psychological stresses from the short-term “white coat syndrome” to long-term effects of urban lifestyles all increase blood pressure [67, 159, 194, 192, 193].

The effects of stress are generally synergistic with other BP-altering factors such as age and gender, and are strongly correlated with deleterious behaviours such as overeating, overconsumption of alcohol, and poor sleep. Even in the absence of chronic stress, there persist that concerns that short-term stress-related spikes in blood pressure may cumulatively produce an increase in lifetime cardiovascular risk. Interestingly, the age-independence of blood pressure in patients with Down’s Syndrome has been attributed to their reduced perception of stress [136].

1.2.6 Obesity and diet

In Western countries, over 50% of hypertensives are overweight or obese [23, 76, 115, 204, 90]. With intervention, blood pressure typically decreases with weight loss, and this decrease is augmented by increased exercise and physical activity [204, 116]. Additional dietary factors such as fibre levels, vegetarianism, and fish intake can also produce small but significant decreases in blood pressure [8, 119], while alcohol and salt consumption can lead to increases [113, 7, 9, 31]. It is the effects of salt that will be the primary focus of this thesis.

1.3 The role of salt in hypertension

Over the past century, the role of salt in hypertension and cardiovascular mortality has been the subject of intense scientific research. It is now generally agreed that excessive salt consumption, primarily in the form of sodium chloride, is independently associated with elevated blood pressure and the development of hypertension and cardiovascular disease. Today, the World Health Organisation (WHO) cites reduction of dietary salt as
one of the most cost-effective measures for combating non-communicable disease generally, and cardiovascular disease in particular. In most countries, the average per-person salt intake is 9-12g daily, double the recommended 5g per day. Responsible for 13.5% of all premature deaths (7.6 million annually), this excessive salt consumption is a crucial target for reduction of hypertensive incidence and treatment of hypertensive disease.

When dietary sodium intake exceeds the excretory capacity of the kidneys, sodium accumulates in the body. This accumulation induces cardiovascular changes that increase blood pressure and cardiovascular risk. Interestingly, changes are typically seen only when dietary intake is “excessive.” Essential hypertension is virtually absent in populations that consume less than 50 mmol sodium (3g salt) per day, but becomes a prominent disease in populations consuming over 100 mmol sodium (6g salt) daily.

One of the most comprehensive studies investigating the effect of salt on blood pressure followed the effects of salt over three decades. Examining 10,000 subjects in 32 countries, the International Study of Salt and BP (INTERSALT) study showed that difference in daily sodium consumption of 50 mmol (3g salt) resulted in a 4.5 mmHg/2.3 mmHg difference in systolic/diastolic blood pressure and a significant difference in cardiovascular risk. The Dietary Approaches to Stop Hypertension (DASH) trial produced similar findings, demonstrating that reducing daily salt from 8g to 4g reduced blood pressure by 10 mmHg/4.5 mmHg (SBP/DBP). Generally speaking, the acute and chronic effects of salt on blood pressure have been demonstrated repeatedly in a variety of small- and large-scale clinical studies.

One particular finding that has emerged from such extensive investigation is that while populations as a whole demonstrate blood pressure increases in response to salt, this is not the case for all, or even most, individuals. It is estimated that only 25-35% of people undergo “salt-sensitive” blood pressure changes. A number of studies [59, 60, 216, 215], including the DASH trial, have shown that women are more salt-sensitive than men, blacks are more salt-sensitive than other ethnic groups, and, perhaps unsurprisingly, hypertensives are more likely to be salt-sensitive than normotensives. However, despite this extensive evidence, the physiological basis of salt-sensitivity and the mechanisms by which salt affects blood pressure remain largely unknown.

1.4 Guyton’s theory of hypertension

Nearly 40 years ago, A. Guyton developed a complex model to explain both normal and abnormal blood pressure control that was so detailed it still exerts considerable influence today [56, 211]. The theory states that all hypertension stems from chronic salt load in excess of renal excretory capacity, and that the autonomic nervous system plays no role in hypertension. This mechanism is diagrammed in Figure 1.1. Briefly, this theory states that when salt intake exceeds renal excretory capacity, salt is retained in the body. This salt causes osmotic retention of a compensatory volume of water. The increase in
water increases extracellular fluid volume (ECVF), which increases blood volume (BV). Greater blood volume creates a greater venous return (Ven Ret) and cardiac output (CO), elevating blood pressure (BP). Guyton suggested that in healthy individuals, disturbances in the fluid-sodium balance are corrected within hours or days. It is only in individuals with renal abnormalities that attempts to restore this balance result in hypertension.

1.4.1 Challenges to Guyton’s theory

Despite the complexity of his model and its continued prevalence today, discrepancies between the Guyton theory and experimental evidence have been slowly building. In fact, Guyton himself was first to demonstrate inconsistencies between its predictions and experimental data. To verify his hypothesis, Guyton surgically reduced the renal mass of dogs to about 25-30% of normal [27]. Remarkably, this did not affect blood pressure until dietary salt intake was increased two- to threefold, after which severe hypertension developed. Furthermore, while blood pressure initially increased with increasing BV (as predicted), a delayed rise in TPR eventually accounted for the bulk of the blood pressure increase. This is in contrast to the initial model depicted in Figure 1.1, which suggests that a salt-induced rise in blood pressure is solely a result of changes in BV and CO, not changes in TPR. Guyton chose to explain this discrepancy by adding an additional mechanism, “long-term auto-regulation,” to his model.

Despite this addition, more recent studies have highlighted similar issues. A study by Kirkendall et al in 1976 [82] showed that when eight normotensive men were put on consecutive low, moderate, and high salt diets for 4 weeks each, there was no significant increase in total body water (TBW) with dietary salt content. This suggests that, in contrast to Guyton’s model, increased salt consumption does not necessarily cause water retention. Similarly, in 2000 Heer et al [62] randomly allocated 32 male subjects to NaCl intakes of 50, 200, 400, or 500 mEq/day. Again, contrary to Guyton’s model, increased salt consumption did not cause water retention, with TBW and body weight (BW) remaining constant.

A study by Titze et al explored this relationship between salt intake and water retention more thoroughly [197]. Titze et al compared changes in total body sodium ($\Delta$TBS) and changes in body weight ($\Delta$BW) for three subjects kept on a metabolic ward for 135 days. Guyton’s model would suggest a direct relationship between $\Delta$TBS and $\Delta$BW. Instead, during Days 0-30, two of the three subjects actually showed a decrease in BW despite increased TBS. Between Days 30-80, all three subjects showed the predicted parallel increase, but after Day 80, BW plateaued despite a continued increase in TBS.

Together, these studies suggest that dietary salt intake in excess of renal excretion does not, of necessity, cause water retention, and may explain why only 25-35% of the population exhibit salt-sensitivity. The existence of a salt-sensitive subset of the human population encourages reexamination of the Guyton model and an exploration of whether this subset in particular has a decreased capacity for “non-osmotic” sodium accumulation.
Critical feedback circuit in Guyton’s theory of hypertension. (1) The relationship of arterial blood pressure (AP) to urinary output of salt and water (UO). As AP increases, so does UO. (2) Calculation of net salt change. UO is subtracted from salt and water intake. If UO exceeds intake, then net salt is lost; if intake exceeds UO, then net salt is gained. (3) The behaviour of extracellular fluid volume in response to net salt changes. If net salt is lost, then ECFV decreases through compensatory water loss; if net salt is gained, then ECFV increases. (4) Relationship between ECFV and blood volume (BV). ECFV and BV are directly related such that a drop or rise in ECFV causes a related drop or rise in BV. (5) Relationship between BV and mean systolic blood pressure (MSP, mmHg). MSP increases or decreases with associated changes in BV. (6) Calculation of the gradient for venous return (Ven Ret). MSP is subtracted from right atrial pressure (RAP, mmHg) to determine Ven Ret. (7) Calculation of cardiac output (CO, L/min). Ven Ret is divided by resistance to venous return (R Ven Ret) to determine CO. (8) Calculation of AP. A multiplier of CO times total peripheral resistance (TRP) equals AP. [87]
1.5 Non-osmotic sodium storage

The intracellular fluid (ICF) and extracellular fluid (ECF) both have an osmolalities of 275-290 mOsmol/kg. To maintain this osmolality, 0.22kg of water are necessary to osmotically neutralise each 1g (43.5 mEq) of additional Na\(^+\). In the traditional “two-compartment model” of sodium storage, sodium is located either intracellularly or extracellularly, with the extracellular space further sub-divided into the interstitial and intravascular spaces (Figure 1.2). Water and sodium can move freely between these compartments, but a consistent osmolality is maintained as water follows and osmotically neutralises free sodium ions.

An interesting phenomenon arises when the work of Kirkendall et al [82], Heer et al [63, 62], and Titze et al [197] are examined with this in mind. Heer et al [63, 62] will be used as an example, but the findings equally apply to Kirkendall et al and Titze et al. In 1993, Heer et al subjected healthy participants to three consecutive eight-week periods of stepwise increase in dietary NaCl (220 to 440 to 660 mEq/day). At the end of the study, TBS was increased by \(1,704.2 \pm 309.8\) mEq Na\(^+\). If this sodium were entirely unbound and osmotically active, then participants would need to have retained \(7.05 - 10.2\) kg of water to preserve fluid osmolality. But despite these calculations the researchers found no significant increase in BW, while serum sodium and serum osmolality remained unchanged.

The necessary explanation adopted by researchers is that this sodium must not only exist in the ICF and ECF, where it exerts osmotic effects, but also at other sites in the body where it is osmotically inactive. Potential osmotically inactive sodium reservoirs may include cartilage, bone, and skin. Three studies by Titze et al [199, 200, 198] began exploring sodium accumulation at these sites in 2002. Focusing primarily on bone and skin, they showed that while bone sodium content cannot account for differences in osmotically active sodium storage between control and salt-sensitive rat strains, salt loading of rats does lead to an accumulation of osmotically inactive sodium in the skin, with skin sodium concentration increasing from 155 mM to 187 mM.

While accumulation of sodium in the skin was a novel finding, excessive sodium accumulation has long been a known feature of cartilage, with homeostatic cartilage sodium concentrations in humans ranging from 250-350 mmol/L [100] with extracellular osmolalities of 350-450 mOsmol/kg [207]. In cartilage, this excessive osmolality is maintained by a high osmotic gradient drawing water in, with compressive forces preventing osmotic equilibration. Extensive research on cartilage has shown that the disproportionately high sodium concentration is due to the prevalence of polyanionic proteoglycans and glycosaminoglycans (GAGs) in cartilage tissue. Recently, Titze et al [198] and Schaffluhuber et al [175] showed that these same proteoglycans and GAGs may be similarly responsible for accumulation and osmotically inactive storage of sodium in the skin.
Figure 1.2: Two compartment model of water and ion storage. Water and ions are located in either the intracellular or the extracellular space, with the extracellular space further sub-divided into the interstitial and intravascular spaces. Water and ions are free to move between these spaces, maintaining a constant osmolality of $275 - 290$ mOsm/kg. A healthy adult male weighting 70 kg will contain approximately 42 L of fluid, with 28 L in the intracellular space and 14 L in the extracellular space.
1.6 Proteoglycans and glycosaminoglycans

Proteoglycans are prolific in the extracellular matrix (ECM) of a variety of tissues, including cartilage, skin, and blood vessels. They are a family of molecules in which one or more glycosaminoglycan chains are covalently bound to a core protein (Figure 1.3). Glycosaminoglycans (GAGs) are linear polysaccharides comprised of a repeating disaccharide unit. These disaccharide units are made up of an amino sugar derivative (N-acetylglucosamine, glucosamine with various N substitutions, or N-acetylgalactosamine) and a uronic acid derivative (glucuronic acid or iduronic acid) or galactose. There are six major types of GAGs in mammals, differentiated by the composition of their repeating disaccharide subunits: hyaluronan (HA), chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS), heparan sulphate (HS), and heparin (Hep) (Table 1.1). These names reflect the tissues from which each was originally isolated. Each GAG can be further classified by the location of sulfate groups on the disaccharide subunits.

Proteoglycans result when GAGs are attached to core proteins via the trisaccharide Gal-Gal-Xyl (with the exception of keratan sulphate) at the reducing end of the GAG chain, allowing xylose to form an O-glycosidic bond with a serine reside on the core protein. Enzymes affecting the synthesis of GAGs can be seen in Figure 1.4. Different proteoglycans result from different combinations of core proteins and GAG side-chains. Hyaluronan is unique among the glycosaminoglycans in that it is not known to covalently bind core proteins. Instead, hyaluronan interacts noncovalently with proteoglycans via hyaluronan-binding motifs or link proteins.

A single proteoglycan can contain anywhere from one (decorin) to over 150 (aggrecan) GAG side changes. There is also evidence that, for some proteoglycans such as aggrecan, the GAG-binding sites are not perpetually saturated and under certain stimuli additional GAGs can be attached to an existing core protein [169]. Multiple types of GAGs can bind a single core protein and the length of a each GAG (the number of disaccharide repeats it contains) is highly variable, with most containing ~80 sugar residues. This allows for a wide diversity of proteoglycans with various GAG content and length. Due to the large size and number of GAGs associated with a single core protein, glycosaminoglycan chains tend to dominate the chemical properties of proteoglycans.

Proteoglycans have distinct physical properties which can be attributed to their “bottle brush” structure, caused by multiple linear GAGs extending from a single core protein. The high charge density present around each glycosaminoglycan creates a highly expanded and hydrophilic structure that occupies a large volume within the solution which other macromolecules cannot occupy, but which is freely permeable to small molecules. The attraction of water to proteoglycans is mediated by the sulphate and carboxylic acid groups on the GAG side chains. Classically, it is thought that the volume occupied by proteoglycans can only be reduced under pressure, by the thermodynamically unfavourable release of bound water. This water can then be slowly taken back up as pressure is eased, allowing proteoglycan aggregates to function as molecular shock absorbers.
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Figure 1.3: General proteoglycan and glycosaminoglycan structure. Proteoglycans are composed of one or more glycosaminoglycans attached to a core protein via the Gal-Gal-Xyl linkage (with the exception of keratan sulfate, which lacks this linkage region). Each of the six types of glycosaminoglycans has a unique disaccharide subunit whose length can vary dramatically even between glycosaminoglycans attached to the same core protein. Hyaluronan is the only glycosaminoglycan that exists free from a core protein.
### Table 1.1: Classification of common glycosaminoglycans

<table>
<thead>
<tr>
<th>Type</th>
<th>Amino sugar derivative</th>
<th>Uronic acid derivative</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronan (HA)</td>
<td>GlcUA</td>
<td>GlcNAc</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Chondroitin sulphate (CS)</td>
<td>GlcUA</td>
<td>GalNAc(4S)</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GalNAc(6S)</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GalNAc(4S,6S)</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Keratan sulphate (KS)</td>
<td>Gal</td>
<td>GlcNAc(6S)</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Heparan sulphate (HS)</td>
<td>IdoUA</td>
<td>GlcNAc</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Heparin (Hep)</td>
<td>GlcUA</td>
<td>GlcNAc</td>
<td><img src="image" alt="Structure" /></td>
</tr>
</tbody>
</table>

Gal = β-D-galactose, Gal(6S) = 6-O-sulfo-β-D-galactose, GalNAc = β-D-N-acetylgalactosamine, GalNAc(4S) = β-D-N-acetylgalactosamine-4-O-sulfate, GalNAc(6S) = β-D-N-acetylgalactosamine-6-O-sulfate, GalNAc(4S,6S) = β-D-N-acetylgalactosamine-4-O, 6-O-sulfate, GlcNAc = α-D-N-acetylglucosamine, GlcNS = α-D-N-sulfoglucosamine, GlcNS(6S) = α-D-N-sulfoglucosamine-6-O-sulfate, GlcUA = β-D-glucuronic acid, GlcUA(2S) = 2-O-sulfo-β-D-glucuronic acid, IdoUA = α-L-iduronic acid

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Figure 1.4: Synthesis of glycosaminoglycans. The synthesis of GAGs is a multi-step process controlled by a wide variety of enzymes. The main steps of chondroitin sulfate (CS), dermantan sulfate (DS), and heparan sulfate (HS) are presented here. Black text indicates the proteins associated with each step, and red text details the genes coding for each protein.

**Gal** = β-D-galactose **Gal(6S)** = 6-O-sulfo-β-D-galactose, **GalNAc** = β-D-N-acetylgalactosamine, **GalNAc(4S)** = β-D-N-acetylgalactosamine-4-O-sulfate, **GalNAc(6S)** = β-D-N-acetylgalactosamine-6-O-sulfate, **GalNAc(4S,6S)** = β-D-N-acetylgalactosamine-4-O, 6-O-sulfate, **GlcNAc** = α-D-N-acetylglucosamine, **GlcNS** = α-D-N-sulfo glucosamine, **GlcNS(6S)** = α-D-N-sulfo glucosamine-6-O-sulfate, **GlcUA** = β-D-glucuronic acid, **GlcUA(2S)** = 2-O-sulfo-β-D-glucuronic acid, **IdoUA** = α-L-iduronic acid
1.6.1 Glycosaminoglycans and osmotically inactive sodium storage

The high negative charge density of GAGs functionally attracts cations such as Na\(^+\) to GAG-rich tissues like cartilage, bathing chondrocytes in an extracellular Na\(^+\) concentration of 350-450 mmol/L [100] and an extracellular osmolality of 250-350 mOsmol/kg [207]. Given the extensive documentation on the role of GAGs in attraction of sodium to cartilage, Titze et al proposed that osmotically inactive sodium storage in the skin might be a process that, similar to cartilage, is dependent on the GAG content.

To examine this hypothesis, the group placed Sprague-Dawley rats on either a low (0.1%) or high (8%) NaCl diet for eight weeks, then examined water, sodium, proteoglycan, and GAG content in the skin [198]. By assuming that rats fed a low salt diet would have no osmotically inactive sodium storage, they estimated that in rats fed a high salt diet nearly 20% of the sodium in the skin was stored as in an osmotically inactive manner. The group also found that in rats fed a high salt diet, there was no significant change in proteoglycan core protein content, but a large increase in GAG content. Real-time PCR analysis confirmed an increased expression of GAG synthetic enzyme mRNA as well as mRNA coding for enzymes which attach newly-synthesised GAG side chains to the proteoglycan core proteins. This suggests that while the number of proteoglycans did not increase, either 1) the length of existing GAG chains increased, 2) more GAG chains of the same length or longer were added to existing core proteins, or 3) both. This evidence supports the idea that GAG content is more important for sodium storage than simply the concentration of core proteins, which had previously been targeted for quantification.

Schafflhuber et al [175] performed a follow-up study in which rats were salt depleted as opposed to salt loaded. This group found that in ten-week-old rats fed a low salt (0.1%) diet for four weeks, total body sodium decreased by 9%. Half of this sodium loss was osmotically inactive and originated largely from the skin. This salt deprivation was also associated with decreased GAG content in the skin. These findings complement those of Titze et al [198], and together suggest that 1) the skin is the primary site of osmotically inactive sodium storage, and 2) that GAG content in the skin is directly influenced by and correlated with dietary sodium content.

1.6.2 Glycosaminoglycans and tissue hydration

Although associated with sodium storage, this is not believed to be the primary physiological function of glycosaminoglycans. GAGs are highly charged, and as such they generate an osmotic pressure that plays a major role in determining tissue hydration. This osmotic pressure is central to a wide range of physiological functions, enabling cartilage to withstand high compressive loads, maintaining the transparency of the cornea [42], and influencing solute exchange through the interstitium [120].

In solution, the sulfate and carboxylic acid groups of glycosaminoglycans contribute
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to the overall fixed charge density (FCD) of the tissue, with the FCD of healthy articular cartilage ranging from 0.05 – 0.35 mEq/mL H₂O [120, 208]. However, a number of studies have shown that the osmotic pressure of GAGs decreases with increasing ion concentration [38, 22, 5]. In Ehrlich et al [38], the osmotic pressure of a chondroitin sulfate solution increased with chondroitin sulfate concentration (expressed as FCD) and decreased with increasing salt concentration. Above 1.0 M NaCl, the osmotic pressure changes were negligible despite further increases in NaCl concentration due to saturation. These findings support the idea that sodium can alter the biochemical properties of GAGs, and deviation from homeostatic concentrations may be detrimental to tissue biomechanics. With respect to blood pressure, salt-induced changes in GAG content or GAG biochemical behaviour could affect the stiffness of blood vessels and lead to chronic hypertension.

1.7 Arterial biomechanics and glycosaminoglycans

How glycosaminoglycans interact with sodium and how sodium influences GAG synthesis have been predominantly studied in cartilage and skin. Linking this GAG-sodium relationship to hypertension is more complex, although Titze et al [199] showed that spontaneously hypertensive rats had reduced osmotically-inactive sodium storage in the skin. Perhaps the predominant role of GAGs in hypertension is to buffer free body sodium, minimising salt-dependent blood pressure changes. In this way, GAGs play an indirect role in cardiovascular homeostasis.

GAGs may play a more direct role as well. As it has been shown that GAG content in the skin increases with salt loading, perhaps salt loading also induces increased synthesis of GAGs in the arterial wall. Such changes in wall composition may then have a direct impact on the biomechanics of the vessel and lead to increased blood pressure. To elucidate this latter mechanism, it is important to first establish how how stiffness is controlled in healthy arteries and the normal role of GAGs in the vessel wall.

1.7.1 Structure of the arterial wall

Arteries can be divided into two categories: elastic and muscular. Elastic arteries, like the aorta, generally have larger diameters and are located closer to the heart, while muscular arteries are located at the periphery. The mechanical behaviour of elastic arteries is dominated by the composition and structural arrangements of the vessel wall [50, 195]. The wall is composed of three concentric layers (Figure 1.5). The innermost layer, the intima, is characterised by a continuous monolayer of endothelial cells (ECs) lining the vessel lumen and an underlying basement membrane, ending at the internal elastic lamina. Surrounding the intima is the thickest layer, the media, comprised of vascular smooth muscle cells (VSMCs), proteoglycan matrix (commonly called “ground substance”), elastin, and collagen. Despite its heterogenous composition, the media tends to behave as a cohesive
unit. The outermost layer, then adventitia, is composed of matrix, fibroblasts, and nerve cells.

*In vivo* adjustments to vascular tone are accomplished via communication between ECs and VSMCs. Directly exposed to the lumen, ECs are responsible for sensing changes in pressure or shear stress and communicating this information to the VSMCs in the media via signalling molecules and physical contact [105]. However, while VSMCs do contribute to vascular tone and long-term structural remodelling, they are not themselves regarded as a structurally important material. Instead, the mechanical properties of the wall are predominantly governed by elastin and collagen in the media.

### 1.7.2 Biomechanics, haemodynamics, and stiffness

Elastin and collagen are arranged in circumferential lamellae which expand and relax with variations in blood pressure. These lamellae comprise the majority of the arterial wall and are responsible for its elastic properties. At physiological pressures collagen in kinked and unstretched, and elastin dominates the mechanical properties of the wall. It is only when pressure increases that the collagen gradually straightens and provides rigidity, acting as a final safeguard against wall failure. This bimodal dominance of stiffness makes the elastic properties of arterial walls non-linear with pressure (Figure 1.6). As people age these elastin fibres decay, shifting primary load-bearing to the stiffer collagen fibres. Due to the low turnover of elastin, these broken fibres are generally not repaired or replaced. In contrast, collagen content increases with age, further shifting biomechanical dominance onto collagen [176].

Young, healthy arteries behave like elastic tubes, expanding and contracting with pulsatile blood flow from the heart. During systole, blood is ejected from the left ventricle and the elastic arteries, primarily the aorta, distend to accommodate the rapid volume increase. This distention creates a “reservoir” of stored blood and potential energy. As systole ends the arteries recoil, and this stored energy continues the forward propulsion of blood without ventricular contraction during diastole. The overall effect of this elastic recoil is to transform pulsatile flow from the heart into a smooth, continuous flow, protecting the more distal vessels from large fluctuations in pressure and blood supply. This mechanistic smoothing is known as the “Windkessel effect,” after Windkessel vessels of the eighteenth century which fulfilled a similar function in fire engines of that time.

Wave reflection further contributes to the efficiency of this system. Intermittent ventricular ejection from the heart creates pressure waves which travel away from the heart and towards the periphery, where they are reflected back towards the heart and sum with subsequent incidental (forward-moving) waves. In healthy vessels, these reflected waves arrive back in the ascending aorta during diastole, effectively boosting diastolic pressure and assisting coronary artery perfusion. This means that any measured waveform in the vascular system will be a composite of both incidental and reflected waves. However, the efficiency of this system is decreased if the timing of the reflected waves is
Figure 1.5: **Structure of the elastic artery.** (A) Elastic arteries are comprised of three concentric layers, the intima, media, and adventitia. The intima lines the lumen of the vessel. (B) Biological composition of each larterial layer. [225] (C) Alcian blue staining with Lugol’s Iodine counterstaining for GAGs (blue) and elastin (black). Aorta is from human donor TS175.

Figure 1.6: **Effect of pressure on collagen recruitment and stiffness.** At most physiological pressures, collagen recruitment is low and elastin dictates the stiffness of the vessel wall. As pressure increases, collagen fibres unkink and contribute towards stiffness. At high pressures, the stiffness of the vessel wall is dominated by collagen resistance.
offset, as happens during arterial stiffening.

When the elasticity of the arterial wall is diminished, the artery becomes stiffer. Aortic stiffness, the reduced ability of an artery to expand and contract in response to pressure changes, has long been recognised as a significant predictor of cardiovascular morbidity and mortality. Such stiffening reduces the buffering capacity of large arteries during systole and allows premature arrival of reflected waves, reducing the efficient smoothing of pulsatile flow and increasing pulsatile stress and sheer stress on the vessel walls. Stiffness is a dynamic contributor towards overall cardiovascular health, and can both influence and be influenced by other cardiovascular predictors such as left ventricular hypertrophy, cardiac output, pulse wave velocity, and blood pressure. Stiffening of the aorta generally leads to isolated systolic hypertension, which accounts for two thirds of essential hypertension in people over 60 years of age.

1.7.3 Glycosaminoglycans and the biomechanical properties of the aorta

Elastin and collagen have been the focus of most vascular stiffness studies, but there is growing interest in the contribution of proteoglycans and GAGs to stiffness. Distributed throughout the arterial wall (Figure 1.5C), the impact of proteoglycans on aortic stiffness is highlighted by a group of metabolic disorders collectively referred to as mucopolysaccharidoses (Table 1.2). Mucopolysaccharidosis is caused by the absence or disfunction of one or more of the lysosomal enzymes necessary for glycosaminoglycan degradation. Without these enzymes, GAGs build up in the extracellular matrix, resulting in progressive and permanent damage that affects appearance, physical ability, organ function, and mental development.

A number of studies on individuals with mucopolysaccharidosis show that the disease can result in increased intima-media thickness, reduced cross-sectional compliance, and greater incremental elastic modulus (stiffness) in the carotid artery [16]; increased arterial stiffness index and reduced distensibility in the ascending aorta [138]; and a higher incidence of systemic hypertension generally attributed to arterial narrowing [189, 220]. In contrast, aortic aneurysms - which result from excessively distensible arterial walls - are associated with decreased glycosaminoglycan content [93, 191, 190]. There is even recent work suggesting an increase in GAGs at the site of aortic dissections. Though reliant on extreme GAG disregulation, these diseases clearly demonstrate that arterial GAG content can influence cardiovascular health.
Table 1.2: Main mucopolysaccharidoses

<table>
<thead>
<tr>
<th>MPS type</th>
<th>Enzyme deficiency</th>
<th>GAG affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPS I (Hurler)</td>
<td>$\alpha$-L-iduronidase</td>
<td>DS, HS</td>
</tr>
<tr>
<td>MPS II (Hunter)</td>
<td>$\alpha$-L-iduronidase</td>
<td>DS, HS</td>
</tr>
<tr>
<td>MPS III (Sanfilippo)</td>
<td>heparan sulfamidase</td>
<td>HS</td>
</tr>
<tr>
<td></td>
<td>N-acetyl-$\alpha$-D-glucosaminidase</td>
<td>HS</td>
</tr>
<tr>
<td></td>
<td>acetyl-CoA-$\alpha$-glucosaminidase</td>
<td>HS</td>
</tr>
<tr>
<td></td>
<td>N-acetyltransferase</td>
<td>HS</td>
</tr>
<tr>
<td></td>
<td>N-acetylglucosamine-6-sulfatase</td>
<td>HS</td>
</tr>
<tr>
<td>MPS IV (Morquio)</td>
<td>N-acetylgalactosamine-6-sulfatase</td>
<td>CS, KS</td>
</tr>
<tr>
<td></td>
<td>$\beta$-galactosidase</td>
<td>CS, KS</td>
</tr>
<tr>
<td>MPS VI (Maroteaux-Lamy)</td>
<td>N-acetylgalactosamine-4-sulfatase</td>
<td>DS</td>
</tr>
<tr>
<td>MPS VII (Sly)</td>
<td>$\beta$-D-glucuronidase</td>
<td>CS, DS, KS</td>
</tr>
<tr>
<td>MPS IX (Natowiez)</td>
<td>Hyaluronidase</td>
<td>CS, HA</td>
</tr>
</tbody>
</table>

DS = dermatan sulfate, CS = chondroitin sulfate, HS = heparan sulfate, KS = keratan sulfate
1.7.4 Genetic basis of salt-induced glycosaminoglycan production and hypertension

In rats, there is evidence to suggest that the relationship between glycosaminoglycan production and hypertension has a genetic basis. While Titze et al [198] and Schafflhuber et al [175] only examined GAGs in the skin of Sprague-Dawley rats, Reynertson et al [163, 164] compared GAGs in the aortae of spontaneously hypertensive (SH) rats and control Wistar Kyoto (WKY) rats. They showed that the aortae of SH rats have a higher CS content, altered rates of proteoglycan synthesis and/or degradation, and altered proteoglycan structure compared to the aortae of WKY rats. This study encourages investigation into whether salt-sensitive expression of GAGs may also have a genetic basis in humans, and could suggest an explanation for why only 25-35% of the human population has blood pressure sensitive to dietary salt intake.

1.7.5 Combined influence of glycosaminoglycans and ions on the biomechanical properties of the aorta

A number of studies have shown that the concentrations of cations – especially sodium – in the arterial wall and cartilage are actually higher than in the plasma. In 1960, Headings et al [61] showed that for sodium, this difference was so great that some Na$^+$ must be stored in an osmotically inactive “third space,” rather than osmotically in the intracellular or extracellular fluid. This third space is popularly theorised to represent association of cations with the negatively charged GAGs, as described in Section 1.5. Palaty et al [153] took this hypothesis further, estimating the amount of sodium bound to the carboxylic acid vs. sulphate groups (which together establish the anionic character of GAGs) in the arterial wall and demonstrating that these groups seem to have equal affinity for calcium and magnesium.

The interplay between GAGs and the ionic composition of the matrix may be associated with stiffness and vascular disease. Studies in the 1960s and 70s examined rats with deoxycorticosterone acetate (DOCA)-induced hypertension and found that after hypertensive onset, both aortic sodium content and aortic GAG content were increased [153, 89]. Similarly, a recent study has shown that vascular calcification is enhanced in mice with overexpression of GAGs, indicating a possible role for GAGs in facilitating and sustaining calcium deposition in murine chronic kidney disease [160].

Together, these studies suggest that GAGs in the arterial wall may interact significantly with extracellular ions – in particular, with sodium and calcium – in the progression of stiffness-associated cardiovascular diseases. Combined with studies showing that increased ionic content decreases the osmotic pressure of GAGs, it is suggested that association with these ions may alter the elastic properties of the arterial wall by altering the ability of GAGs to attract water and confer tissue flexibility and resilience.

Furthermore, studies by Titze et al [198] and Schafflhuber et al [175] have asserted
that the association between GAGs and ions is not just casual, but also causative. They showed that dietary salt-loading or salt-depletion of rats will raise or lower, respectively, the expression of GAG synthetic enzyme mRNA in the skin. It is therefore also suggested that, like in skin, ion concentrations in the aorta can themselves influence GAG content, consequently influencing stiffness. Interactions between ions or GAGs and other structural components of the arterial matrix, such as collagen and elastin, have also been noted [184, 71, 46, 104]. It is possible, then, that alteration of one component could create significant downstream changes in structural content or modelling.

1.8 Summary

There is substantial evidence showing that excessive dietary salt can lead to vascular stiffening and hypertension. However, the conventional volume-dependent mechanism by which this occurs has recently been challenged. Instead, new research suggests that excessive salt can be stored non-osmotically via binding to negatively-charged glycosaminoglycans in the skin. In turn, the excessive salt then stimulates synthesis of more glycosaminoglycans. It is not clear whether arterial glycosaminoglycans are similarly affected by salt.

In this thesis, we will investigate the non-osmotic binding of salt to glycosaminoglycans, how salt influences glycosaminoglycan synthesis in both the skin and the aorta, how glycosaminoglycan content in the aorta influences vascular stiffness, and how salt influences vascular stiffness generally.
Key questions of this thesis

Key question 1. To what extent does the skin, by way of glycosaminoglycans, act as an osmotically inactive sodium reservoir for buffering against salt-induced haemodynamic changes? Does dietary salt content induce the same changes in arterial glycosaminoglycan content that it does in the skin?

Titze *et al* [198] and Schafflhuber *et al* [175] have suggested that in rats, the glycosaminoglycan content of skin was correlated with salt intake such that the more dietary salt a rat ingested, the greater the expression of glycosaminoglycan synthetic enzymes in the skin, but the aortae were not similarly examined. It must be noted that salt handling in rats differs drastically from salt handling in humans, and the high salt diets fed to rats in these experiments possessed salt contents far in excess of normal human consumption. Therefore, it is important to determine first whether the salt-associated GAG changes seen in the skin are also seen in the aorta, and second to determine if these changes which are seen in rats are also seen in humans.

Key question 2. Excluding extreme cases such as mucopolysaccharidoses, is arterial glycosaminoglycan content associated with vessel stiffness and blood pressure? In salt-induced hypertensive models, is any change in the arterial glycosaminoglycan content caused directly by increased body sodium (as seems to be the case in skin), or are these changes secondary to salt-induced changes in blood pressure?

Cardiovascular changes have been noted in extreme cases of GAG dysfunction, such as mucopolysaccharidoses. However, the extent to which more moderate ranges of GAG content correlate with blood pressure or incidence of cardiovascular risk has not been studied. Moreover, it is not clear whether the dietary salt consumption can directly influence arterial GAG content (as it seems to in the skin), or if arterial changes in GAG content in salt-induced hypertensive models are secondary to salt-induced changes in blood pressure.
Key question 3. Are some types of glycosaminoglycans more strongly associated with these processes than others?

There are six major types of glycosaminoglycans in mammals (Table 1.1). Because GAGs dominate the chemical properties of proteoglycans, and because the same GAGs are found in different quantities and combinations on different proteoglycan core proteins, the differential distribution of GAGs is of more interest than the distribution of proteoglycans. Determining whether certain GAGs are more strongly correlated with sodium storage, sodium buffering, or vessel stiffness would be useful devising possible therapeutic targets.

Thesis overview

To answer these questions, three major studies were conducted. First, a large collection of human aortae were examined to determine relationships between hypertension, cardiovascular disease, aortic GAGs, and aortic sodium content without direct intervention. Second, a salt-loading study in rats was performed to discover if salt-induced changes seen in skin GAGs are similarly found in the aorta and to assess to what extent changes seen during salt loading are salt-dependent or pressure-dependent. Finally, an interventional trial in humans was conducted to study the effects of salt reduction on sodium, water, and GAGs in the skin. The latter two studies provide additional perspectives on the mechanism of non-osmotic skin sodium storage.
Chapter 2  

General methods  

2.1 Physiological measurements  

2.1.1 Human  

2.1.1.1 Height and weight  

Height was measured using a wall-mounted stadiometer. Weight and total body water content were measured using calibrated Tanita scales, assuming 1 kg of clothing weight. Individuals were measured wearing one layer of clothing and no shoes.

2.1.1.2 Blood sampling  

Up to 20 mL venous blood were drawn from the cubital fossa. Blood for electrolyte and urea analysis was drawn into S-Monovette® 4.7 mL Z-Gel tubes (03.1524, Sarstedt, Germany), for renin and aldosterone analysis into S-Monovette® 2.6 mL K3E tubes (04.1901, Sarstedt, Germany), for hyaluronan content into S-Monovette® 7.5 mL K3E tubes (01.1605.001, Sarstedt, Germany), and any future analysis into S-Monovette® 9 mL Z tubes (02.1063, Sarstedt, Germany). Monovette® 4.7 mL Z-Gel tubes and S-Monovette® 2.6 mL K3E tubes were sent to Cambridge University Hospitals Pathology Partnership (Addenbrooke’s Hospital, Cambridge, United Kingdom) for analysis. S-Monovette® 7.5 mL K3E tubes and S-Monovette® 9 mL Z tubes were centrifuged at 4000 g, 40°C for 15 minutes and the plasma and serum stored at -80°C.

2.1.1.3 Blood pressure  

At clinical visits, seated and supine systolic and diastolic blood pressures were measured using fully-automatic oscillometric devices. Study participants were given five minutes rest in either the seated or supine position before measurement. Each measurement was performed in triplicate, and the average of the second and third readings reported.

To obtain 24-hour ambulatory blood pressure measurements, participants each wore a validated, automated ambulatory blood pressure monitor for a period of 24 hours. The
monitor took readings every 30 minutes during the day, and every 60 minutes overnight.

2.1.1.4 Pulse wave velocity

Pulse wave velocity (PWV) was measured using applanation tonometry and the SphygmoCor® Cardiovascular Management Suite (CvMS) (AtCor Medical, Sydney, Australia), in conjunction with a simultaneous three-lead electrocardiogram (ECG) recording.

Using SphygmoCor® CvMS, carotid-to-femoral PWV (cfPWV\textsubscript{CvMS}) in supine participants was measured by attaching a three-lead ECG to an individual, and alternately flattening the carotid and femoral arteries with a hand-held tonometry probe for 10-12 acquisitions each. The distance travelled by the pulse wave was defined as the surface distance from the supra-sternal notch to the femoral probe site (d\textsubscript{femoral}) minus the distance from the supra-sternal notch to the carotid probe site (d\textsubscript{carotid}) as determined by tape measure (carotid site) or calliper (femoral site). The foot of the pulse wave was defined as the beginning of the sharp systolic up-stroke. The transit time of each pulse wave (tt\textsubscript{carotid} or tt\textsubscript{femoral}) was determined relative to the R wave of the ECG (Figure 2.1A).

PWV was calculated as the distance the pulse wave travels divided by transit time. To calculate cfPWV\textsubscript{CvMS}, the sternal-carotid distance was subtracted from the sternal-femoral distance to compensate for parallel transmission along the brachiocephalic and carotid arteries, and around the aortic arch. The transit time is similarly corrected:

\[
\text{cfPWV}_{CvMS} = \frac{d_{femoral} - d_{carotid}}{tt_{femoral} - tt_{carotid}}
\]

In the few instances where an individual’s PWV fell below the calibration range of the SphygmoCor CvMS, the SphygmoCor® XCEL PWA and PWV Device was used instead (AtCor Medical, Sydney, Australia). With this instrument, a cuff around the individual’s thigh captures the femoral waveform while a tonometry probe captures the carotid waveform. Distance was defined as the surface distance from the supra-sternal notch to the top of the thigh cuff (d\textsubscript{cuff}) minus the distance from the supra-sternal notch to the carotid probe site (d\textsubscript{carotid}). Without an accompanying ECG, transit time (\(\Delta T\)) was defined as the delay between the foot of the carotid pulse wave and the foot of the femoral pulse wave (Figure 2.1B). The cfPWV\textsubscript{XCEL} is calculated as:

\[
\text{cfPWV}_{XCEL} = \frac{d_{cuff} - d_{carotid}}{\Delta T}
\]

All calculations were performed automatically by the SphygmoCor® software. PWV was measured in triplicate. Measurements were discarded if the standard deviation was above 0.5 m/s.
Figure 2.1: Determination of carotid-to-femoral pulse wave velocity using SphygmoCor® CvMS (cfPWV<sub>CvMS</sub>) or SphygmoCor® XCEL PWA and PWV (cfPWV<sub>XCEL</sub>). (A) Carotid and femoral arterial waveforms were sequentially measured using applanation tonometry. Pulse transit times (tt<sub>carotid</sub> and tt<sub>femoral</sub>) were calculated from the electrocardiogram R-wave to the foot of each waveform. Over-body measurements from the supra-sternal notch to the carotid and femoral applanation sites (d<sub>carotid</sub> and d<sub>femoral</sub>) estimated the travel distance of each wave. cfPWV<sub>CvMS</sub> is computed by subtracting d<sub>carotid</sub> from d<sub>cuff</sub>, and dividing by the difference between transit times; (B) Simultaneous acquisition of the carotid pulse waveform by tonometric probe and femoral pulse waveform by pressurised cuff were used to calculate the transit time between waves (ΔT). Distances were measured from the supra-sternal notch to the carotid tonometry site (d<sub>carotid</sub>) or top of the thigh cuff (d<sub>cuff</sub>). cfPWV<sub>XCEL</sub> is computed by subtracting d<sub>carotid</sub> from d<sub>cuff</sub>, and dividing by ΔT.

Figure 2.2: Pulse wave analysis (PWA) and augmentation index (Aix) using SphygmoCor® CvMS. Radial waveforms were captured using applanation tonometry. Pulse pressure (PP) is defined as the pressure difference between peak systolic pressure (SBP<sub>1</sub>) and diastolic pressure (DBP). Aix is calculated by subtracting the second systolic peak (SBP<sub>2</sub>) from SBP<sub>1</sub>, as a percentage of pulse pressure.
2.1.1.5 Pulse wave analysis and augmentation index

Pulse wave analysis (PWA) allows visualisation of peripheral pressure waveforms and generation of the central waveform for analysis. PWA in supine participants was measured using SphygmoCor® CvMS after calibration to brachial cuff SBP and DBP. An individual’s right radial artery was flattened with a hand-held tonometry probe for 10-12 acquisitions. The aortic pulse pressure waveform was derived from the radial artery wave via mathematical transfer function, and represents a composite of both the forward pressure wave and reflected wave. Augmentation index (Aix), a measure of systemic arterial stiffness, was calculated as the difference between the first and second systolic peaks (SBP_2 and SBP_1), expressed as a percentage of the pulse pressure (PP) (Figure 2.2). All calculations were performed automatically by the SphygmoCor® software. PWA measurements were done in triplicate.

2.1.1.6 Cardiac output

Cardiac output was estimated using the non-invasive Innocor® inert gas re-breathing system (Innovision, Denmark). Individuals were asked to breathe in a gas mixture (1% sulphur hexafluoride, 5% nitrous oxide, 94% oxygen) for approximately five breaths, at a breathing rate of 20 breaths per minute. Expired gas was continuously sampled and analysed by an infrared photo-acoustic gas analyser. The rate of disappearance of the blood-soluble nitrous oxide is proportional to the blood flow through the lungs. This pulmonary blood flow is equal to cardiac output, in the absence of any significant intrapulmonary shunting.

2.1.1.7 Heart rate variability and autonomic function

Heart rate variability (HRV) was measured with the SphygmoCor® CvMS using a three-lead ECG. One ten-minute recording was performed with the individual in a supine position, maintaining a relaxed breathing pattern, and awake for the duration. The variability in R-to-R intervals was used to calculate heart rate variability. Spectral analysis of successive R-to-R intervals was used to quantify sympathetic and vagal influences on the heart. All calculations were performed automatically by the SphygmoCor® software.

2.1.1.8 Skin biopsy

5 mm skin biopsies were taken from the upper buttock region. Biopsies were performed by medically qualified members of the Division of Experimental Medicine and Immunotherapeutics.

Participants lay in a prone position for the procedure. The skin was cleaned with a ChloraPrep® scrub (270400, Insight Health Limited, United Kingdom) before administration of up to 20 mL of custom-ordered sodium-and-potassium-free lignocaine anaesthetic (Tayside Pharmaceuticals, Australia). The ChloraPrep® solution and lignocaine
anaesthetic were both tested for sodium and potassium content, and found not to be significant sources of contamination (3.1.2). Some doctors chose to mark the area of skin with a marker pen to visually identify a region for lignocaine administration. In these cases, the doctors were careful to ensure that their markings did not contaminate the biopsy site, due to the sodium and potassium content of the marking pens.

Biopsy procedures were performed using General OP Surgical Packs from Addenbrooke’s Hospital Surgical Services. After ensuring proper anaesthesia, biopsies were taken using a 5 mm punch (BP-50F, Kai Medical) and immediately snap-frozen on dry ice. Sites were closed using two Vicryl Rapide sutures (W9911, Ethicon), cleaned with a second ChloraPrep® scrub, and dressed with SteriStrips (E4547, 3M Health Care) and water-proof OPSITE POST-OP plasters (66000708, Smith&Nephew). After snap-freezing, biopsies were stored at -80ºC. Samples were cut in thirds for future analyses. Cutting was performed on a frozen metal platform resting on dry ice to prevent thawing.

2.1.1.9 Skin sodium using $^{23}$Na MRI

All scanning and analysis were performed in Addenbrooke’s Hospital Department of Radiology by Dr. Frank Reimer and Dr. Mary McLean, with assistance from Dr. Josh Kaggi. Participants lay in a supine position for imaging using a clinical 3T system (GE MR750, GE Healthcare, Waukesha, WI) with a bespoke $^{23}$Na T/R coil of 2 cm diameter placed under the lower back on either the right or left side. The location of the coil corresponded to the location of subsequent skin biopsy. Skin biopsies were taken within 60 minutes following completion of the MRI.

Sodium imaging was performed using a 3D cones sequence with the following imaging parameters: echo time (TE) = 0.46 ms, repetition time (TR) = 100 ms, field of view (FOV) = 16 cm, nominal resolution $1\times1\times10$ mm, 10 NEX, 14730 total readouts, 250,000 Hz full receiver bandwidth, 24.5 minutes acquisition duration. The imaging slab was positioned sagittally on the lower back. Sodium images were corrected for receive sensitivity. A phantom replacement method was used to create a calibration curve against five NaCl in 4% agar phantoms varying between 7 and 160 mmol/L NaCl and this was applied to the in vivo data to create quantitative sodium maps. Sodium images were reconstructed in Matlab 2016b (MathWorks, Natick, MA) using the Image Reconstruction Toolbox (IRT) and iterative density compensation. Regions of interest were drawn in Matlab, care was taken to avoid partial volume with the air surface and deeper layers of tissue such as fat.

2.1.2 Rat

2.1.2.1 Body size

Weight was measured using a traditional laboratory scale, and nose-to-tail length was measured using a ruler.
2.1.2.2 Water consumption, food consumption, and urine output

Water consumption, food consumption, and urine output were measured using metabolic cages. Rats were housed individually in metabolic cages for a period of 24 hours.

2.1.2.3 Tail-cuff blood pressure

Conscious systolic blood pressure was measured non-invasively using a tail-cuff apparatus. Rats were gently placed inside the animal holder and the end plate attached, leaving the tail to protrude freely. The nose cone was adjusted to cover the rat’s face and prevent turning around in the holder. The rat and holder were then placed on a 37°C warming platform and left to rest for five minutes. The occlusion tail cuff and sensor cuff were threaded onto the tail, and the occlusion cuff automatically inflated to allow blood pressure measurement. All data was gathered automatically.

2.2 Biomechanical measurements

2.2.0.1 Tensile testing in rat aortae

Rat aortae were freshly dissected and unbranched 3-4 mm aortic ring segments cut from the superior-most regions on the thoracic and abdominal aortae. Rings were stored in cold saline for no more than 2 hours before testing. Rings were mounted on two 26-gauge pins in a custom mounting block and fixed to a tensile testing apparatus (model 5542, Instron; equipped with 10 N load cell) for stretching (Figure 2.3A). The outer diameter ($d_o$) and wall thickness ($h$) of each ring were measured using a microscope graticule. Rings were pre-cycled 10 times at 2 mm/min between 0 and $(2 \times 200 \times 133.3 \times w \times r \times N)$ (200 mmHg equivalent), before stretching to failure. Measured load for each extension of the aorta was used to calculate Young’s elastic modulus as a measure of stiffness. All calculations were performed in the Anaconda 5.0 Python distribution [Python 3.6].

2.2.0.2 Calculating Young’s elastic modulus in rat aortae

To calculate Young’s elastic modulus ($E_m$), the raw load and extension data from the Instron were first transformed into a stress-strain curve (Figure 2.4).

$E_m$ is defined as tensile stress ($\sigma$) over tensile strain ($\varepsilon$) in the elastic (initial, linear) portion of a stress-strain curve:

$$E_m = \frac{\sigma}{\varepsilon}$$

where

$$\sigma = \frac{F}{A}$$
Figure 2.3: **Tensile testing of rat aortic rings.** (A) Unbranched aortic rings were fitted to two custom mounting plates by threading needles through the lumen. (B) Plates were machined with a 475 μm separation from the outer edge of each needle to the inner edge of the plate. (C) Extension ($l$) was defined as the separation between the inner edges of the needles, while separation between the outer edges of the needles was defined as length ($L$). (D) The width ($w$), wall thickness ($h$), and outer diameter ($d_o$) were measured using a microscope graticule, with $h$ and $d_o$ used to calculate lumen diameter ($d_i$).
\[ \varepsilon = \frac{\Delta L}{L_0} \]

\( F = \) force (N)

\( A = \) cross-sectional area (m\(^2\))

\( \Delta L = \) change in length (m)

\( L_0 = \) initial length (m)

Instron outputs of load and extension were converted to tensile stress and tensile strain using a hollow cylindrical model with a cross-sectional area of \( 2 \times w \times h \):

\[ \sigma = \frac{F}{2 \times w \times h} \]

\[ \varepsilon = \frac{L - L_0}{L_0} \]

\( F = \) load (N) (Instron output)

\( w = \) ring width (m)

\( h = \) wall thickness (m)

\( L = l + 9.5 \times 10^{-4} \) (m)

\( L_0 = d_i \) (m)

where \( l \) is extension (Instron output) and \( 9.5 \times 10^{-4} \) m equals the needle diameters plus initial separation (Figure 2.3B). \( L_0 \) was set as the lumen diameter (\( d_i \)) to remove data points for which the ring was not being deformed.

Incremental elastic modulus (\( E_{\text{inc}} \)) is defined as the slope of the stress-strain curve at a specific strain. \( E_{\text{inc}} \) at strains corresponding to physiological pressures were examined to obtain relevant results.

Gaussian smoothing was applied to stress data to minimise noise in the stress-strain curve (Figure 2.5). A quadratic polynomial was fitted to the stress-strain curve, and the derivative of the fit line used to produce a graph of \( E_{\text{inc}} \) (\( d\sigma/d\varepsilon \)) vs strain (Figure 2.6). Quadratic polynomial fits were superior to linear fits according to least squares analysis.

The stress corresponding to a particular pressure (\( \sigma_P \)) was calculated as:

\[ \sigma_P = \frac{F}{2 \times w \times h} = \frac{2 \times P \times r_i \times w}{2 \times w \times h} = \frac{P \times r_i}{h} \]

where \( P \) is the pressure of interest in N. The corresponding strain value (\( \varepsilon_P \)) was extracted from the stress-strain curve. The \( E_{\text{inc}} \) vs strain fit line was then used to calculate \( E_{\text{inc}} \) corresponding to \( \varepsilon_P \) at all pressures of interest.
Figure 2.4: Producing the stress-strain curve. Load vs extension (left) and stress vs strain (right) for rat HSTx7.

Figure 2.5: Smoothing the stress-strain curve. Raw stress vs strain (left) and stress vs strain after stress smoothing (right) for rat HSTx7.
Figure 2.6: Producing the modulus-strain curve. The stress vs strain graph for rat HSTx7 with a quadratic polynomial fit (left). The derivative of the fit line produces $E_{inc} (d\sigma/d\varepsilon)$ vs strain (right).
2.3 Biochemical measurements

2.3.1 Elemental quantification using ICP-OES

This inductively coupled plasma-optical emission spectroscopy (ICP-OES) protocol was validated and optimised in Section 3.1. All measurements were conducted at the Medical Research Council (MRC) Elsie Widdowson Laboratory (Cambridge, United Kingdom; formerly MRC Human Nutrition Research). Analysis of human aortae was performed with assistance from Liliana Pedro at the MRC Human Nutrition Research.

All calculations assume complete breakdown of the sample into its component elements by acid digestion and subsequent exposure to a plasma flame. If breakdown was incomplete or detection inhibited by the binding state of the element, matrix effects would be apparent during analysis. For human and rat skin and aorta no matrix effects were detected (see Section 3.1.5), so it was assumed that any elemental binding in these tissues did not affect quantification.

2.3.1.1 Human aortae

25-60 mg human aortae were briefly rinsed with phosphate-buffered saline (PBS), blotted dry, and freeze-dried (Mini Lyotrap, LTE scientific Ltd) in Eppendorf tubes with perforated caps for 72 hours until they reached constant weight. The freeze-dried samples were weighed to determine tissue water content and digested using a closed-vessel microwave assisted acid digestion (MWAD) system (Milestone UltraWAVE™) and Si-free, pre-cleaned standard polytetrafluoroethylene (PTFE) vials with 500 µL 35% (v/v) nitric acid (HNO₃). The microwave heating program was performed in three steps: (1) 5 minute ramp from 23°C to 120°C; (2) 5 min ramp from 120°C to 230°C; and (3) 10 min at 230°C; followed by a cooling step for 20 minutes. The microwave power was limited to a max of 1000 W and pressure was limited to a maximum of 150 bar. All samples were digested to completeness and diluted 1/10 by weight in ultra high purity (UHP) water for the quantification of the Ca, P, Na, and K. Blanks for the control of the digestion process and pooled sample for quality control were prepared in a similar manner and analysed collectively with the samples using inductively coupled plasma-optical emission spectroscopy (ICP-OES). The ICP-OES (JY Ultima 2c, Horiba Scientific) running conditions for the digested aortae sample analysis are summarised in Table 2.2.

2.3.1.2 Human skin, rat skin, rat aortae

5-10 mg human skin, 10-15 mg rat skin, or 2-6 mg rat aortae were defatted, briefly rinsed with PBS, blotted dry, and freeze-dried (Alpha 1-2 LDplus, Christ) in Eppendorf tubes with perforated caps for 24 hours until they reached constant weight. The freeze-dried samples were weighed to determine tissue water content and digested using 150 µL 1:1 30% H₂O₂:69% HNO₃ for 24 hours at ambient temperature followed by 24 hours at 40°C
in a water-bath, with occasionally venting. All samples were digested to completeness
and diluted 1/40 by weight with UHP water for quantification of Na\(^+\) and K\(^+\). Blanks
for the control of the digestion process and skin from a skin reduction patient for quality
control were prepared in a similar manner and analysed collectively with the samples.

The ICP-OES (JY-2000, Horiba Scientific) running conditions for the digested skin and
aortae sample analysis are summarised in Table 2.2. Drift check solutions were carried
out after every block of approximately 10 samples.

2.3.1.3 Standards and elemental peaks

A standard curve was prepared by plotting the raw intensity of each element-specific
wavelength detected by the ICP-OES against the known elemental concentrations of
standard solutions.

Multi-element standards were prepared by weight from individual 1,000 ppm aqueous
stock solutions, with final concentrations for each element ranging from 0 to 10 ppm in an
acid solution matched to the digested samples, i.e. 0.17% (v/v) HNO\(_3\) for human aortae
samples and 0.86% (v/v) HNO\(_3\) for human skin, rat skin, and rat aortae samples. Peak
profiles were analysed prior to each ICP-OES run and the polychromator was centred in
the Mg wavelength (Table 2.3).

2.3.1.4 Calculation of tissue element concentrations

For a digested sample, the raw intensity of each element-specific wavelength was com-
pared against a standard curve to obtain the concentration of that element in the digest.
This concentration was then multiplied by the dilution factor of each digest and the un-
diluted volume of digest, then divided by the wet or dry mass of the sample to obtain the
concentration of that element relative to the weight of the sample:

\[
[E]_{\text{sample}} = \frac{[E]_{\text{digest}} \times K \times V_{\text{digest}}}{m_{\text{sample}}}
\]

\[
[E]_{\text{sample}} = \text{concentration of element in the sample (μg/mg)}
\]

\[
[E]_{\text{digest}} = \text{concentration of element in the sample (μg/mL)}
\]

\[
K = \text{dilution factor, } V_{\text{dilution}} / V_{\text{digest}} \text{ (unit-less)}
\]

\[
V_{\text{dilution}} = \text{volume of diluted digest (mL)}
\]

\[
V_{\text{digest}} = \text{volume of undiluted digest (mL)}
\]

\[
m_{\text{sample}} = \text{mass of undigested sample, either wet or dry (mg)}
\]

Dry sample weights were determined after freeze-drying for 24 hours. Elemental con-
centrations were also expressed relative to the water content of the sample:

\[
[E]_{\text{aqueous}} = \frac{[E]_{\text{digest}} \times K \times V_{\text{digest}} \times m_a}{m_{\text{water}}}
\]
Table 2.1: Running conditions of ICP-OES for aortae samples using JY Ultima 2c

<table>
<thead>
<tr>
<th>Analytical conditions</th>
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<tbody>
<tr>
<td>RF power (W)</td>
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<tr>
<td>Plasma gas (L/min)</td>
</tr>
<tr>
<td>Sheath gas (L/min)</td>
</tr>
<tr>
<td>Auxiliary gas (L/min)</td>
</tr>
<tr>
<td>Speed pump (rates/min)</td>
</tr>
<tr>
<td>Nebulisation flow rate (L/min)</td>
</tr>
<tr>
<td>Plasma stabilisation time (s)</td>
</tr>
<tr>
<td>Number of replicates</td>
</tr>
</tbody>
</table>

Table 2.2: Running conditions of ICP-OES for aortae and skin samples using JY-2000

<table>
<thead>
<tr>
<th>Analytical conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF power (W)</td>
</tr>
<tr>
<td>Plasma flow (L/min)</td>
</tr>
<tr>
<td>Sheath gas (L/min)</td>
</tr>
<tr>
<td>Auxiliary gas (L/min)</td>
</tr>
<tr>
<td>Speed pump (rates/min)</td>
</tr>
<tr>
<td>Nebulisation flow rate (L/min)</td>
</tr>
<tr>
<td>Nebuliser pressure (mmHg)</td>
</tr>
<tr>
<td>Number of replicates</td>
</tr>
</tbody>
</table>

Table 2.3: Atomic spectroscopic information for the multi-element analysis of human and rat skin and aortae samples by ICP-OES

<table>
<thead>
<tr>
<th>Element</th>
<th>Chromator type</th>
<th>Wavelength (nm)</th>
<th>Standard range (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>Monochromator</td>
<td>317.933</td>
<td>0-10</td>
</tr>
<tr>
<td>P</td>
<td>Monochromator</td>
<td>177.440</td>
<td>0-10</td>
</tr>
<tr>
<td>K</td>
<td>Monochromator</td>
<td>766.490</td>
<td>0-5</td>
</tr>
<tr>
<td>Na</td>
<td>Monochromator</td>
<td>589.592</td>
<td>0-10</td>
</tr>
</tbody>
</table>
where \([E]_{\text{aqueous}}\) is the aqueous concentration of the element in mmol/L, \(m_a\) is the atomic mass of the element, and \(m_{\text{water}}\) is the mass of the water in the sample as the difference between wet and dry sample weights. This latter calculation, while historically popular, should be viewed cautiously in this work as it implies that the element is completely dissolved in solution and that there exists a direct relationship between elemental content and water content. Because this thesis advocates the concept of non-osmotic binding, the assumption of complete solvation is violated. Nevertheless, it is included for comparison against existing literature.

### 2.3.2 Sulphated glycosaminoglycan quantification

Sulphated glycosaminoglycan content was assayed using the Blyscan Sulphated Glycosaminoglycan Assay (B1000, BioColor). Skin and aorta were digested with papain (P3125, Sigma Aldrich) according to manufacturer protocol. Papain digests were diluted 1/2 (skin) or 1/4 (aortae) with water before assaying according to manufacturer protocol.

### 2.3.3 Hyaluronan quantification

10-25 mg aorta were digested in 1 mL of 0.01 mmol/L Tris-HCl with 0.2 mmol/L EDTA and 1 mg protease from *Streptomyces griseus* (P5147, Sigma Aldrich) at 55°C for 12 hours with agitation. Samples were then heated to 80°C for 30 minutes to denature the protease. Samples were then centrifuged at 15,000 rpm for 30 minutes and the supernatant diluted 1/150 with water for analysis. Serum samples were diluted 1/2 with water before analysis.

Hyaluronan content was assayed using the Hyaluronan DuoSet ELISA (DY3614, R&D Systems) according to manufacturer protocol. It should be noted that in early 2016, the kits began producing inverse concentration curves (i.e. lower concentrations produced higher signals) when analysing digested tissue samples and could no longer be used for tissue analysis. Contact with the manufacturer could not resolve the issue. However, the kits continued to work as expected for serum samples.

### 2.3.4 qPCR analysis

Rat trunk skin was defatted, shaved with a scalpel, and cut into pieces < 0.5 cm². Samples were stored in RNA later™ Stabilisation Solution (AM7020, Thermo Fisher Scientific) for 24 hours, before storage at −80°C until RNA extraction. Human lower back skin from GRENaDE study participants was removed and cut into thirds as described in Section 2.1.1.8. One third was stored in pre-chilled RNA later™-ICE Tissue Transition Solution (AM7030, Thermo Fisher Scientific) for at least 24 hours at −20°C before RNA extraction.

Digestion, extraction, and qPCR analysis protocol were optimised in Section 3.2. 10-15 mg RNA later™-stabilised rat skin or 4-8 mg RNA later™-ICE-stabilised human skin were minced and combined with 800 µL TRIzol® Reagent (15596026, Thermo Fisher Scientific) and 5 mm steel beads. Samples were broken down using a TissueLyser LT
(69980, Qiagen) for 30 minutes at 30 Hz (rat skin) or 3 hours at 50 Hz (human skin). RNA from digests was extracted using the RNeasy Plus Universal Mini Kit (73404, Qiagen) (rat) or the PureLink™ Pro 96 total RNA Purification Kit (12173011A, Thermo Fisher Scientific) (human). Extraction was carried out according to manufacturer protocols, with DNase digestion (79254, Qiagen). RNA concentration quality were measured using a NanoDrop Spectrophotometer.

RNA was reverse transcribed to cDNA using the Promega Reverse Transcription System (A3500, Promega) with custom protocol to accommodate low RNA concentrations. RNase-free water and RNA extracts were combined to a final volume of 8.9 µL and RNA concentration of 1 µg (rat) or 150 µg (human). 1 µL of Random Primers and 1 µL of Oligo(dT)$_{15}$ Primers were added to each sample for incubation at 70°C for 10 minutes. Each sample was then combined with 4 µL MgCl$_2$, 2 µL dNTP, 2 µL RT buffer, 0.5 µL RNasin, and 0.6 µL AMV Reverse Transcriptase, and amplified at:

1. 22°C for 15 minutes
2. 42°C for 60 minutes
3. 95°C for 5 minutes
4. 4°C for 5 minutes

Rat samples were diluted 1/4 and human samples 1/2.5 with DNase-free water. 25 ng rat cDNA or 12.5 ng human cDNA was amplified in duplicate using TaqMan® Gene Expression Assays (Tables 2.4 and 2.5, Thermo Fisher Scientific) in TaqMan® Gene Expression Master Mix (4369016, Thermo Fisher Scientific) according to manufacturer protocol. Reactions had a final volume of 20 µL.
Table 2.4: Rat TaqMan® Gene Expression Assays

<table>
<thead>
<tr>
<th>Gene</th>
<th>Dye</th>
<th>TaqMan Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>FAM</td>
<td>Hs99999901</td>
</tr>
<tr>
<td>Chpf</td>
<td>FAM</td>
<td>Rn01400272</td>
</tr>
<tr>
<td>Chsy1</td>
<td>FAM</td>
<td>Rn01478125</td>
</tr>
<tr>
<td>Dse</td>
<td>FAM</td>
<td>Rn02109126</td>
</tr>
<tr>
<td>Epas1</td>
<td>FAM</td>
<td>Rn00576515</td>
</tr>
<tr>
<td>Ext1</td>
<td>FAM</td>
<td>Rn00468764</td>
</tr>
<tr>
<td>Glce</td>
<td>FAM</td>
<td>Rn01417963</td>
</tr>
<tr>
<td>Vegfc</td>
<td>FAM</td>
<td>Rn01488076</td>
</tr>
<tr>
<td>Xylt1</td>
<td>FAM</td>
<td>Rn01755138</td>
</tr>
</tbody>
</table>

Table 2.5: Human TaqMan® Gene Expression Assays

(a) Monoplexing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Dye</th>
<th>TaqMan Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHPF</td>
<td>FAM</td>
<td>Hs00226041</td>
</tr>
<tr>
<td>EPAS1</td>
<td>FAM</td>
<td>Hs01026149</td>
</tr>
</tbody>
</table>

(b) Duplexing

<table>
<thead>
<tr>
<th>Duplex pair</th>
<th>Gene 1</th>
<th>Dye 1</th>
<th>Taqman Assay ID 1</th>
<th>Gene 2</th>
<th>Dye 2</th>
<th>TaqMan Assay ID 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18S</td>
<td>FAM</td>
<td>Hs99999901</td>
<td>XYLT1</td>
<td>VIC</td>
<td>Hs00544498</td>
</tr>
<tr>
<td>2</td>
<td>GLCE</td>
<td>FAM</td>
<td>Hs00392011</td>
<td>CHSY1</td>
<td>VIC</td>
<td>Hs00208704</td>
</tr>
<tr>
<td>3</td>
<td>VEGFC</td>
<td>FAM</td>
<td>Hs01099203</td>
<td>EXT2</td>
<td>VIC</td>
<td>Hs00181158</td>
</tr>
<tr>
<td>4</td>
<td>DSE</td>
<td>FAM</td>
<td>Hs01897959</td>
<td>HYAL1</td>
<td>VIC</td>
<td>Hs00201046</td>
</tr>
</tbody>
</table>
Chapter 3

Validation and optimisation of ICP-OES and qPCR

When investigating the influence of salt on haemodynamics and glycosaminoglycan content, a range of assays are necessary to construct a complete physiological picture. Some of these assays have already been validated and are accepted in standard use, but others must of tailored for a specific species, tissue, or target. Presented here are the methodological investigations used to construct effective protocols for 1) quantification of Na\(^+\), K\(^+\), Ca\(^{2+}\), and P\(^3-\) using inductively couple plasma-optimal emission spectroscopy (ICP-OES) in rat and human skin and aorta, and 2) qPCR analysis of glycosaminoglycan- and salt-sensitive enzymes in rat and human skin.

3.1 Tissue elemental analysis using ICP-OES

3.1.1 Background

When studying the physiological effects of salt, scientists frequently rely on indirect quantification of body sodium content. Until relatively recently, radioactive isotopes were used as a direct measure, but increasing concerns about safety effectively curtailed their use, particularly in humans [45, 44, 43, 218, 15, 26, 78]. In contrast, indirect measurements of body sodium remain popular for their relative simplicity and minimal invasiveness. The two most common indirect measures are dietary sodium intake and urinary sodium excretion. Manipulation of dietary intake is often used to direct qualitative changes in body sodium content, but it does not provide proof of sodium accumulation or depletion. Moreover, it can be be prohibitively expensive to do in-house diet control studies in humans, and self-reporting is notoriously unreliable. Similarly, while measuring urinary sodium excretion provides a direct quantitative measure of sodium loss, it is complicated by active kidney adaptation to sodium changes, does not directly measure accumulation or depletion, and, in humans, relies heavily on outpatient compliance.

Where possible, direct analysis of tissue samples can provide more detailed informa-
tion on sodium homeostasis. Some of the most popular techniques for tissue elemental analysis include sequential precipitation, flame photometry, and atomic absorption spectroscopy (AAS) [18, 19, 187, 230, 69, 85, 43]. Recently, there has been growing interest in using inductively coupled plasma-optical emission spectrometry (ICP-OES; also called inductively coupled plasma atomic emission spectroscopy, ICP-AES). ICP-OES works by misting a liquid sample directly into a 7000K plasma flame, exciting the component elements of the sample mist. As the excited elements return to ground state, they emit element-specific wavelengths for quantification. While AAS is superior for trace element analysis, ICP-OES has a higher sensitivity for metals and enables measurement of non-metals. And while slower on a per-measurement basis, a significant advantage of ICP-OES is its ability to measure multiple elements simultaneously rather than sequentially. This makes ICP-OES superior when many elements must be analysed or when working with very small samples. It is this latter benefit that makes ICP-OES of particular interest for studies in living humans, where only small amounts of tissue can be removed.

A full protocol for human aortae and skin from excision to ICP-OES analysis does not currently exist in the literature, so one needed to be established and optimised. A number of specific procedural steps had to be investigated for their effects on result reliability and reproducibility including tissue-handing, rinsing, drying, and ICP-OES matrix effects.

### 3.1.2 Tissue handling and rinsing

It was important to ensure that the elemental content of a tissue was not accidentally altered during removal or preparation. Common sources of contamination include physiological and experimental contaminants and storage and rinse solutions (Table 3.1).

Human aortae, studied in Chapter 4, were obtained from organ donors at Addenbrooke’s Hospital. Generally, the entire aortic tree and associated organs were removed from the donor and placed in Wisconsin solution (WS) for live tissue preservation before transplant. Once all organs were removed from the aortic tree (6-12 hours), the leftover aortae were given to the lab. This meant that aortae obtained in this way were potentially contaminated by WS. In select cases, aortae were obtained directly after removal from the organ donor, but these aortae were necessarily contaminated with blood. Blotting may not sufficient for blood removal while rinsing with water or phosphate-buffered saline (PBS) could alter ion content.

Human skin biopsies, studied in Chapter 7, faced similar issues. Skin biopsies were obtained under local anaesthesia with 1% lidocaine solution. Because commercial 1% lidocaine solutions contain 300 mM NaCl to control tonicity, custom Na-free lidocaine was requested from Tayside Pharmaceuticals (Dundee, Australia). The custom Na-free lidocaine contained 3.5% glucose in lieu of NaCl, and was formulated by Dr. Viknesh Selvarajah. The sodium content of each batch was be tested. The effects blotting or rinsing to remove blood from skin were also examined. In these experiments, aortic samples weighed 25-80 mg and skin samples weighed 5-15 mg.
### Table 3.1: Elemental composition of potential tissue contaminants

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Source</th>
<th>Na&lt;sup&gt;+&lt;/sup&gt; (mmol/L)</th>
<th>K&lt;sup&gt;+&lt;/sup&gt; (mmol/L)</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt; (mmol/L)</th>
<th>P&lt;sup&gt;3-&lt;/sup&gt; (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na-free lidocaine</td>
<td>experimental</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Blood (serum)</td>
<td>physiological</td>
<td>135-145</td>
<td>3.5-5.0</td>
<td>1.5-2.5</td>
<td>1.0-2.0</td>
</tr>
<tr>
<td>Blood (erythrocytes)</td>
<td>physiological</td>
<td>5-10</td>
<td>120-140</td>
<td>30-60 x 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>30-60</td>
</tr>
<tr>
<td>Wisconsin solution (WS)</td>
<td>storage</td>
<td>29</td>
<td>125</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Deionised water</td>
<td>rinsing or storage</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Phosphate-buffered saline (PBS)</td>
<td>rinsing or storage</td>
<td>147</td>
<td>4.5</td>
<td>0</td>
<td>11.8</td>
</tr>
</tbody>
</table>
3.1.3 Methodology

3.1.3.1 Methodology

Blood

Bloodless skin samples from breast reduction surgeries (n = 3) and bloodless aortae samples from organ donors (n = 3) were halved, and 100 µL blood was added to one half and removed via blotting, while the other half of the sample was analysed without intervention.

Wisconsin solution (WS)

Aortae (n = 3) were quartered and each piece submerged in WS for 0, 5, 30, or 60 minutes.

Water

Skin samples from breast reduction patients (n = 3) were quartered, with one piece left unaltered and the other three exposed to either a brief rinse, 5 minute submersion, or 60 minute submersion in water.

Phosphate-buffered saline (PBS)

Skin samples from breast reduction patients (n = 3) were quartered, with one piece left unaltered and the other three exposed to either a brief rinse, 5 minute submersion, or 60 minute submersion in PBS.

Na-free lidocaine

Each batch of Na-free lidocaine was directly analysed for elemental content. All tissue samples were blotted dry before freeze-drying for 48 hours (aortae) or 24 hours (skin). Dried samples were then digested in a 1:1 30% H₂O₂:69% HNO₃ solution for 24 hours at ambient temperature, followed by 24 hours at 40°C. Sample digests were diluted 1/40 with ultra-pure water and analysed using a JY2000 ICP-OES at the MRC Elsie Widdowson Laboratory (Cambridge, United Kingdom; formerly MRC Human Nutrition Research).

Statistics

For each experiment, samples were compared using ANOVA analysis with post-hoc Student’s t tests. All statistical analyses were performed using IBM SPSS (version 25). Graphing was performed in the Anaconda 5.0 Python distribution [Python 3.6]. All values are shown as mean ± SEM. A p < 0.05 was considered statistically significant.
3.1.3.2 Results

The effects of blood, WS, water, and PBS contamination can be seen in Figure 3.1. For aortae, soaking in WS caused a significant increase in K⁺ content and decrease in Na⁺ and Ca²⁺ content after just one hour (p < 0.001). After 12 hours, a decrease in P³⁻ content became significant as well (p < 0.01). Rinsing skin samples with water caused a small but significant reduction in Na⁺ (-7%, p < 0.01) and K⁺ (-10%, p < 0.01), which rose substantially with as little as 5 minutes soaking. Rinsing skin samples with PBS caused no significant change in Na⁺, but did cause a small decrease in K⁺ (-8%, p = 0.012). After soaking for 5 minutes, Na⁺ content significantly increased (+47%, p < 0.001) while K⁺ content significantly decreased (-53%, p < 0.001). This effect was further exaggerated after 60 minutes. For both skin and aortae, removal of blood via blotting still left significant residual K⁺ contamination (up to +78%, p < 0.001).

All batches of Na-free lidocaine showed no significant Na⁺ or K⁺ content (data not shown).

3.1.3.3 Conclusions

Aortae

Sodium, potassium, and calcium values are rapidly altered by submersion in WS, but phosphorus may be viable if the submersion is one hour or less. Aortae should be analysed without submersion in WS, however this is generally not feasible due to transplant logistics. If fresh aortae are received, blotting is not sufficient to remove blood contamination, and rinsing with PBS is recommended.

Skin

Na-free lidocaine was found to be sufficiently sodium- and potassium-free, and so should not alter sodium and potassium content in skin biopsies. Blotting was not sufficient to remove blood contamination, so rinsing of tissues is required. While rinsing with PBS was better than water at maintaining tissue elemental content, both may be considered acceptable. Tissues should not be exposed to either for more than a brief rinse.

3.1.4 Tissue drying

For accurate analysis of tissue water content, the tissue must be precisely and completely dried. Common drying techniques include oven-drying and freeze-drying (lyophilisation). The choice of which to use is often determined by what resources a research group has access to, and what type and volume of tissue is being dried. Groups also differ when choosing a tube type for drying samples, with the most common choices being traditional Eppendorf conical tubes or in specialised cryovials. It is particularly common for snap-frozen samples, such as those from GRENaDE Study participants, to be left in cryovials.
**Figure 3.1: Effect of contaminants on Na⁺, K⁺, Ca²⁺, and P³⁻ quantification.** Aortae or skin samples were intentionally contaminated with Wisconsin Solution (WS), blood, water, or phosphate-buffered saline (PBS). All samples were blotted dry after exposure, freeze-dried, and digested in 1:1 H₂O₂:HNO₃ for ICP-OES analysis. All lines represent individual donors. *p < 0.01, **p < 0.001
during freeze-drying, for simplicity. Both drying methods and tube type were examined to determine the most appropriate choice for aortae and skin. In these experiments, aortic samples weighed 25-80mg and skin samples weighed 10-25mg.

3.1.4.1 Methodology

Drying method

Human aortae from organ donors (n = 9) or skin samples from breast reduction patients (n = 4) were divided in half, with one half dried in an oven at 40°C for up to 31 hours and the other half dried in a freeze-dryer for up to 72 hours, with regular weighing. 1.5mL eppendorfs were used for both drying conditions.

Tube type

Empty cryovials (n = 4) and eppendorf tubes (n = 4) were freeze-dried for 24 hours.

Statistics

Groups were compared using independent Student’s t-tests.

3.1.4.2 Results

A comparison of oven-drying vs freeze-drying for both skin and aortae can be seen in Figure 3.2. Aortic weights stabilised between 5 and 24 hours (oven-drying) and 55 and 72 hours (freeze-drying). Skin weights stabilised between 6 and 12 hours (oven-drying) and 12 and 24 hours (freeze-drying). For the same sample, oven-drying caused significantly greater weight loss compared to freeze-drying in both aortae and skin (aortae: 72.7 ± 7% vs 74.5 ± 7%, p = 0.018; skin: 69.8 ± 3% vs 85.3 ± 4%, p < 0.001).

The effect of freeze-drying on cryovial and eppendorf tube weights was analysed as a percentage change in weight before and after drying. Freeze-drying had no effect on eppendorf tube weight (0.00 ± 0.01%, p > 0.05), but decreased cryovial weight by a small but significant amount (-0.03 ± 0.01%, p < 0.001).

3.1.4.3 Conclusions

Aortae and skin both required 12-24 hours in a 40°C oven to reach a constant weight. When freeze-drying, aortae required up to 72 hours and skin up to 24 hours. Aortae showed slightly higher weight loss when dried in an oven compared to freeze-drying (75% vs 73%, p < 0.01). This effect was greatly exacerbated in skin samples (85% vs 70%, p < 0.001). Similar oven-drying vs freeze-drying results were found by Dr. Viknesh Selvarajah when analysing VARSITY Study skin biopsies (data not published).

Studies by other groups have found human skin water content to be 55-72%,[43, 230, 214] in line with these freeze-drying results, suggesting that oven-drying causes anomal-
Figure 3.2: Oven-drying vs freeze-drying in skin and aorta. Aortae (n = 9) or skin (n = 4) samples were dried using 40°C oven or freeze-dryer, and the percentage weight loss plotted against drying time. Individual lines represent different tissue donors.
ously high weight loss. This excess weight loss when oven-drying may be due to the loss of volatile organic compounds that are not lost when freeze-drying. This effect may be further exaggerated in these skin samples given their small size (5-25mg), resulting in a high surface area-to-volume ratio. In this light, freeze-drying is recommended for both tissues, but essential for small skin samples.

After freeze-drying, Eppendorf tubes remained the same weight. In contrast, cryovials showed significant weight loss, suggesting that the tube itself dries out. This is likely due to a rubber O-ring present in the cryovial cap. Therefore, samples snap-frozen in cryovials must be transferred to eppendorf tubes for freeze-drying. It should be noted that this effect constitutes a ∼0.15mg weight loss, and may be considered negligible for large samples.

3.1.5 ICP-OES matrix effects

As a general rule, when samples are digested for elemental analysis they will contain the aqueous elements of interest (in this case, sodium and potassium), as well as additional components such as water, organic compounds, acids, salts, and dissolved solids. These additional components are known as the “matrix.” This matrix can sometimes interfere with accurate measurement of elemental components, causing a “matrix effect.” To safeguard the reliability of any new ICP-OES sample preparation, the presence of matrix effects must be investigated and corrected for if necessary. With respect to this thesis, matrix effects caused by non-osmotic sodium binding were of particular interest.

3.1.5.1 Methodology

Matrix effects were explored using the standard addition method. Four human skin samples from two breast reduction patients and two Sprague Dawley rat aortae samples were rinsed with PBS, blotted dry, and freeze-dried for 24 hours. The samples were then digested in a 1:1 30%H$_2$O$_2$:69%HNO$_3$ solution for 24 hours at ambient temperature, and a further 24 hours at 40ºC. Sample digests were diluted 1/40 with ultra-pure water and evenly combined to create a matrix. Four known dilutions of an aqueous standard (20ppm Na$^+$, 10ppm K$^+$) were spiked with a fixed volume of this matrix, and the intensities compared to an unspiked standard curve. Samples were run in triplicate.

3.1.5.2 Results

The difference in measured Na$^+$ and K$^+$ intensity between matrix-spiked standards and unspiked standards can be seen in Figure 3.3. If no matrix effects were present, the measured intensity for each spiked standard ($I_i$) would be equal to the standard intensity ($I_{STD}$) plus the pure matrix intensity ($I_m$). To quantitatively determine the effect of the matrix on Na$^+$ or K$^+$ intensity, the recovery rate is calculated:
recovery rate = \((100) \times \left[ \frac{(I_{STD}) - (I_i - I_m)}{(I_{STD})} \right] \)

This recovery rate represents the measured intensity of a matrix-spiked sample compared to its expected intensity. The recovery rates of Na\(^+\) were within ±10%, while the recovery rates of K\(^+\) were within ±6%. Matrix effects are considered significant if they alter the recovery rate by more than ±20%.

3.1.5.3 Conclusion

Skin and aortae digestion in H\(_2\)O\(_2\):HNO\(_3\) does not produce significant matrix effects on sodium or potassium measurement in the concentration range of interest.

3.1.6 Finalised protocol

Skin and aortae samples should not be stored in WS, PBS, or any other solution. Samples should have any blood removed via rinsing with PBS, and be blotted dry before freeze-drying in 1.5mL Eppendorf tubes for 24 hours (skin) or 72 hours (aortae). Once dry, samples should be digested with 1:1 H\(_2\)O\(_2\):HNO\(_3\) for 24 hours at ambient temperature, followed by 24 hours at 40\(^\circ\)C. Digested samples are diluted 1/40 with ultra-pure water for ICP-OES analysis, without correction for matrix effects. Samples should be run in triplicate.
Figure 3.3: **Analysis of matrix effects using standard addition.** Five standards of known $\text{Na}^+$ and $\text{K}^+$ concentration were spiked with a fixed volume of matrix derived from human skin. $\text{Na}^+$ and $\text{K}^+$ intensities in matrix-free and matrix-spiked samples were compared, and recovery rates calculated. Red dashed lines show the acceptable limits of recovery. STD = standard.
3.2 RNA extraction and qPCR analysis

In 2004, Titze et al investigated the link between dietary sodium and skin GAGs in rats using qPCR analysis [198]. Comparing mRNA expression levels for GAG synthetic enzymes between control rats and those fed a high salt diet, they found that a high salt diet significantly increased mRNA expression. In 2009, Heer et al performed a similar salt-dependent qPCR analysis of skin GAG synthetic enzymes in humans, with comparable finding [64]. Building on these works, qPCR analyses in rat skin (see Chapter 5) and human skin (see Chapter 7) were performed. To ensure accurate results before analysis, RNA extraction and qPCR analysis were optimised, and an appropriate housekeeping gene selected.

3.2.1 Tissue digestion and RNA extraction

Two digestion buffers and three RNA extraction kits were compared. Digestion buffers were either phenol-free (PureLink™ Pro 96 total RNA Purification Lysis Buffer) or contained phenol (TRIzol®). RNA was extracted using either the RNeasy Plus Universal Mini Kit (73404, Qiagen), PureLink™ Pro 96 kit (12173011A, Thermo Fisher Scientific), or Direct-zol (R2061, Zymogen Research). The RNeasy Plus Universal Mini Kit is the standard for RNA extraction, offering high yields and excellent 260/280 and 260/230 values. However, it is relatively expensive, the protocol is lengthy, and the samples must be processed individually. In comparison, the PureLink™ Pro 96 kit is cheaper, the protocol shorter, and allows up to 96 samples to be processed simultaneously. Finally, the relatively new Direct-zol kit has a very short protocol and is relatively cheap, but requires samples to be processed individually.

3.2.1.1 Methodology

Rat skin samples were prepared as in described in Section 5.2.2.3. Samples of 5-30 mg were minced and added to either 800μL PureLink™ Pro 96 Lysis Buffer or TRIzol® extraction reagent. Using 5 mm stainless steel beads, samples were broken down using a TissueLyser LT (69980, Qiagen) until no solid particles were visible. RNA was extracted using the RNeasy Plus Universal Mini Kit, PureLink™ Pro 96 kit, or Direct-zol kit, according to manufacturer protocols with DNase treatment (79254, Qiagen). RNA concentration, 260/280 values, and 260/230 values were analysed using a NanoDrop spectrophotometer. Extraction efficiency was assessed as ngRNA/mg tissue.

3.2.1.2 Results

Extraction results can be seen in Table 3.2. Samples digested with PureLink™ Pro 96 Lysis Buffer consistently clogged all kits, completely preventing extraction. When examining TRIzol®-digested samples, the RNeasy Plus Universal Mini Kit was the most
efficient at extracting RNA, and produced the best 260/280 and 260/230 values. The PureLink™ Pro 96 kit was marginally less effective at extracting RNA compared to the RNeasy Plus Universal Mini Kit (p = 0.023) but no different than the Direct-zol kit (p > 0.05). The Direct-zol kit produced highly variable results, with some extractions having 260/280 values of 1.91 ± 0.05 and others having 260/280 values of 1.48 ± 0.03. Direct-zol 260/230 values were similarly variable, ranging from 1.75 ± 0.03 to 0.75 ± 0.04.

3.2.1.3 Conclusions

PureLink™ Pro 96 Lysis Buffer is not sufficiently aggressive to break down rat skin, possibly due to the presence of small but numerous hair fragments. Phenol-based lysis buffers such as TRIzol® are required. Of the three kits examined the RNeasy Plus Universal Mini Kit was marginally more efficient and consistent at extracting RNA. However, because the RNeasy Plus Universal Mini Kit protocol is comparatively lengthy and expensive, it is not ideal for large numbers of samples. The PureLink™ Pro 96 provided a slightly lower RNA yield but allowed up to 96 samples to be extracted at once, making it ideal for processing large numbers. The Direct-zol kit is not recommended due to the high variability of results. Investigations by other groups have similarly shown a slight advantage of the RNeasy kits over the PureLink TM Pro 96 kits[171] as well as the variability of the Direct-zol kit.[3]

For experiments with less than 50 samples the RNeasy Plus Universal Mini Kit is recommended. For experiments with more than 50 samples the PureLink™ Pro 96 is recommended. In both cases a phenol-based lysis buffer is required for rat skin.

3.2.2 Choosing cDNA quantity and housekeeping gene

When the native expression levels of a gene of interest (“target gene”) are unknown, or the efficiency of a qPCR probe are unknown, it is important to produce dilution curves to aide in choosing an optimal cDNA quantity for qPCR analysis. The optimal cDNA quantity should yield Ct values between 10 and 36 while still complying with manufacturer guidelines (usually 5-100 ng per reaction). Because cDNA quantity may still differ between samples, a “housekeeping gene” (also called a “reference gene”) is used to standardise expression of a target gene to total cDNA content. It is important that the housekeeping gene produce a Ct value between 10 and 25 at the chosen cDNA concentration and not be affected by the experimental conditions.

Here, the dilution curves of eight target genes and three common housekeeping genes (18S, ACTB, and GAPDH) were examined for suitability in salt-dependent rat (see Chapter 5) and human (see Chapter 7) experiments. Furthermore, because human RNA is necessarily limited by small sample size, human genes were analysed using qPCR duplexing. For these assays, it must be shown that simultaneous gene amplification does not interfere with the amplification of each gene individually.
Table 3.2: Comparison of RNA extraction kits

<table>
<thead>
<tr>
<th>RNA Extraction</th>
<th>RNA concentration nL RNA/mg sample ± SEM</th>
<th>260/280</th>
<th>260/230</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNeasy Plus Universal Mini Kit</td>
<td>539 ± 29</td>
<td>2.02 ± 0.01</td>
<td>1.85 ± 0.04</td>
</tr>
<tr>
<td>PureLink Pro 96</td>
<td>495 ± 35</td>
<td>2.03 ± 0.02</td>
<td>1.80 ± 0.05</td>
</tr>
<tr>
<td>Direct-zol</td>
<td>515 ± 50</td>
<td>1.69 ± 0.04</td>
<td>1.27 ± 0.10</td>
</tr>
</tbody>
</table>

Table 3.3: qPCR genes of interest

<table>
<thead>
<tr>
<th>Gene code</th>
<th>Gene product</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td><em>no product</em></td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>(housekeeping gene)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTB</td>
<td>beta-actin</td>
<td>cell motility, structure, and integrity</td>
</tr>
<tr>
<td>(housekeeping gene)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gapdh</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>glycolysis</td>
</tr>
<tr>
<td>(housekeeping gene)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chpf</td>
<td>chondroitin polymerising factor</td>
<td>chondroitin sulfate chain elongation</td>
</tr>
<tr>
<td>Chsyl1</td>
<td>chondroitin sulfate synthase 1</td>
<td>biosynthesis of chondroitin sulfate</td>
</tr>
<tr>
<td>Dse</td>
<td>dermatan sulfate epimerase</td>
<td>converts chondroitin sulfate to dermatan sulfate</td>
</tr>
<tr>
<td>Ext1 and 2</td>
<td>exostosin glycosyltransferase 1</td>
<td>heparin sulfate chain elongation</td>
</tr>
<tr>
<td>Glce</td>
<td>glucuronic acid epimerase</td>
<td>heparin sulfate chain elongation</td>
</tr>
<tr>
<td>Xytl1</td>
<td>xylosyltransferase 1</td>
<td>attachment of glycosaminoglycan chains to proteoglycan backbone</td>
</tr>
<tr>
<td>Vegfc</td>
<td>vascular endothelial growth factor C</td>
<td>promotes angiogenesis</td>
</tr>
</tbody>
</table>
3.2.2.1 Methodology

Rat skin samples were prepared as in Section 5.2.2.3. Samples of 10-15 mg were minced and digested with 800 μL TRIzol® extraction buffer. Using 5 mm stainless steel beads, samples were broken down using a TissueLyser until no solid particles were visible. RNA was then extracted using the RNeasy Plus Universal Mini Kit.

Human skin samples from GRENaDE Study participants were prepared as in Section 2.1.1.8. Samples of 4-8 mg were minced, combined with 800 μL TRIzol® extraction buffer, and broken down using 5 mm steel beads and TissueLyser. RNA was extracted using the PureLink™ Pro 96 kit. For both rat and human samples, RNA concentration, 260/280 values, and 260/230 values were analysed using a NanoDrop spectrophotometer.

RNA was reverse-transcribed to cDNA using the Promega Reverse Transcription System (A3500, Promega). The protocol was modified from manufacturer specifications to accommodate lower extracted RNA concentrations (see Section 2.3.4). A total of 1 μg rat RNA or 850 ng human RNA per sample was used for reverse transcription. The resulting cDNA was serially diluted to 100 ng (rat only), 50 ng, 25 ng, 12.5 ng, 6.25 ng, and 3.13 ng per assay before amplification using the TaqMan gene expression system. To identify the efficient cDNA concentration range, Ct values were plotted against the log of the cDNA concentration, and the linear region identified.

12.5 ng human cDNA per qPCR assay were used to compare Ct values of duplexed assays to their monoplexing counterparts with paired Student’s t tests. Table 3.4 shows the duplexed pairings and the dye for each gene.

3.2.2.2 Results

Ct values for monoplexed rat and human housekeeping and target genes were plotted against log([cDNA]) (Figure 3.4). In rats, the plot of log([cDNA]) vs Ct value was most linear between 6.25 and 50 ng cDNA for both housekeeping genes and target genes, while in humans linearity was greatest between 6.25 and 25 ng cDNA.

In both rats and humans, housekeeping gene 18S produced the lowest Ct values at each concentration compared to ACTB and GAPDH. 18S also showed no significant difference in expression between rats fed a normal diet and rats fed a high salt diet, while ACTB and GAPDH showed significantly increased expression (p_{ACTB} = 0.012, p_{GAPDH} = 0.032).

In humans, there was no significant difference in Ct value between monoplexed and duplexed assays (p > 0.10).

3.2.2.3 Conclusions

Due to the comparatively low Ct values and salt-independence demonstrated in rats, 18S should be used as the housekeeping gene in both rat and human experiments. Titze et
Table 3.4: Duplex pairings for human qPCR analysis

<table>
<thead>
<tr>
<th>Duplex pair</th>
<th>Gene 1</th>
<th>Dye 1</th>
<th>Gene 2</th>
<th>Dye 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18S</td>
<td>FAM</td>
<td>Xylt1</td>
<td>VIC</td>
</tr>
<tr>
<td>2</td>
<td>Glce</td>
<td>FAM</td>
<td>Chsy1</td>
<td>VIC</td>
</tr>
<tr>
<td>3</td>
<td>Vegfc</td>
<td>FAM</td>
<td>Ext2</td>
<td>VIC</td>
</tr>
<tr>
<td>4</td>
<td>Dse</td>
<td>FAM</td>
<td>Hyal1</td>
<td>VIC</td>
</tr>
</tbody>
</table>

Figure 3.4: Ct value vs log([cDNA]) in rat and human housekeeping and target genes. cDNA concentrations of 3.13-100 ng (rat) or 3.13-50 ng (human) were amplified using the TaqMan gene expression system. Ct values are shown ± SDEV.
al [198] and Heer et al [64] used 18S as the housekeeping gene in their respective works, allowing more direct comparison of results.

Because it is important to choose a cDNA concentration in the middle of the linear portion of the Ct vs log([cDNA]) curve to reduce the impact of concentration on qPCR efficiency, 25 ng rat cDNA and 12.5 ng human cDNA are recommended. Duplexing of human genes did not affect amplification efficiency.
Chapter 4

Biomechanical properties and glycosaminoglycan content of the human aorta

4.1 Background

The biomechanical properties of large arteries, particularly the aorta, are key determinants of cardiovascular haemodynamics and development of cardiovascular risk. A healthy artery is elastic: it expands to accommodate the systolic stroke volume before recoiling and pushing the blood forward during diastole, effectively transforming the pulsatile blood flow from the heart to a “smoothed” continuous flow. This “Windkessel effect” diminishes as the elastic arteries become stiffer and less compliant, like they do in aging. In stiffened vessels expansion and recoil are limited, leading to an increased systolic pressure and decreased diastolic pressure for a given stroke volume. The resulting increase in pulse pressure (systolic pressure less diastolic pressure) is a major predictor of cardiovascular morbidity and mortality [10, 132]. Such stiffening also causes an increase in the speed of pulse wave travel through the vessel, called the pulse wave velocity (PWV). A higher PWV allows faster return of reflected peripheral waves, causing earlier summation with the incident wave from the heart, further augmenting pressure in late systole and decreasing end-diastolic pressure. In general, increased arterial stiffness may indicate vascular changes relating to or predisposing the development of vascular diseases such as hypertension, aortic dissection, and aneurysm.

Arterial stiffness can be analysed globally or regionally. For convenience, global analyses are much more common than regional analyses, and are some of the most common in vivo measurements of stiffness (Table 4.1). However, though they provide well-documented information about the effects of cardiovascular disease on the overall properties of the arterial system, global analyses do not provide data regarding localised effects on vascular properties. In 1990, Ting et al provided the first investigation into the regional aortic effects of hypertension, concluding that some localised effects of essential
hypertension are independent of blood pressure and suggest fundamental differences in the mechanical properties of the aortic wall [196]. Since then, other studies have confirmed and expanded upon these findings, but work has generally been hindered by the difficulty of measuring regional stiffness in vivo and the limited ability to obtain ex vivo human aortae for study.

Because the distensibility of an artery can be modelled as an elastic tube [219], its stiffness can be characterised using a property called Young’s elastic modulus (E$_m$). In engineering, E$_m$ describes the resistance of an elastic object to deformation when stress is applied. In the context of arteries, E$_m$ describes how much an artery will deform in response to blood pressure. Because stiff arteries will resist deformation, they are described as having a high E$_m$. However, the pressure-deformation (or stress-strain) relationships of blood vessels are strongly nonlinear, so it is impossible to assign a single E$_m$ value to a vessel. Instead, it has become common to use the incremental elastic modulus (E$_{inc}$) to describe stiffness [21, 96, 123, 133]. Introduced by Bergel in 1961 [13, 12], E$_{inc}$ represents the slope of a vessel’s stress-strain curve at a specific pressure, such as 100mmHg. In this way, the mechanical stiffness of the vessel at physiological pressures can be analysed. E$_{inc}$ can be further used to quantify the effect of this stiffness on PWV using the Moens-Korteweg equation. Most measurements of E$_{inc}$ in humans have been performed in vivo, with few ex vivo measurements of aortic rings presently available (Table 4.2).

The mechanical stiffness of the arterial wall is primarily determined by the vascular media and its biological components, including elastin, collagen, vascular smooth muscle cells, and “ground substance.” Elastin and collagen are the principal elastic components of the wall. At healthy physiological pressures, collagen fibres are generally kinked and do not contribute substantially to stiffness, so the behaviour of the tissue at these pressures is due primarily to elastin. It is only at higher pressures that the collagen fibres straighten and become directly stressed, further stiffening the arterial wall. In contrast, smooth muscle cells exhibit vasoactive behaviour in vivo but do not contribute extensively to passive mechanical stiffness.

The remaining ground substance has garnered continuing interest over the past few decades. This ground substance is rich in glycosaminoglycans (GAGs) and associated proteoglycans whose water- and ion-binding potential may affect wall mechanics either directly or indirectly. However, the actual contribution of medial GAGs to arterial stiffness remains largely unknown.

Research on disease models such as mucopolysaccharidoses, aortic aneurysms, and aortic dissections suggest that extremes of GAG content are correlated with altered elasticity, however it remains to be seen whether more moderate changes in GAG content are similarly correlated. To evaluate the interplay between cardiovascular disease, blood pressure, biomechanical stiffness, and GAG content in the general human population, the aortae of human organ donors were analysed.
Hypothesis: Aortae from donors with hypertension will have a higher elastic modulus, HA content, sulphated GAG (sGAG) content, and different sGAG distribution than aortae from normotensive donors.
Table 4.1: Published in vivo measurements of pulse wave velocity

<table>
<thead>
<tr>
<th>PWV site</th>
<th>Age (y)</th>
<th>Gender</th>
<th>n</th>
<th>Method</th>
<th>PWV (m/s)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotid-femoral (global PWV)</td>
<td>27.8 ± 0.4</td>
<td>male</td>
<td>102</td>
<td>noninvasive pressure recordings</td>
<td>5.9 ± 0.10</td>
<td>[142]</td>
</tr>
<tr>
<td></td>
<td>28.2 ± 0.90</td>
<td>male</td>
<td>240</td>
<td>SphygmoCor</td>
<td>6.5 ± 1.5</td>
<td>[148]</td>
</tr>
<tr>
<td></td>
<td>28.2 ± 0.90</td>
<td>female</td>
<td>284</td>
<td>SphygmoCor</td>
<td>5.5 ± 1.3</td>
<td>[148]</td>
</tr>
<tr>
<td></td>
<td>&lt;30</td>
<td>ns</td>
<td>1455</td>
<td>intersecting tangent algorithm</td>
<td>6.2 ± 1.6</td>
<td>[186]</td>
</tr>
<tr>
<td></td>
<td>30-39</td>
<td>ns</td>
<td>1455</td>
<td>intersecting tangent algorithm</td>
<td>6.5 ± 2.7</td>
<td>[186]</td>
</tr>
<tr>
<td></td>
<td>49-49</td>
<td>ns</td>
<td>1455</td>
<td>intersecting tangent algorithm</td>
<td>7.2 ± 2.4</td>
<td>[186]</td>
</tr>
<tr>
<td></td>
<td>41 ± 16</td>
<td>ns</td>
<td>89</td>
<td>SphygmoCor</td>
<td>7.37 ± 1.75</td>
<td>[124]</td>
</tr>
<tr>
<td></td>
<td>43.4 ± 11.4</td>
<td>female</td>
<td>48</td>
<td>noninvasive pressure recordings</td>
<td>8.8 ± 1.7</td>
<td>[11]</td>
</tr>
<tr>
<td></td>
<td>45.3 ± 11.1</td>
<td>male</td>
<td>80</td>
<td>noninvasive pressure recordings</td>
<td>9.1 ± 1.7</td>
<td>[11]</td>
</tr>
<tr>
<td></td>
<td>50-59</td>
<td>ns</td>
<td>1455</td>
<td>intersecting tangent algorithm</td>
<td>8.3 ± 3.8</td>
<td>[186]</td>
</tr>
<tr>
<td></td>
<td>60-69</td>
<td>ns</td>
<td>1455</td>
<td>intersecting tangent algorithm</td>
<td>10.3 ± 5.4</td>
<td>[186]</td>
</tr>
<tr>
<td></td>
<td>70+</td>
<td>ns</td>
<td>1455</td>
<td>ultrasound</td>
<td>8.2 ± 2.1</td>
<td>[99]</td>
</tr>
<tr>
<td></td>
<td>64.3 ± 12.1</td>
<td>ns</td>
<td>51</td>
<td>ultrasound</td>
<td>8.65 ± 2.43</td>
<td>[112]</td>
</tr>
<tr>
<td></td>
<td>78.6 ± 4.3</td>
<td>male</td>
<td>166</td>
<td>ultrasound</td>
<td>8.37 ± 2.41</td>
<td>[112]</td>
</tr>
<tr>
<td></td>
<td>77.9 ± 4.1</td>
<td>female</td>
<td>190</td>
<td>ultrasound</td>
<td>8.37 ± 2.41</td>
<td>[112]</td>
</tr>
<tr>
<td>Ascending thoracic (regional PWV)</td>
<td>29 ± 9</td>
<td>ns</td>
<td>18</td>
<td>micro-manometer and mingograph</td>
<td>3.87 ± 0.87</td>
<td>[126]</td>
</tr>
<tr>
<td>Descending thoracic (regional PWV)</td>
<td>58.4 ± 8.3</td>
<td>ns</td>
<td>62</td>
<td>ultrasound</td>
<td>3.94 ± 0.57</td>
<td>[154]</td>
</tr>
<tr>
<td></td>
<td>18-77</td>
<td>ns</td>
<td>162</td>
<td>cine phase contrast MRI</td>
<td>5.5 ± 2.0</td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td>18-77</td>
<td>ns</td>
<td>162</td>
<td>cine phase contrast MRI</td>
<td>5.7 ± 2.3</td>
<td>[66]</td>
</tr>
<tr>
<td>Abdominal (regional PWV)</td>
<td>18-77</td>
<td>ns</td>
<td>162</td>
<td>Cine phase contrast MRI</td>
<td>6.1 ± 2.9</td>
<td>[66]</td>
</tr>
</tbody>
</table>

PWV = pulse wave velocity, ns = not specified
### Table 4.2: Published values for aortic $E_{\text{inc}}$

<table>
<thead>
<tr>
<th>Region</th>
<th>Age (y)</th>
<th>Gender</th>
<th>n</th>
<th>Method</th>
<th>$E_{\text{inc}}$ (MPa)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascending thoracic</td>
<td>29 ± 9</td>
<td>ns</td>
<td>18</td>
<td>micro-manometer and mingograph</td>
<td>0.28 ± 0.09</td>
<td>[126]</td>
</tr>
<tr>
<td></td>
<td>58.4 ± 8.3</td>
<td>ns</td>
<td>62</td>
<td>ultrasound</td>
<td>0.73 ± 0.19</td>
<td>[154]</td>
</tr>
<tr>
<td>Descending thoracic</td>
<td>14-79</td>
<td>male</td>
<td>33</td>
<td>transesophageal echocardiography</td>
<td>0.76 ± 0.28</td>
<td>[156]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>female</td>
<td>20</td>
<td>transesophageal echocardiography</td>
<td>0.68 ± 0.34</td>
<td>[156]</td>
</tr>
<tr>
<td></td>
<td>26.2 ± 3.6</td>
<td>ns</td>
<td>16</td>
<td>ultrasonic, phase-locked echo-tracking system</td>
<td>0.52 ± 0.17</td>
<td>[181]</td>
</tr>
<tr>
<td></td>
<td>43.8 ± 3.3</td>
<td>ns</td>
<td>11</td>
<td>ultrasonic, phase-locked echo-tracking system</td>
<td>0.84 ± 0.27</td>
<td>[181]</td>
</tr>
<tr>
<td></td>
<td>61.4 ± 3.6</td>
<td>ns</td>
<td>11</td>
<td>ultrasonic, phase-locked echo-tracking system</td>
<td>1.12 ± 0.42</td>
<td>[181]</td>
</tr>
<tr>
<td></td>
<td>68.6 ± 2.9</td>
<td>ns</td>
<td>9</td>
<td>ultrasonic, phase-locked echo-tracking system</td>
<td>1.17 ± 0.32</td>
<td>[181]</td>
</tr>
<tr>
<td></td>
<td>40-95</td>
<td>male</td>
<td>30</td>
<td>estimation, $\frac{P_{\text{inc}}}{h}$</td>
<td>0.62 ± 0.03</td>
<td>[227]</td>
</tr>
<tr>
<td>Abdominal</td>
<td>0-19</td>
<td>ns</td>
<td>9</td>
<td>ultrasonic, phase-locked echo-tracking system</td>
<td>0.453</td>
<td>[77]</td>
</tr>
<tr>
<td></td>
<td>20-39</td>
<td>ns</td>
<td>11</td>
<td>ultrasonic, phase-locked echo-tracking system</td>
<td>0.755</td>
<td>[77]</td>
</tr>
<tr>
<td></td>
<td>40-59</td>
<td>ns</td>
<td>10</td>
<td>ultrasonic, phase-locked echo-tracking system</td>
<td>0.857</td>
<td>[77]</td>
</tr>
<tr>
<td></td>
<td>60+</td>
<td>ns</td>
<td>9</td>
<td>ultrasonic, phase-locked echo-tracking system</td>
<td>1.27</td>
<td>[77]</td>
</tr>
<tr>
<td></td>
<td>25.3 ± 4.0</td>
<td>ns</td>
<td>20</td>
<td>ultrasonic, echo-tracking system</td>
<td>0.69 ± 0.21</td>
<td>[94]</td>
</tr>
<tr>
<td></td>
<td>46.4 ± 4.4</td>
<td>ns</td>
<td>9</td>
<td>ultrasonic, echo-tracking system</td>
<td>1.44 ± 0.60</td>
<td>[94]</td>
</tr>
<tr>
<td></td>
<td>60.1 ± 3.3</td>
<td>ns</td>
<td>7</td>
<td>ultrasonic, echo-tracking system</td>
<td>2.20 ± 0.43</td>
<td>[94]</td>
</tr>
<tr>
<td></td>
<td>71.1 ± 4.9</td>
<td>ns</td>
<td>15</td>
<td>ultrasonic, echo-tracking system</td>
<td>3.37 ± 1.01</td>
<td>[94]</td>
</tr>
<tr>
<td></td>
<td>40-95</td>
<td>male</td>
<td>30</td>
<td>estimation, $\frac{P_{\text{inc}}}{h}$</td>
<td>0.55 ± 0.04</td>
<td>[227]</td>
</tr>
</tbody>
</table>

$E_{\text{inc}}$ = incremental elastic modulus, $ns$ = not specified
4.2 Methodology

All aortae samples were obtained with the ethical approval of the National Health Service Research Ethics Committees (references LREC03/2/074 and 05/MRE04/7). Where available, the medical history of the donor and aortic region of the sample were recorded. In the medical records, incidence of a diseased state, such as hypertension, was recorded in a no/yes format. Blood pressures and heart rates prior to terminal hospital admission were not available.

4.2.1 Aortae collection

Human aortae \( n = 218 \) were sourced from terminal organ donors at Addenbrooke’s Hospital, Cambridge, United Kingdom. During transplant, the aortic tree and associated organs were removed from the donor and immersed in commercial Wisconsin Solution (Preservation Solutions) for up to 72 hours. Once all transplant organs were removed, the remaining aortae were collected for analysis (Division of Experimental Medicine and Immunotherapeutics, University of Cambridge, Cambridge, United Kingdom). Excess blood and fat were removed, and where possible the aortae divided into:

1. 1 cm cross-sectional rings, frozen at \(-80^\circ\text{C}\);
2. 0.25-0.5 cm\(^2\) strips, frozen at \(-80^\circ\text{C}\);
3. 4 mm punch biopsies, stored in RNA\textit{later}® solution (Sigma-Aldrich) at 4\(^\circ\text{C}\) for up to 48 hours, then washed with phosphate-buffered saline (PBS) and stored at 4\(^\circ\text{C}\); and
4. 0.25-0.5 cm\(^2\) sections and 4 mm punch biopsies, stored in formalin for up to 5 years.

In some cases, donor serum was provided and stored at \(-80^\circ\text{C}\).

4.2.2 Tensile testing

Because tensile testing measurements required intact aortic rings, only 199 out of the total 218 aortae were considered suitable for analysis. Tensile testing was measured in collaboration with Professor Ashraf Khir at Brunell University (London, United Kingdom). Sample rings were shipped on dry ice before being thawed to room temperature. Width \( w \) and wall thickness \( h \) were measured five times at various points using vernier callipers, and the averages recorded. Circumference was measured five times using wet suture, and the lumen radius \( r \) calculated. Rings were kept moist by periodic spray application of PBS and mounted in a tensile test machine (model 5542, Inston) for stiffness measurements (Figure 4.1). Samples were cycled five times between 0 mmHg and \( (2 \times 180 \times 133.3 \times w \times r) \) (180 mmHg equivalent) at a rate of 10 mm \( \cdot \text{min}^{-1} \), then...
Chapter 4. Biomechanical properties and glycosaminoglycan content of the human aorta

stretched to failure. The resulting stress-strain curve was used to calculate the incremental elastic modulus \( (E_{inc}) \) at 30, 60, 100, 160, and 200 mmHg equivalent by finding the slope of the tangent line at each equivalent stress value:

\[
E_{inc} = \frac{\Delta\text{stress}}{\Delta\text{strain}} \quad \text{(Hooke's Law)}
\]

For a detailed description of elastic modulus calculations see Section 2.2.0.2.

Pulse wave velocity \((PWV)\) was calculated from \(E_{inc}\) using the Moens-Korteweg equation, assuming a blood density \((\rho)\) of 1040 kg/m\(^3\):

\[
PWV = \sqrt{\frac{E_{inc} \times h}{2 \times r \times \rho}}
\]

4.2.3 Glycosaminoglycan analysis

Small subpopulations of aortae were randomly selected for GAG analysis. Aortic sulphated GAG (sGAG) content \( (n=17) \), aortic hyaluronan (HA) content \( (n=38) \), and serum HA content \( (n=13) \) were assayed according to Sections 2.3.2 and 2.3.3. Briefly, sGAG aortae samples were digested with papain \((P3125, \text{Sigma Aldrich})\) for 24 hours at 37\(^\circ\)C, and diluted 1/4 with water before assaying with the Blyscan Sulphated Glycosaminoglycan Assay \((B1000, \text{BioColor})\). HA aortae samples were digested with protease \((P5147, \text{Sigma Aldrich})\) for 12 hours at 55\(^\circ\)C, and diluted 1/150 with water before assaying with the Hyaluronan DuoSet ELISA \((DY3614, \text{R&D Systems})\). HA serum samples were diluted 1/2 with water before assaying with the Hyaluronan DuoSet ELISA.

4.2.4 Histology staining

A small subpopulation of aortae was randomly selected for histological analysis. Formalin-fixed samples \( (n=13) \) were embedded in paraffin wax and sectioned at 20\(\mu\)m thickness by Dr. Nichola Figg (Cambridge Cardiovascular, University of Cambridge, Cambridge, United Kingdom). Sections were deparaffinised and rehydrated to distilled water, then stained with Alcian Blue and counterstained with Nuclear Fast Red. Image analysis was performed using the Anaconda 5.0 Python distribution [Python 3.6].

4.2.5 Contributions of others

Aortae were retrieved from organ donors by the transplant team at Addenbrooke’s Hospital (Cambridge, United Kingdom). After all associated organs were removed, the aortae were collected and processed by Sarah Cleary (Division of Experimental Medicine and Immunotherapeutics, University of Cambridge, Cambridge, United Kingdom). All tensile testing measurements and elastic modulus calculations were performed by students in the
Figure 4.1: **Tensile testing of human aorta.** Aortae from human organ donors (A) were cut into rings (B) and the width (w), wall thickness (h) and radius (r) measured (C). Rings were mounted in an Instron tensile testing machine (D) and cycled five times between 0 and 180 x 133.3 x w x r N (180 mmHg equivalent) at a rate of 10 mm · min⁻¹ before being stretched to failure. The resulting stress-strain curve (E) was used to calculate elastic modulus. Stress-strain curve shown is for sample TS001.
4.2.6 Statistical analysis

Aortic groupings were compared using the Mann-Whitney U test. Correlations were performed using Spearman’s rank. Associations between categorical variables were evaluated using Pearson Chi-Square test. To investigate covariates and interactions, ANCOVA and ANOVA were used due to the lack of non-parametric equivalent. This was considered acceptable due to the large sample size of the data set. Differences in stiffness between hypertensive and normotensive aortae at various pressures were compared using repeated measures ANOVA with history of hypertension as a Between Subjects Factor. All statistical analyses were performed using IBM SPSS (version 25). Graphing was performed in the Anaconda 5.0 Python distribution [Python 3.6]. All values are reported as mean ± SEM. A p < 0.05 was considered statistically significant.

4.3 Results

A total of 218 aortae (126 female, 92 male; mean age 55 years, range 17-83 years) were collected for analysis, with 199 tested for stiffness. The origins of most aortae were unknown, so the collection was not sub-divided by aortic region.

For the collection as a whole, age was significantly correlated with aortic diameter ($r = 0.620$, $p < 0.001$), wall thickness ($r = 0.471$, $p < 0.001$) and stiffness at 60 mmHg and above ($r_{E60\text{mmHg}} = 0.273$, $r_{E100\text{mmHg}} = 0.457$, $r_{E160\text{mmHg}} = 0.663$, $r_{E200\text{mmHg}} = 0.704$, $p < 0.001$ for all; $r_{PWV60\text{mmHg}} = 0.295$, $r_{PWV100\text{mmHg}} = 0.510$, $r_{PWV160\text{mmHg}} = 0.698$, $r_{PWV200\text{mmHg}} = 0.731$, $p < 0.001$ for all). Age was significantly higher in donors with hypertension (HTN, $p < 0.001$) and donors with diabetes ($p < 0.01$).

After controlling for age, $E_{100\text{mmHg}}$ was significantly correlated with diameter ($r = 0.434$, $p < 0.001$) and wall thickness ($r = -0.220$, $p < 0.01$). HTN aortae had higher $PWV_{100\text{mmHg}}$ ($p = 0.027$), and trended towards increased $E_{100\text{mmHg}}$ ($p = 0.76$). Aortae from diabetics and smokers trended towards increased dry sGAG content ($p_{\text{diabetes}} = 0.059$, $p_{\text{smoking}} = 0.062$). Aortae from diabetics had higher $E_{100\text{mmHg}}$ ($p = 0.020$) and $PWV_{100\text{mmHg}}$ ($p = 0.044$), but this significance disappeared after controlling for age.

Differences between genders can be seen in Table 4.3. There was no difference in history of HTN or diabetes between genders, but women were more likely to smoke than men ($p = 0.023$). While female aortae were significantly smaller than male aortae ($p = 0.039$), this difference disappeared when controlling for age. Similarly, there were no differences between genders for serum HA, aortic HA, aortic sGAG, aortic diameter, or aortic stiffness at any pressure, even after controlling for age. However, $E_{\text{inc}}$ at 160 mmHg and 200 mmHg approached significance when controlling for age ($p_{E_{160\text{mmHg}}} = 0.054$, $p_{E_{200\text{mmHg}}} = 0.055$).
### Table 4.3: Gender differences in donor demographics and affect on aortic properties

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total ((n))</th>
<th>Female ((n))</th>
<th>Male ((n))</th>
<th>(p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no : yes (Pearson Chi-Squared)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTN</td>
<td>194:75:39:50:30</td>
<td></td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>Diabetes</td>
<td>192:103:10:71:8</td>
<td></td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>Smoking</td>
<td>177:49:55:47:26</td>
<td></td>
<td></td>
<td>0.023</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55.4±1.0(218)</td>
<td>52.9±1.5(126)</td>
<td>58.7±1.4(92)</td>
<td>0.014</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>13.62±0.32(218)</td>
<td>13.59±0.04(126)</td>
<td>13.67±0.05(92)</td>
<td>ns</td>
</tr>
<tr>
<td>Wall thickness (mm)</td>
<td>1.51±0.02(201)</td>
<td>1.46±0.03(116)</td>
<td>1.58±0.04(85)</td>
<td>0.039</td>
</tr>
<tr>
<td>(E_{inc}) at 30 mmHg (MPa)</td>
<td>0.104±0.004(199)</td>
<td>0.107±0.006(115)</td>
<td>0.100±0.005(84)</td>
<td>ns</td>
</tr>
<tr>
<td>(E_{inc}) at 60 mmHg (MPa)</td>
<td>0.126±0.005(199)</td>
<td>0.132±0.008(115)</td>
<td>0.119±0.007(84)</td>
<td>ns</td>
</tr>
<tr>
<td>(E_{inc}) at 100 mmHg (MPa)</td>
<td>0.169±0.007(199)</td>
<td>0.172±0.010(115)</td>
<td>0.166±0.011(84)</td>
<td>ns</td>
</tr>
<tr>
<td>(E_{inc}) at 160 mmHg (MPa)</td>
<td>0.327±0.017(199)</td>
<td>0.328±0.023(115)</td>
<td>0.325±0.026(84)</td>
<td>ns</td>
</tr>
<tr>
<td>(E_{inc}) at 200 mmHg (MPa)</td>
<td>0.441±0.023(199)</td>
<td>0.440±0.030(115)</td>
<td>0.443±0.035(84)</td>
<td>ns</td>
</tr>
<tr>
<td>PWV at 30 mmHg (m/s)</td>
<td>3.05±0.06(199)</td>
<td>3.04±0.08(115)</td>
<td>3.07±0.08(84)</td>
<td>ns</td>
</tr>
<tr>
<td>PWV at 60 mmHg (m/s)</td>
<td>3.37±0.06(199)</td>
<td>3.36±0.09(115)</td>
<td>3.35±0.09(84)</td>
<td>ns</td>
</tr>
<tr>
<td>PWV at 100 mmHg (m/s)</td>
<td>3.85±0.08(199)</td>
<td>3.82±0.10(115)</td>
<td>3.91±0.11(84)</td>
<td>ns</td>
</tr>
<tr>
<td>PWV at 160 mmHg (m/s)</td>
<td>5.22±0.12(199)</td>
<td>5.13±0.17(115)</td>
<td>5.36±0.18(84)</td>
<td>ns</td>
</tr>
<tr>
<td>PWV at 200 mmHg (m/s)</td>
<td>6.05±0.14(199)</td>
<td>5.92±0.19(115)</td>
<td>6.24±0.20(84)</td>
<td>ns</td>
</tr>
<tr>
<td>Serum HA ((\mu g/mL))</td>
<td>44.7±3.3(13)</td>
<td>44.7±3.3(7)</td>
<td>35.4±5.7(6)</td>
<td>ns</td>
</tr>
<tr>
<td>Aortic HA ((\mu g/mg wet tissue))</td>
<td>163.0±21.5(38)</td>
<td>163.0±21.5(19)</td>
<td>182.4±21.6(19)</td>
<td>ns</td>
</tr>
<tr>
<td>Aortic sGAG ((\mu g/mg wet tissue))</td>
<td>7.47±1.00(17)</td>
<td>7.47±1.00(12)</td>
<td>6.14±1.93(5)</td>
<td>ns</td>
</tr>
</tbody>
</table>

\(E_{inc}\) = incremental elastic modulus, PWV = pulse wave velocity, HA = hyaluronan, sGAG = sulphated glycosaminoglycan, ns = not significant.
Differences between normotensives and hypertensives can be seen in Table 4.4. Hypertensive donors had a higher incidence of both diabetes ($p = 0.02$) and smoking ($p = 0.030$) than normotensives, as well as larger aortic diameters ($p < 0.001$) and thicker aortic walls ($p = 0.030$). However, differences in diameter and wall thickness disappeared after controlling for age. Aortae from HTN donors had significantly higher $E_{inc}$ and PWV than aortae from normotensive donors at or above 100 mmHg ($p < 0.001$), and this difference increased with increasing pressure (Figure 4.2, Pressure*HTN interaction $p < 0.001$ for both). These interactions persisted when controlling for age and gender. There were also a significant pressure interaction with smoking (Pressure*Smoking interaction $p < 0.001$) for both $E_{inc}$ and PWV. There was no significant interaction between pressure and gender on stiffness.

Aortic HA, serum HA, and aortic sGAG content were not significantly correlated with age, gender, history of HTN, or history of diabetes. Histology showed an sGAG gradient through the media towards the intima, with no sGAG staining in the adventitia. In HTN aortae, there was a sharply defined delineation of sGAG content in the intima, in contrast to the more gradual gradient seen in normotensive aortae (Figure 4.3). This delineation was statistically significant according to Python image analysis ($p < 0.05$).
Table 4.4: Hypertensive differences in donor demographics and aortic properties

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total</th>
<th>Normotensive</th>
<th>Hypertensive</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
<td></td>
</tr>
<tr>
<td><strong>Gender (f:m)</strong></td>
<td>194</td>
<td>75:50</td>
<td>39:30</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Diabetes (n:y)</strong></td>
<td>187</td>
<td>114:7</td>
<td>55:11</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Smoking (n:y)</strong></td>
<td>171</td>
<td>56:58</td>
<td>38:19</td>
<td>0.030</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>55.4±1.0</td>
<td>50.8±1.4</td>
<td>63.3±1.3</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>(218)</td>
<td>(125)</td>
<td>(69)</td>
<td></td>
</tr>
<tr>
<td><strong>Diameter (mm)</strong></td>
<td>13.62±0.32</td>
<td>13.19±0.36</td>
<td>14.43±0.63</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>(218)</td>
<td>(125)</td>
<td>(69)</td>
<td></td>
</tr>
<tr>
<td><strong>Wall thickness (mm)</strong></td>
<td>1.51±0.02</td>
<td>1.46±0.03</td>
<td>1.57±0.04</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>(201)</td>
<td>(117)</td>
<td>(63)</td>
<td></td>
</tr>
<tr>
<td><strong>E\text{inc} at 30mmHg (MPa)</strong></td>
<td>0.104±0.004</td>
<td>0.097±0.004</td>
<td>0.107±0.007</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(199)</td>
<td>(115)</td>
<td>(63)</td>
<td></td>
</tr>
<tr>
<td><strong>E\text{inc} at 60mmHg (MPa)</strong></td>
<td>0.126±0.005</td>
<td>0.114±0.006</td>
<td>0.133±0.009</td>
<td>0.082</td>
</tr>
<tr>
<td></td>
<td>(199)</td>
<td>(115)</td>
<td>(63)</td>
<td></td>
</tr>
<tr>
<td><strong>E\text{inc} at 100mmHg (MPa)</strong></td>
<td>0.160±0.007</td>
<td>0.144±0.007</td>
<td>0.198±0.016</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>(199)</td>
<td>(115)</td>
<td>(63)</td>
<td></td>
</tr>
<tr>
<td><strong>E\text{inc} at 160mmHg (MPa)</strong></td>
<td>0.327±0.017</td>
<td>0.259±0.016</td>
<td>0.410±0.03</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>(199)</td>
<td>(115)</td>
<td>(63)</td>
<td></td>
</tr>
<tr>
<td><strong>E\text{inc} at 200mmHg (MPa)</strong></td>
<td>0.441±0.023</td>
<td>0.358±0.023</td>
<td>0.550±0.04</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>(199)</td>
<td>(115)</td>
<td>(63)</td>
<td></td>
</tr>
<tr>
<td><strong>PWV at 30mmHg (m/s)</strong></td>
<td>3.05±0.06</td>
<td>2.96±0.06</td>
<td>3.10±0.09</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(199)</td>
<td>(115)</td>
<td>(63)</td>
<td></td>
</tr>
<tr>
<td><strong>PWV at 60mmHg (m/s)</strong></td>
<td>3.37±0.06</td>
<td>3.20±0.06</td>
<td>3.44±0.10</td>
<td>0.037</td>
</tr>
<tr>
<td></td>
<td>(199)</td>
<td>(115)</td>
<td>(63)</td>
<td></td>
</tr>
<tr>
<td><strong>PWV at 100mmHg (m/s)</strong></td>
<td>3.85±0.08</td>
<td>3.57±0.07</td>
<td>4.14±0.14</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>(199)</td>
<td>(115)</td>
<td>(63)</td>
<td></td>
</tr>
<tr>
<td><strong>PWV at 160mmHg (m/s)</strong></td>
<td>5.22±0.12</td>
<td>4.71±0.12</td>
<td>5.80±0.23</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>(199)</td>
<td>(115)</td>
<td>(63)</td>
<td></td>
</tr>
<tr>
<td><strong>PWV at 200mmHg (m/s)</strong></td>
<td>6.05±0.14</td>
<td>5.50±0.15</td>
<td>6.73±0.25</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>(199)</td>
<td>(115)</td>
<td>(63)</td>
<td></td>
</tr>
<tr>
<td><strong>Serum HA (µg/mL)</strong></td>
<td>44.7±3.3</td>
<td>41.2±4.3</td>
<td>37.9±2.0</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(13)</td>
<td>(10)</td>
<td>(3)</td>
<td></td>
</tr>
<tr>
<td><strong>Aortic HA (µg/mg wet tissue)</strong></td>
<td>163.0±21.5</td>
<td>177.1±18.7</td>
<td>163.1±26.3</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(38)</td>
<td>(26)</td>
<td>(12)</td>
<td></td>
</tr>
<tr>
<td><strong>Aortic sGAG (µg/mg wet tissue)</strong></td>
<td>7.47±1.00</td>
<td>8.01±1.16</td>
<td>5.37±1.38</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(17)</td>
<td>(11)</td>
<td>(5)</td>
<td></td>
</tr>
</tbody>
</table>

E\text{inc} = incremental elastic modulus, PWV = pulse wave velocity, HA = hyaluronan, sGAG = sulphated glycosaminoglycan, ns = not significant
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Figure 4.2: Incremental elastic modulus ($E_{\text{inc}}$) in hypertensive and normotensive aortae. The stiffness of aortae from human organ donors analysed using an Instron tensiometer. Aortae were cycled 5 times between 0 mmHg and $(180 \times 133.3 \times w \times r N)$ (180 mmHg equivalent; $w =$ width, $r =$ radius) at a rate of $10 \text{ mm} \cdot \text{min}^{-1}$, then stretched to failure. The resulting stress-strain curve was used to calculate the $E_{\text{inc}}$ at 30, 60, 100, 160, and 200 mmHg equivalent ($\text{top}$). $E_{\text{inc}}$ at each pressure was converted to pulse wave velocity (PWV) using the Moens-Korteweg equation ($\text{bottom}$). Points represent $\bar{x} \pm \text{SEM}$. ** = $p < 0.001$. ANOVA Pressure*HTN interaction $p < 0.001$ for both graphs.
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Figure 4.3: Representative examples human aortae staining with Alcian blue and Nuclear fast red. An sGAG gradient towards the intima can be seen in both normotensive [A (TS180, female, 81 years), C (TS188, female, 38 years)] and hypertensive [B (TS177, female, 58 years), D (TS193, female, 50 years)] aortae. Compared to the more gradual gradient in normotensive aortae, hypertensive aortae appear to have a clear sGAG delineation at the media-intima boundary (arrows).
4.4 Discussion

This study did not find many of the gender-based differences reported by previous studies. Common gender differences such as aortic diameter [97, 155, 212, 66], wall thickness [181, 170, 227, 103], history of HTN [41, 162, 34], and history of cardiovascular disease (CVD) [137, 125, 162, 34] were not found. The contrasting findings for diameter and thickness may be because measurements in this study were taken without applying load to the aortae, rather than during application of physiological pressure. There are mixed findings regarding gender differences in aortic stiffness, with some groups finding a difference aortic stiffness [81, 161] and others not [66, 150].

Age correlations were more consistent with existing literature. Similar to other groups, age was correlated with aortic diameter [181, 94, 97, 155, 212], wall thickness [170, 103], and aortic stiffness [181, 94, 212, 98, 81]. Age was also correlated with history of HTN [34, 41, 162] and history of diabetes [34, 137, 125].

This work represents one of the few analyses of ex vivo Young’s elastic modulus and pulse wave velocity in human aortae, and the only one of this scale. Therefore, comparison of findings to existing ex vivo work is limited. Instead, comparisons to in vivo work are considered. Despite similar values for wall thickness, average aortic diameters in this study (13.67 ± 0.52 mm) were notably smaller than diameters reported in other studies (19.8 ± 0.7, 20.3 ± 0.9, and 21.0 ± 0.8 mm) [94, 227, 66]. This may be because the measurements were taken on relaxed aortae, as previously stated.

While literature on the elastic modulus of ex vivo human aortic rings is scarce, there are a number of studies examining ex vivo uniaxial and biaxial elastic modulus in strips of human aortae. Work by Zarins et al. estimated ex vivo elastic modulus in thoracic rings at 0.62 ± 0.03 MPa, but did not measure stiffness directly [227]. Similarly, in vivo measurements suggest elastic modulus in the descending thoracic aortae is between 0.5 and 1.2 MPa [156, 181]. These values are of the same order of magnitude as our ex vivo measurements, but are generally higher overall. It is possible these differences result from the difference in measurement techniques, and the handling and freezing of samples before tensile testing. There is conflicting literature on the effect of freezing on uniaxial and biaxial tensile testing results, so the sourcing and preparation of tissues should be kept in mind [33, 147, 172, 185].

One particular consideration when using elastic modulus to characterise the stiffness of non-homogenous materials such as aortae is an inherent overestimation of material thickness. At lower physiological pressures the mechanical properties of the aorta are predominantly controlled by elastin, with the contribution of collagen increasing non-linearly with pressure. However, when measuring the thickness of a sample for $E_{inc}$ calculations, it is traditional to use the full wall thickness without correcting for the thickness of collagen or other non-contributing components. The result is an overestimation of the relevant thickness, and consequently an underestimation of elastic modulus.

Engineers have suggested the use of nano-indentation and atomic force microscopy
(AFM) to separate the independent elastic moduli in non-homogeneous materials [106, 107], and recent biological studies have used these techniques to assess the biomechanics of the aortic microstructure [79, 1, 2]. However, assessing the individual stiffnesses of aortic components and determining their contributions to gross elastic modulus at various pressures remains technically challenging. Instead, the effect of thickness overestimation of aortic elastic modulus has historically been assumed negligible. However, one potential solution may lie in the conversion of elastic modulus to PWV. When converting elastic modulus to PWV via the Moens Kortweg equation, the terms for thickness \( h \) cancel out, effectively removing this source of error from the stiffness calculation:

\[
PWV = \sqrt{\frac{E_{inc} \cdot h}{2 \cdot \tau \cdot \rho}} = \sqrt{\frac{\rho \cdot PWV}{2 \cdot \tau \cdot \rho}}
\]

For this reason, it may be more meaningful to use PWV when discussing aortic stiffness rather than elastic modulus.

Regardless, HTN aortae were still found to have higher elastic modulus and PWV compared to normotensive aortae above 60 mmHg, consistent with general findings on arterial stiffness in hypertension [126]. This difference was found to be pressure-dependent, and became more pronounced with increased pressure. Marque et al. found similar pressure-dependent divergence when comparing aortae of 15-month old spontaneously hypertensive (SH) rats to normotensive Wistar-Kyoto (WKY) controls, but no difference in 3- or 9-month old rats [121]. They suggest this age-associated divergence represents a fundamental change in the composition of the aortic wall that is independent of elastin or collagen content.

Similar findings have been observed in humans. In young individuals with hypertension, there was no significant difference in PWV after correcting for blood pressure [130]. However, in elderly individuals with hypertension, this PWV difference remained even after blood pressure was taken into account [131, 223]. One possibility for these pressure-independent differences in stiffness is a change in the composition or arrangement of glycosaminoglycan content in the arterial wall associated with hypertension.

Unfortunately, there were no correlations between total sGAG or HA content and aortic stiffness. Unpublished work by Dr. Kaisa Maki-Petaja (Division of Experimental Medicine and Immunotherapeutics, University of Cambridge) found no change in the elastic modulus of rat aortae before and after removal of HA with hyaluronidase, reaffirming older studies [152]. Similarly, other groups have shown that digestion of sulphated proteoglycans does not alter stiffness in human aortae [70] but may increase stiffness in mesenteric arteries [48]. Preliminary work by this group in uniaxial tensile testing after chondroitinase digestion suggests this may also be true in human aortae (unpublished). Interestingly, simultaneous digestion of both HA and sGAG in porcine aortae may marginally decrease stiffness [6].

It is possible that glycosaminoglycans and proteoglycans influence stiffness not by
their quantity but rather their distribution - for example, histological examination of aortic dissections found a buildup of sGAG at the sites of rupture [117]. Histological analysis in this study showed that while the total sGAG content between hypertensive and normotensive aortae did not differ, the distribution did. In both groups, there was a gradient of sGAG towards the intima, but in hypertensive aortae there was a visible delineation of sGAG buildup in the intima.

A similar sGAG gradient was described by Engel et al in animal aortae in 1971[39], but comparisons to more recent literature are complicated by counterstaining that obscures qualitative sGAG distribution [6]. Increased sGAG content in response to arterial coarctation has been noted previously [109, 206], and work in vascular smooth muscle cells suggests this localised buildup may be the result of blood pressure changes on VSMC proteoglycan production [167, 166]. Whether this buildup directly influences the stiffness differences is unclear.

When discussing the influence of glycosaminoglycans on vessel mechanics, discussions have generally focused on the “ground substance” in the media. However, there is now a growing interest in the role of glycosaminoglycans that comprise the endothelial surface layer, or “glycocalyx,” located on the luminal side of the endothelium. The most prevalent glycosaminoglycan in the glycocalyx is heparan sulfate, which combines with a variety of protein cores to form heparan sulfate proteoglycans (HSPGs).

HSPGs are known to be pressure-sensitive: they transition from a coiled conformation to a linear conformation when blood pressure and sheer stress increase. This linear conformation exposes numerous negatively charged side chains that attract large concentrations of positively charged sodium ions, triggering membrane hyperpolarization and vasodilatory effects [179, 180]. Hollander et al have shown that an increase in vascular sodium follows aortic coarctation, demonstrating a direct influence of pressure on vascular sodium accumulation [72]. Work by Ueda et al took this mechanism a step further, showing that the glycocalyx of bovine endothelial cells increases in thickness as sheer stress increases [205]. Thus, while sodium may not directly alter vascular stiffness, it could trigger a build-up of HSPGs in the intima during chronically elevated blood pressure in an effort to maintain lower vascular tone. However, while this redistribution may be indicative of hypertension, it does not, itself, necessarily affect mechanical stiffness.

There is some evidence that in large quantities sodium may actually damage this glycocalyx and cause glycocalyx shedding, with downstream effects on blood pressure. Once compromised, uncontrolled sodium leakage into the endothelium can impair nitric oxide production and lead to endothelial stiffening. The damaged glycocalyx is also unable to protect against immune cell infiltration, which can trigger vascular inflammation. Vascular glycocalyx damage has been observed in patients with diabetes and chronic kidney disease, and erythrocyte glycocalyx damage has been noted in patients with hypertension.

In this work, it was not clear whether glycosaminoglycan remodelling caused the hypertension, was caused by caused by hypertension, both, or neither. Regardless, this work
provides a unique look and the relationship between hypertension, vascular stiffness, and arterial glycosaminoglycans.

**Limitations**

This sample population was necessarily limited and skewed towards the elderly and those with advanced disease states. It was therefore not possible to compare young and older hypertensives, for example. Furthermore, tensile testing of \textit{ex vivo} arteries faces notable limitations. Loss of VSMC viability and muscle tone mean that tensile testing cannot predict VSMC contribution to the mechanical stiffness, and storage and preparation may alter tissue properties. Furthermore, because this collection was not subdivided by aortic region, there is the possibility that correlations which may be significant in one region were shown as insignificant, or less significant, when aortae from all regions were analysed together. The effects of the tensile testing apparatus must also be considered, as the vessel will be weakened at grip locations. However, these limitations are difficult to overcome in a real-world setting, and the aim should be to control these factors rather than eliminate them.

**Future directions**

It would be beneficial to further explore this sGAG gradient, possibly through sGAG and HA quantification in the individual layers of the aortae. It would also be beneficial to analyse stiffness in aortae whose elemental content has not been compromised, and compare regional stiffness, HA content, and sGAG content in different regions of the aorta.
Chapter 5

The effect of salt loading on glycosaminoglycans and vascular stiffness in rats

5.1 Background

Understanding general vascular changes in response to increased blood pressure is an integral part of hypertension research. During chronic hypertension, the aorta undergoes extensive remodelling, including increased collagen content, smooth muscle cell apoptosis, and changes in the activity of matrix metalloproteinases which regulate degradation of laminin, fibronectin, and proteoglycans. The cumulative effect of these changes is a general increase in vessel wall thickness and reduction of the lumen diameter as a result of inward hypertrophy (Figure 5.1). While limited rat work has shown increased chondroitin sulfate content in the arterial wall of spontaneously hypertensive rats [164, 163], the actual interplay between arterial glycosaminoglycans (GAGs) and blood pressure remains largely unknown.

Results from the analysis of human aortae (Chapter 4) showed that while GAG content was not necessarily increased in aortae from hypertensive human donors, its distribution in the aorta was be altered. However, these results only allow for limited interpretation. The study provided correlational information, but no causational data. It is not clear, for example, if the GAG remodelling seen in the hypertensive aortae was initiated by a tertiary factor (such as dietary salt) and then contributed to rising blood pressure in these donors, or if higher blood pressure, regardless of source, initiated the GAG remodelling. Or, perhaps, they are completely independent of each other but have a shared common cause.

Looking beyond the aorta, GAGs have also been implicated in total body sodium buffering via osmotically inactive sodium storage in the skin, and by extension may influence the onset and progression of salt-sensitive hypertension. Rat studies by Titze et al and Schafflhuber et al suggest that dietary salt can change the sodium content of the skin
as well as alter GAG synthesis [202, 175]. By increasing GAG content in response to high dietary salt, the skin could provide a non-osmotic sodium reservoir for dietary salt in excess of the kidney’s excretory capacity. Perhaps this sequestering could serve the purpose of buffering the body against salt-induced haemodynamic changes.

To better understand the role of skin GAGs in non-osmotic sodium storage and whether arterial GAG remodelling is salt-dependent or pressure-dependent, an interventional rat study was designed. Three groups of rats were used in this study. The first group was fed standard rodent chow and served as a control for dietary salt content. The second group was fed a high salt diet, serving to elucidate salt-dependent effects. The third group was fed a high salt diet with simultaneous antihypertensive treatment, to differentiate salt-dependent and pressure-dependent effects.

When choosing an antihypertensive for this study, it was important to choose one whose mechanism of action does not involve sodium handling. This meant that diuretics and ACE inhibitors were necessarily unsuitable. Instead, amlodipine was selected. Amlodipine is a calcium channel blocker which prevents movement of calcium into vascular smooth muscle cells, inhibiting contraction. The resultant vasodilation and reduction in peripheral vascular resistance causes blood pressure reduction. In this way, pressure-dependent effects could be explored without disrupting the pathogenesis of salt-dependent effects.

Hypothesis: We will see a salt-dependent increase in blood pressure as well as salt-dependent increases in both skin and arterial GAGs. Arterial stiffness will be both salt- and BP-dependent, but may be independent of GAG content, as seen in the human aortae.
Figure 5.1: **Hypertensive remodelling in the aorta.** A number of factors influence the vascular tone and physical stiffness of the aorta as a result of increased blood pressure and chronic hypertension. Figure reproduced from Intengan and Schiffrin (2001) [74].
5.2 Methodology

This study was conducted in collaboration with Professor Alberto Avolio at Macquarie University, Sydney, Australia. It was approved by the Macquarie University animal ethics committee.

5.2.1 Study design

24 male Sprague Dawley rats (Animal Resource Centre, Perth, Australia) were housed at Macquarie University Central Animal Facility from four weeks of age. The rats were divided into three groups: “control” (C, n=8), “high salt” (HS, n=8), and “high salt with antihypertensive” (HSTx, n=8). C rats were fed a standard rodent chow with 0.15% sodium and 0.40% potassium (AIN93G, Standard Rodent Diet, Specialty Feeds, Australia), while HS and HSTx rats were fed a high-salt content rodent chow with 3.10% sodium (8% NaCl) and 0.40% potassium (SF03-10 diet, 8% Salt Modification of AIN93G Rodent Diet, Specialty Feeds, Australia). The elemental content of each diet can be found in Table 5.1. Commencing at five weeks of age, HSTx rats were given subcutaneous injections of 5mg/kg amlodipine in peanut oil (15mg/mL amlodipine besylate) every second day. In contrast, C and HS rats received peanut oil injections without amlodipine.

All rats were given ad-libitum access to chow and drinking water for approximately three months. Water consumption, food consumption, urine output, and faecal output were measured twice using metabolic cages. Conscious systolic blood pressure was measured weekly. At 14-17 weeks of age, rats underwent non-recovery anaesthesia for invasive cardiovascular measurements and tissue sample collection. Only one rat was anaesthetised per day. In total, it took four weeks to sacrifice all 24 rats, creating an age difference of 27 days between the first and last rat (Table 5.2).
Chapter 5. The effect of salt loading on glycosaminoglycans and vascular stiffness in rats

Table 5.1: **Elemental content of rodent diets (w/w)**

<table>
<thead>
<tr>
<th></th>
<th>NaCl</th>
<th>Ca</th>
<th>P</th>
<th>Mg</th>
<th>Na</th>
<th>Cl</th>
<th>K</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard rodent diet</td>
<td>0.26%</td>
<td>0.47%</td>
<td>0.35%</td>
<td>0.08%</td>
<td>0.15%</td>
<td>0.16%</td>
<td>0.40%</td>
<td>0.23%</td>
</tr>
<tr>
<td>High salt rodent diet</td>
<td>8%</td>
<td>0.47%</td>
<td>0.30%</td>
<td>0.09%</td>
<td>3.10%</td>
<td>4.90%</td>
<td>0.40%</td>
<td>0.23%</td>
</tr>
</tbody>
</table>

Table 5.2: **Schedule of rat procedures**

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Rats weaned at Animal Resource Centre, Perth, Australia</td>
</tr>
<tr>
<td>4</td>
<td>Rats arrived at Central Animal Facility Macquarie University, Sydney, AustraliaC rats started on standard rodent chowHS and HSTx rats started on high salt rodent chow</td>
</tr>
<tr>
<td>5</td>
<td>HSTx rats started on amlodipine in peanut oil injectionsC and HS rats started on peanut oil injections</td>
</tr>
<tr>
<td>10</td>
<td>Metabolic cage measurements</td>
</tr>
<tr>
<td>13</td>
<td>Metabolic cage measurements</td>
</tr>
<tr>
<td>14-15</td>
<td>End-point experiments, one rat per day, 6 rats total</td>
</tr>
<tr>
<td>15-16</td>
<td>End-point experiments, one rat per day, 6 rats total</td>
</tr>
<tr>
<td>16-17</td>
<td>End-point experiments, one rat per day, 6 rats total</td>
</tr>
<tr>
<td>17-18</td>
<td>End-point experiments, one rat per day, 6 rats total</td>
</tr>
</tbody>
</table>
5.2.2 Measurements

5.2.2.1 Conscious procedures

All measurements were performed as described in Section 2.1.2.

Weight and body size

Animal weight and nose-to-tail length were measured every second day, and the weights were used to calculate amlodipine dose. Animal body mass index (\(BMI\)) was calculated as:

\[
BMI = \frac{\text{weight (g)}}{\text{length}^2 (\text{cm}^2)}
\]

Metabolic cage measurements

Water consumption, food consumption, urine output, and faecal output were measured using metabolic cages at the Central Animal Facility at Macquarie University. Rats were housed individually in metabolic cages for a period of 24 hours. This measurement was performed at weeks 10 and 13.

Tail-cuff blood pressure

Tail-cuff blood pressure was measured weekly, and performed as described in Section 2.1.2.3. Briefly, rats were secured inside a cylindrical animal holder with the tail protruding freely. The rat and holder were then placed on a 37°C warming platform and left undisturbed for five minutes. The occlusion tail cuff and sensor cuff were threaded onto the tail, and the occlusion cuff automatically inflated for blood pressure measurements. Measurements for weeks 4 and 5 were excluded from analysis because the tail diameter of the young rats prevented accurate occlusion with the tail cuff, and unfamiliarity with the procedure caused exacerbated movement during measurements.

5.2.2.2 Anaesthetised procedures

Anaesthesia and instrumentation

At 14-17 weeks of age, rats underwent non-recovery anaesthetisation using 1.3 g/kg urethane (0.1 g/mL in 0.9% saline), intraperitoneal injection. Top-up doses of urethane were given at 10% initial dose by intravenous cannula, as required. Rats were secured in a supine position on a 37°C heating pad to maintain body temperature.

Instrumentation was performed as in Figure 5.2. Electrocardiogram (ECG) leads were placed for continuous heart rate monitoring, and a cannula inserted into the femoral vein for drug administration. The trachea was carefully exposed and opened with a transverse cut to allow insertion of a tracheal tube. This tube ensured unobstructed free-breathing.
during the procedure. Supplemental oxygen was provided via a small oxygen mask placed close to the face. Two high-fidelity pressure-sensing catheters (Scisense catheter diameter 1.6 F) were inserted via the carotid artery (proximal catheter) and femoral artery (distal catheter), and an electromagnetic flow probe (Transonic) positioned around the aorta superior to the renal arteries. Finally, an ultrasound probe (Esaote) was positioned against the skin to image the aorta immediately superior to the flow probe.

Invasive *in vivo* data (heart rate, blood pressure) were recorded at 500 Hz during monitoring periods and at 10 kHz data acquisition periods using the CED 1401 data acquisition system (Cambridge Electronic Design, UK). These recordings were retained to serve as anaesthesia monitoring logs.

### Aortic stiffness measurements

The proximal catheter was initially positioned in the left ventricle to construct left ventricular pressure-volume loops. It was then moved to the descending thoracic aorta, slightly inferior to the heart, and the distal catheter positioned inferior to the renal arteries for pulse wave velocity (PWV) and pulse pressure amplification (PPA) measurements (Figure 5.2). The distance \( d \) between the proximal and distal probes was determined post-mortem.

Blood pressure was artificially raised and lowered using alternating intravenous infusions of phenylephrine (30 μg/mL at 30 μg/mL/min) and sodium nitroprusside (30 μg/mL at 30 μg/mL/min), respectively. This allowed for measurement of PWV and PPA between 70 mmHg and 140 mmHg.

PWV was calculated as the distance between proximal and distal catheters over the transit time \( tt \) between the foot of the carotid pulse wave and the foot of the femoral pulse wave:

\[
PWV = \frac{d}{tt}
\]

Aortic pulse amplification was calculated as the ratio of the thoracic aortic pulse pressure to the abdominal aortic pulse pressure:

\[
PPA = \frac{PP_{\text{thoracic}}}{PP_{\text{abdominal}}}
\]

#### 5.2.2.3 Post-mortem procedures

Animals were typically kept under anaesthesia for 10-12 hours. After complete data collection, animals were sacrificed with 0.5 mL 3 M intravenous potassium chloride. Death was confirmed using ECG recordings, blood pressure recordings, pinch-tests, and ocular reflex tests.
Rats were anaesthetised with 1.3 g/kg urethane (0.1 g/mL in 0.9% saline), i.p. A tracheal tube and supplemental oxygen allowed unobstructed free-breathing. A cannula was inserted into the femoral vein, and pressure-sensing probes into the carotid artery and femoral artery. A flow probe was fitted around the abdominal aorta, and imaged using an ultrasound probe. Pulse wave velocity (PWV) was calculated as the distance between pressure-sensing probes (d) over the pulse wave transit time (tt). Pulse pressure amplification (PPA) was calculated as the ratio of pulse pressures at the proximal (PP_{proximal}) and distal (PP_{distal}) probes.

**Figure 5.2: Instrumentation of the anaesthetised rat.** Rats were anaesthetised with 1.3 g/kg urethane (0.1 g/mL in 0.9% saline), i.p. A tracheal tube and supplemental oxygen allowed unobstructed free-breathing. A cannula was inserted into the femoral vein, and pressure-sensing probes into the carotid artery and femoral artery. A flow probe was fitted around the abdominal aorta, and imaged using an ultrasound probe. Pulse wave velocity (PWV) was calculated as the distance between pressure-sensing probes (d) over the pulse wave transit time (tt). Pulse pressure amplification (PPA) was calculated as the ratio of pulse pressures at the proximal (PP_{proximal}) and distal (PP_{distal}) probes.
Sample collection and shipment

Immediately preceding KCl infusion, 1-3 mL blood were collected from the carotid artery using needle and syringe. The blood was centrifuged for 10 minutes at 4000 g and 4°C, and the serum frozen at −80°C.

Post-mortem, the descending aorta was exposed and the distance between the two pressure-sensing catheters measured in triplicate using thread. The heart and aorta were then removed together, cleaned of connective tissue, and separated into heart, aortic arch, descending thoracic aorta and abdominal aorta. Unbranched segments of approximately 3-4 mm length were cut from the superior-most portions of the descending aorta and abdominal aorta and stored in saline for tensile testing measurements. 10 mm segments of the thoracic and abdominal aortae were removed and immersed in RNA later™ Stabilisation Solution (AM7021, Thermofisher) at 4°C for 24 hours, blotted dry, and stored at −80°C. Aortic segments of 3-5 mm were removed and fixed in formalin at 4°C for 24 hours, transferred to 70% EtOH, and stored at 4°C until embedding in paraffin wax. The remaining aortic arch, descending thoracic aorta, and abdominal aorta were snap frozen in liquid nitrogen and stored at −80°C.

The left ventricle of the heart was excised, weighed, and oven-dried at 60°C. The ventricle was weighed daily until weight stabilised (typically 7-14 days). Kidneys were similarly removed and dried to a stable weight.

A section of skin was shaved and excised from the trunk of the rat. Care was taken to ensure no ultrasound gel contaminated the site. Multiple 0.5 mm² pieces were immersed in RNA later™ at 4°C for 24 hours, blotted dry, and stored at −80°C. Additional 0.5 mm² pieces were fixed in formalin at 4°C for 24 hours, transferred to 70% EtOH, and stored at 4°C until embedding in paraffin wax. Untreated skin was snap frozen in liquid nitrogen and stored at −80°C. The right femur of each rat was excised, snap frozen in liquid nitrogen, and stored at −80°C.

Serum, aorta, skin, and bone samples were shipped from Macquarie University, Sydney, Australia to Cambridge University, Cambridge, United Kingdom by courier (CitySprint, United Kingdom) under authorisation IMP/GEN/2014/04. All tissues were shipped at −80°C, except paraffin-embedded samples which were shipped at ambient temperature.

Tensile testing and calculation of elastic modulus

Ex vivo stiffness was measured using tensile testing. Testing was performed as described in Section 2.2. Briefly, unbranched 3-4 mm aortic ring segments were mounted on two 26-gauge pins in a custom mounting block and fixed to an Inston tensile testing apparatus (model 5542) (Figure 2.3A). The outer diameter (dₒ) and wall thickness (h) of each ring were measured using a microscope graticule. Rings were pre-cycled 10 times at 2 mm/min from 0 to 200 mmHg equivalent load, then stretched to failure. Measured load for each extension of the aorta was used to calculate Young’s elastic modulus at a variety
of pressures. All calculations were performed in the Anaconda 5.0 Python distribution [Python 3.6].

**Sodium and potassium analysis**

Snap-frozen skin and aortae samples were analysed according to Section 2.3.1.2. Briefly, skin and aortae samples were rinsed with phosphate-buffered saline (PBS), blotted dry, and freeze-dried for 24 hours. Skin samples had all noticeable fur and fat removed before rinsing. When dry, samples were digested with 1:1 HNO$_3$:H$_2$O$_2$ at ambient temperature for 24 hours, and at 40$^\circ$C for an additional 24 hours. Digested samples were diluted 1/40 with ultra-pure water and analysed using inductively-coupled plasma optical emission spectrometry (ICP-OES).

**Sulphated glycosaminoglycan analysis**

Snap-frozen skin and aorta samples were dried in a desiccator at ambient temperature for 24 hours before being digested with papain (Section 2.3.2). Sulphated glycosaminoglycan (sGAG) content of the digest was quantified using the Blyscan$^\text{TM}$ Glycosaminoglycan Assay (Biocolor, Northern Ireland).

**qPCR analysis**

RNeater$^\text{TM}$-stabilized skin and aorta samples were used to analyse the RNA content of seven genes relevant to GAG synthesis and salt handling (Table 5.3). Based on preliminary work, 18S was chosen as the housekeeping gene (Section 3.2).

RNA was extracted using RNeasy Universal Plus Mini Kits (Qiagen) and stored at -80$^\circ$C until reverse transcription with the Promega Reverse Transcription System (A3500, Promega, United Kingdom). qPCR analysis was performed using TaqMan Gene Expression Assays (ThermoFisher Scientific) and TaqMan Gene Expression Master Mix (4369542, ThermoFisher Scientific). Changes RNA expression were assessed using using $\Delta\Delta$Ct analysis with fold difference calculation.

### 5.2.3 Contributions of others

Rats were housed at Macquarie University Central Animal Facility (Sydney, Australia) and cared for by qualified husbandry personnel. Dr. Mark Butlin performed routine weight and body size measurements, tail-cuff blood pressures, metabolic cage measurements, and subcutaneous dosing until week 13. Dr. Mark Butlin was responsible for rat anaesthetisation, instrumentation, and monitoring with assistance from Dana Georgevsky. Dr. Mark Butlin and Bart Spronk (Maastricht University, Maastricht, Netherlands) performed in vivo pulse wave velocity and pulse pressure amplification measurements and calculations. Bart Spronk performed ex vivo tensile testing of rat aortae.
Table 5.3: **Genes of interest in the salt-loaded rat**

<table>
<thead>
<tr>
<th>Gene code</th>
<th>Gene product</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S (housekeeping gene)</td>
<td><em>no product</em></td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>Chpf</td>
<td>chondroitin polymerising factor</td>
<td>chondroitin sulfate chain elongation</td>
</tr>
<tr>
<td>Chsy1</td>
<td>chondroitin sulfate synthase 1</td>
<td>biosynthesis of chondroitin sulfate</td>
</tr>
<tr>
<td>Dse</td>
<td>dermatan sulfate epimerase</td>
<td>converts chondroitin sulfate to dermatan sulfate</td>
</tr>
<tr>
<td>Ext1</td>
<td>hypoxia-inducible factor 2-alpha</td>
<td>heparin sulfate chain elongation</td>
</tr>
<tr>
<td>Glce</td>
<td>glucuronic acid epimerase</td>
<td>heparin sulfate chain elongation</td>
</tr>
<tr>
<td>Xylt1</td>
<td>xylosyltransferase 1</td>
<td>attachment of glycosaminoglycan chains to proteoglycan backbone</td>
</tr>
<tr>
<td>Vegfc</td>
<td>vascular endothelial growth factor C</td>
<td>promotes angiogenesis</td>
</tr>
</tbody>
</table>


5.2.4 Statistical analysis

Continuous variables were compared using ANCOVA and post-hoc Student’s t tests as appropriate, with age as a covariate. Correlations were performed using Pearson’s correlation, with partial correlation used to control for the effects of age. Differences in stiffness between groups at various pressures were compared using repeated measures ANOVA with group as a Between Subjects Factor. All statistical analyses were performed using IBM SPSS (version 25). Graphing was performed in the Anaconda 5.0 Python distribution [Python 3.6]. All values are reported as mean ± SEM. A p < 0.05 was considered statistically significant.

5.3 Results

The 8% sodium chloride (NaCl) diet was well tolerated by all rats in this study, however other groups have noted that an 8% NaCl diet can induce diarrhoeal illness [199]. Because only one rat could be examined per day, it took a total of 27 days to sacrifice all rats. This means a roughly one month age difference between the first rat and last rat. Age was significantly correlated with skin Na$^+$ (r = 0.865, p < 0.001) skin K$^+$ (r = -0.736, p < 0.001), skin water content (r = 0.848, p < 0.001), thoracic E$_{100\text{mmHg}}$ (r = -0.649, p < 0.01), abdominal E$_{100\text{mmHg}}$ (r = -0.590, p < 0.01), and thoracic sGAG content (r = 0.440, p = 0.031). Correlations between age and abdominal radius and age and skin sGAG per wet tissue weight (sGAG$_{\text{wet}}$), approached significance ($r_{\text{Ab radius}} = -0.371$, $p_{\text{Ab radius}} = 0.075$; $r_{\text{skin sGAG}} = -0.387$, $p_{\text{skin sGAG}} = 0.061$).

Weight, length, and tail-cuff SBP were measured throughout the study (Figure 5.3). HSTx rats showed decreased food intake at 10 weeks of age (p < 0.01), but this corrected by 13 weeks. HSTx rats also did not gain as much weight or body length as the C rats and HS rats. However, when normalised to body length (BMI) both HS and HSTx rats had lower BMIs than C rats (p < 0.001). HS and HSTx both showed significantly higher water intake and urine output than C rats (p < 0.001). There was no difference in faecal output between groups.

Left ventricular mass and kidney mass were normalised to body weight (Figure 5.4). There was a significant increase in the kidney-to-body mass ratio in HS and HSTx rats, but no statistically significant left ventricular hypertrophy.

In all three groups, the radius of the thoracic aorta was significantly (p < 0.001) larger than in the abdominal aorta. Thoracic wall thickness was also greater than abdominal wall thickness in C rats (p = 0.048) and HS rats (p < 0.001), but not in HSTx rats. Thoracic radius, abdominal radius, thoracic wall thickness, and abdominal wall thickness were not different between groups.

After controlling for age, there was no significant difference between groups with respect to Na$^+$ or K$^+$ in the skin, thoracic aorta, or abdominal aorta (Figure 5.5). Na$^+$ and K$^+$ in the thoracic and abdominal aortae were substantially lower than in the skin,
Figure 5.3: Growth, blood pressure, and metabolic cage values in control, salt-loaded, and salt-loaded antihypertensive rats over three months. Weight and nose-to-tail length were measured every other day, while tail-cuff SBP was measured weekly. Food intake, water intake, urine output, and faecal output were measured twice during this study using 24 hour metabolic cages. Graphs were supplied by Dr. Mark Butlin.
Figure 5.4: Physical dimensions of the rat aorta and tensile testing results. After sacrifice, unbranched aortic rings were examined using a microscope graticule to determine outer radius and wall thickness \( (n_C = 6, n_{HS} = 8, \text{ and } n_{HSTx} = 8) \). Rings were then cyclically stretched and stress-strain data used to calculate incremental elastic modulus (\( E_{inc} \)) at 100mmHg. Data presented as mean ± SEM. \( (* = p < 0.01, \; ** = p < 0.001, \; \text{ns} = \text{not significant} \) )
and 80% lower than aortic Na\(^+\) and K\(^+\) content found in previously-analysed rat aortae (unpublished data). This suggests that there may have been substantial Na\(^+\) and K\(^+\) loss due to sample mishandling and exposure to water following excision.

After controlling for age, there was no significant difference between groups with respect to skin water content or skin Na\(^+\) or K\(^+\) normalised to water content (Figure 5.6). Haemolysis in serum samples caused inaccurate Na\(^+\) and K\(^+\) measurements, so there values were discarded.

Total sGAG content in the skin, thoracic aorta, and abdominal aorta is shown in Figure 5.7. In the skin, there was a significant (p < 0.01) increase in sGAG\(_{\text{wet}}\) in HSTx rats compared to C rats. Once dried (sGAG\(_{\text{dry}}\)), this difference further increased. HSTx rats had significantly greater sGAG\(_{\text{dry}}\) content compared to both C rats (p < 0.01) and HS rats (p < 0.01). In contrast, there was no significant difference in thoracic or abdominal sGAG content between groups. The thoracic aorta had significantly more sGAG\(_{\text{dry}}\) content than the abdominal aorta for all groups (p\(_{\text{C}}\) < 0.01, p\(_{\text{HS}}\) < 0.01, p\(_{\text{HSTx}}\) = 0.016).

RNA could not be usefully extracted from aortic tissue, so qPCR analysis was only performed in the skin (Table 5.4). One HS rat and one HSTx rat were excluded as outliers. In HS rats, all genes showed decreased mRNA expression, with 2.9-5.5 fold reduction. HSTx rats showed greater variability between individuals, but on average also showed reduced expression of all genes, though to a lesser extent than in HS rats (1.3-3.0 fold decrease).

The high salt diet did cause a moderate increase in conscious systolic tail-cuff blood pressure in HS rats (p < 0.01) which was eliminated with antihypertensive treatment (Figure 5.8).

Due to incomplete data capture, PWV and PPA analysis were performed with n\(_{\text{C}}\) = 5, n\(_{\text{HS}}\) = 6, and n\(_{\text{HSTx}}\) = 4. The high salt diet caused significantly higher PWV in HS and HSTx rats compared to controls (p < 0.001), but antihypertensive treatment had no mitigating effect. Similar to PWV, the high salt diet significantly increased PPA in HS rats relative to C rats (p < 0.001). Antihypertensive treatment partially mitigated the effect on PPA, with HSTx rats having a PPA intermediate between HS and C rats. This was true across the blood pressure range.

After sacrifice, thoracic and abdominal aortae were collected for tensile testing (thoracic aortae: n\(_{\text{C}}\) = 6, n\(_{\text{HS}}\) = 8, and n\(_{\text{HSTx}}\) = 8; abdominal aortae: n\(_{\text{C}}\) = 7, n\(_{\text{HS}}\) = 8, and n\(_{\text{HSTx}}\) = 8). After controlling for age, there was no significant difference in thoracic or abdominal E\(_{\text{100mmHg}}\) between the groups. In HSTx rats, E\(_{\text{100mmHg}}\) was significantly higher in the thoracic aorta compared to the abdominal aorta (p = 0.011), but in C and HS rats there was no difference. Thoracic E\(_{\text{100mmHg}}\) was correlated with thoracic wall thickness and radius (r\(_{\text{wall thickness}}\) = -0.460, p\(_{\text{wall thickness}}\) = 0.036; r\(_{\text{radius}}\) = 0.443, p\(_{\text{radius}}\) = 0.044), while abdominal E\(_{\text{100mmHg}}\) was correlated with abdominal wall thickness (r\(_{\text{wall thickness}}\) = -0.722, p\(_{\text{wall thickness}}\) < 0.001).

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Figure 5.5: **Na⁺ and K⁺ concentrations in skin and aorta.** Skin and aorta samples were rinsed with PBS, blotted dry, and freeze-dried for 24 hours. Samples were digested with a 1:1 ratio of HNO₃:H₂O₂ for 24 hours at ambient temperature, then a further 24 hours at 40°C. Digests were diluted 1/40 with ultra-pure water and analysed using ICP-OES. Data presented as mean ± SEM.

Figure 5.6: **Skin water content and Na⁺ and K⁺ concentrations.** Skin water content was assessed by free-drying samples for 24 hours. Na⁺ and K⁺ content used to calculate concentration in mmol/L. Data presented as mean ± SEM.
Figure 5.7: sGAG content of rat skin, thoracic aorta, and abdominal aorta. Skin and aortae samples were dried for 24 hours at ambient temperature in a desiccator before digestion with papain, and the sGAG content of the digests analysed. Data presented as mean ± SEM. (* = p < 0.01, ** = p < 0.001)

Table 5.4: RNA fold change analysis in rat skin, 18S housekeeping gene

<table>
<thead>
<tr>
<th>Gene code</th>
<th>Function</th>
<th>HS fold change (n = 7)</th>
<th>HSTx fold change (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chpf</td>
<td>chondroitin sulfate chain elongation</td>
<td>0.200 ± 0.11</td>
<td>0.332 ± 0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.0 fold decrease)</td>
<td>(3.0 fold decrease)</td>
</tr>
<tr>
<td>Chsy1</td>
<td>biosynthesis of chondroitin sulfate</td>
<td>0.344 ± 0.12</td>
<td>0.472 ± 0.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.9 fold decrease)</td>
<td>(2.1 fold decrease)</td>
</tr>
<tr>
<td>Dse</td>
<td>converts chondroitin sulfate to dermatan sulfite</td>
<td>0.193 ± 0.09</td>
<td>0.662 ± 0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.2 fold decrease)</td>
<td>(1.5 fold decrease)</td>
</tr>
<tr>
<td>Ext1</td>
<td>heparin sulfate chain elongation</td>
<td>0.183 ± 0.11</td>
<td>0.693 ± 0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.5 fold decrease)</td>
<td>(1.4 fold decrease)</td>
</tr>
<tr>
<td>Glce</td>
<td>heparin sulfate chain elongation</td>
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<td>0.782 ± 0.23</td>
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<tr>
<td></td>
<td></td>
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<td>(1.3 fold decrease)</td>
</tr>
<tr>
<td>Xylt1</td>
<td>attachment of glycosaminoglycan chains to proteoglycan backbone</td>
<td>0.300 ± 0.13</td>
<td>0.535 ± 0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.3 fold decrease)</td>
<td>(1.9 fold decrease)</td>
</tr>
<tr>
<td>Vegfc</td>
<td>promotes angiogenesis</td>
<td>0.302 ± 0.07</td>
<td>0.568 ± 0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.3 fold decrease)</td>
<td>(1.8 fold decrease)</td>
</tr>
</tbody>
</table>
Chapter 5. The effect of salt loading on glycosaminoglycans and vascular stiffness in rats

Figure 5.8: Rat conscious tail-cuff SBP, unconscious PWV, and unconscious PPA after salt-loading and antihypertensive treatment. In vivo measurements of tail-cuff blood pressure, pulse wave velocity (PWV) and pulse pressure amplification (PPA) were measured in conscious (tail-cuff BP) or anaesthetised (PWV and PPA) rats after three months on a normal salt diet (Control), high salt diet (High Salt), or high salt diet with antihypertensive treatment (HSTx). After sacrifice, vessel stiffness was measured in the ex vivo aorta with tensile testing to obtain elastic modulus at 100 mmHg ($E_{100 \text{ mmHg}}$). Data presented as mean ± SEM. ($*=p<0.01$, ** =$p<0.001$)
5.4 Discussion

0-14 weeks on an 8% salt diet did not affect growth in male Sprague Dawley rats, however addition of 5 mg/kg amlodipine S.Q. injections did appreciably curtail both weight and length. This amlodipine effect has been previously reported by Dworkin et al. (1996), who noted that high doses of amlodipine in chow could retard growth in male Munich Wistar rats [36]. The diminished growth of HSTx rats may be related to reduced food intake noted at week 10, despite recovery by week 13. The high salt diet increased water consumption and urine output in HS and HSTx rats, as anticipated.

Consistent with increased salt handling, HS and HSTx rats showed increased kidney mass relative to C rats. However, the high salt diet did not induce the expected left ventricular hypertrophy (LVH). While a number of studies on Sprague Dawley and Wistar Kyoto rats have shown that 8-12 weeks on a high salt diet should be sufficient to induce LVH [49, 47, 224], it is possible that LVH would have materialised if rats had remained on the high salt diet for a longer period of time.

After salt-loading, HS rats did show an increase in blood pressure, as expected, and this increase was mitigated by antihypertensive treatment. However, the degree of this increase was relatively muted, with an average rise of 8 mmHg in HS rats compared to controls. This is in contrast to previous salt-loading studies in Sprague Dawley rats which found blood pressure increases of 20-30 mmHg for similar durations of dietary intervention [49, 224, 47].

One potential source of error may be the tail-cuff blood pressure measurement itself. There were issues obtaining accurate blood pressure measurements because the rats generally refused to remain still despite weeks of habituation. This could cause erroneously high blood pressure results for C rats, muting the size of the increase in HS rats. However this type of error would not account for the relatively low average BP of our HS rats (~125 mmHg) compared to salt-loaded rats in other studies (140-150 mmHg). Again, it is possible that rats in this study required more time on the high salt diet to allow greater blood pressure change.

The influence of time was a common theme in the results of this study, with skin Na\(^+\), skin K\(^+\), thoracic E\(^{100}\) mmHg, and abdominal E\(^{100}\) mmHg all significantly correlated with rat age (and relatedly, how much time was spent on each diet). While the one-month difference between the first and last rat was unavoidable, its demonstrable effect on key study parameters is disappointing. This strong time-dependence may also suggest that some rats, particularly the first rats killed, were not allowed enough time on the diets to affect important salt-dependent changes.

Skin sodium did rise with time on the diet, as predicted, but this increase was seen in all groups equally. It was therefore not surprising to see no difference in skin sodium content between groups. The rise of skin water content with time was similarly equal in all groups, and suggests that there was no increase in osmotically inactive sodium storage in rats fed a high salt diet compared to controls.
Other groups studying salt-loading in rats found distinct skin sodium accumulation with high salt diet, in contrast to these results [199, 200, 198, 175]. The reason for this discrepancy is not clear, but a difference in sodium quantification techniques should be noted. In the previous studies, sodium was quantified using atomic absorption spectroscopy (AAS) while in this study skin was quantified using ICP-OES. We contend that both methods are generally considered robust for tissue analysis, and the ability of ICP-OES to detect small changes in elemental composition is presented elsewhere in this thesis (see Chapters 3, 7, and 8). However, despite this validation of ICP-OES in isolation, it may be worth directly comparing these quantification techniques in the future. Regardless, salt-dependent cardiovascular effects were still seen in HS and HSTx rats independent of skin sodium accumulation.

An alternative hypothesis for this discrepancy relies on understanding the pathway by which sodium reaches the skin. As discussed in Chapter 4, the vascular endothelium contains a glycocalyx rich with pressure-sensitive heparan sulfate proteoglycans (HSPGs). In response to increased shear stress, HSPGs convert from a coiled conformation to a linear conformation, exposing negatively-charged side-chains to attract sodium. However, this glycocalyx can be damaged if sodium concentrations are too high, leading to glycocalyx shedding and immune cell infiltration into the vascular wall. Perhaps it is this damage to the vascular wall that allows the excess sodium to “leak” out of the vessels and into the skin, creating the characteristic rise in skin sodium with salt loading. With this in mind, it is possible that the rats presented in the study had sufficient time on the high salt diet to damage the vascular wall and cause in vivo haemodynamic changes, but not enough time for sodium leakage into the skin.

Like tail-cuff SBP, in vivo measurements of arterial stiffness conformed to literature on salt-dependence. PWV was increased in both HS and HSTx rats, and antihypertensive treatment did not mitigate this effect. These results are consistent with literature which suggests that PWV increases with dietary salt in both spontaneously hypertensive rats and Wistar-Kyoto rats [228, 134], and that this salt-dependent increase in PWV precedes blood pressure increases in salt-sensitive Dahl rats [65]. Indeed, treatment of spontaneously hypertensive rats with renin-angiotensin-aldosterone system (RAAS) inhibitors can mitigate these effects [134]. Thus, this increase in PWV can be considered salt-dependent rather than pressure-dependent.

Pulse pressure amplification (PPA) was similarly increased by the high salt diet, but this increase was partially mitigated by amlodipine. PPA is proportional to the length of the arterial tree [140, 149]. In hypertensive rats, the salt-dependent increases in stiffness result in changes in pulse wave reflections which attenuate amplification, causing salt-dependent changes in PPA [174, 173]. However, administration of ACE inhibitors and calcium-channel blockers, independent of dietary salt, has been shown to reduce central pulse pressure with no effect on terminal aortic pulse pressure, leading to changes in PPA [203]. Taken together, these data suggest that PPA is influenced by independent
Chapter 5. The Effect of Salt Loading on Glycosaminoglycans and Vascular Stiffness in Rats

Salt-sensitive and pressure-sensitive mechanisms.

This influence of salt on arterial stiffness changes when the aortae are examined ex vivo. There were no significant differences in thoracic or abdominal $E_{100\text{mmHg}}$ between groups. This can be reconciled with the in vivo stiffness measurements if it is assumed that changes in in vivo stiffness relate to changes in the tone in the living tissue rather than physical remodelling of the vessel wall in a way that alter mechanical stiffness. Interestingly, in HS rats, but not in C or HSTx rats, thoracic and abdominal $E_{100\text{mmHg}}$ were found to decrease with time. This could suggest a salt-dependence, although inverse of what was expected. After controlling for time, thoracic and abdominal $E_{100\text{mmHg}}$ were only correlated with wall thickness and vessel radius. They did not differ between groups and were not correlated with aortic sGAGs. This suggests that vessel stiffness may not be directly related to aortic sGAG content, similar to the findings in human aortae discussed previously.

sGAG content was higher in the thoracic aorta than in the abdominal aorta, in agreement with findings in dogs [180], but there was no difference in aortic sGAG content between groups. sGAG content was lower in the skin than the aorta, also in agreement with the previous literature. sGAG content was higher in the skin of HSTx rats compared to C and HS rats. Interestingly, expression of sGAG synthetic enzyme mRNA was actually slightly reduced in these HSTx rats despite the sGAG accumulation. In contrast, expression was substantially reduced in HS rats without subsequent change in total sGAG content. Although it is generally accepted that changes in mRNA expression of an enzyme do not necessarily translate to changes in the enzyme product, these results do conflict with work by other groups showing increased expression with salt loading [198, 64].

While changes in mRNA expression may not necessarily translate to changes in skin sGAG content, exploring this discrepancy is worth some consideration. Indeed, because only mRNA expression of synthetic enzymes was quantified, it is not known whether sGAG degradation was affected. Perhaps a high salt diet decreased skin sGAG degradation and, in the absence of blood pressure changes, lead to a net sGAG accumulation in HSTx rats. However, the salt-dependent blood pressure and stiffness changes found in HS rats may have caused a proportional decrease in sGAG synthesis, resulting in no net change. It is also possible that, as discussed above, there was not sufficient leaking of sodium from the vasculature into the skin. This lack of skin sodium may be responsible for unexpected sGAG results. Regardless, these findings run counter to work by other groups and it is not immediately clear by what mechanism salt and blood pressure influence skin sGAG content in these rats. It is clear, however, that both salt and pressure do influence skin sGAG content to some extent.

In conclusion, a high salt diet caused a small, but significant, rise in rat SBP which was mitigated by antihypertensive treatment. PWV was found to be salt-dependent and pressure-independent, while PPA was both salt-dependent and pressure-dependent. Ex vivo stiffness of the aorta was independent of both salt and blood pressure, although this could be due to insufficient time for vascular remodelling. Ex vivo stiffness was also independent of sGAG content, similar to the human aortae in Chapter 4.
Skin sodium increased in all groups equally, so there was no salt-dependent accumulation of sodium in the skin. Expression of mRNA coding for GAG synthetic enzymes was decreased with salt-loading, contrary to findings by other groups, and partially mitigated by amlopidine. Skin sGAG content was not similarly decreased by salt-loading or antihypertensive treatment. Instead, sGAG content was unchanged in HS rats, and increased in HSTx rats. This suggests that skin sGAG content may be both salt-dependent and pressure-dependent, and that sGAG degradation may play a primary role in this mechanism.

Limitations

There were three major limitations of this study. The first was a necessary 27-day age difference between the first and last rats analysed. While this could not be avoided, this age difference was significantly correlated with a number of parameters investigated in this work. How to prevent this in the future, however, is not clear as the in vivo measurements are necessarily time-consuming.

The second limitation was highlighted by the lack of LVH and subdued SBP increase seen in HS rats. Both of these changes are considered hallmarks of salt-loading studies, and their diminished appearance here suggest that perhaps the rats needed to be kept on their respective diets for longer to better showcase the influences of BP. The duration of this study was in line with similar works, so it is not clear why this issue arose.

The final limitation, if it can be called so, was the proportional accumulation of sodium in the skin of control rats as well as those on a high salt diet. Why control rats showed skin sodium accumulation, and to the same extent as rats on a high salt diet is not clear, but it may significantly impact interpretation of skin sGAG data and obscured investigation into the role of skin as a salt buffer and osmotically active sodium reservoir.

Future work

Similar to human aortae, there was no direct correlation between aortic ex vivo stiffness and aortic sGAG content. In humans, however, there was evidence of sGAG remodelling. Due to time constraints and supplier issues, histology could not be conducted on the rat aortae before writing of this thesis. However, it would be beneficial to carry out the histological work at a later date for comparison to humans. Similarly, bone sodium was not analysed in these rats, despite collection of femurs, due to time constraint. Some work by Titze et al has previously investigated bone sodium during salt loading, and it may be beneficial to carry out similar analyses for comparison. Finally, this group is still in possession of the RNAlater-sabilised aortae, and it would be worth working up a protocol for mRNA extraction so that synthesis in the aorta can be studies as it was in the skin.
Chapter 6

Measurement of electrolyte excretion in response to Indapamide: The GRENaDE Methods Study

6.1 Background

While controlling dietary salt intake in rats is relatively simple, doing so in humans is far more challenging. A large body of literature has shown that humans are notoriously poor at accurately monitoring their diets, particularly in an outpatient setting. It was therefore decided that in order to study the effects of salt in humans it may be easier to directly alter salt levels using NaCl tablets or diuretics rather than relying on dietary changes. The effect of increased sodium via salt tablet supplementation was previously explored by this group in the VARSITY (VEGF-C And Response to Salt In The Young) Study [178]. Following on this work, we next wanted to examine the effect of diuretic-induced sodium loss.

Before a full study could be conducted, a Methods study was used to determine how long participants should take the chosen diuretic to maximise sodium loss. Because the kidney is remarkably good at regulating urinary sodium excretion, the diuretic had to be taken long enough to induce adequate loss, but not so long that the kidney had time to correct for the loss and possibly increase renal sodium absorption to reverse its effects. As a secondary objective, this pilot also served as a demonstration of participant reliability and compliance with 24-hour urine collections.

A number of common diuretics were considered for use in this work. Ultimately, a thiazide diuretic was chosen over a loop diuretic as thiazides are more commonly recommended and routinely used for treatment of hypertension, are more effective at lowering and producing a sustained reduction in total peripheral resistance and blood pressure, and have a longer half-life. In contrast, loop diuretics require multiple daily administrations, increase renal sodium absorption between doses, and are less well tolerated by patients.

Broadly, thiazide diuretics promote decreased reabsorption of sodium and chloride in
the distal tubule of the kidney, leading to increased sodium excretion. It is generally believed that the kidney will correct for this increased excretion and re-stabilise to pre-treatment excretion rates within two to three days. However, few studies have been done directly demonstrating this, and those that have been done suggest that 1) time to re-stabilisation varies with thiazide, and 2) there may be a period of over-correction, during which the body has lower sodium excretion than before treatment and causing a net sodium retention (Figure 6.1) [17, 75, 83, 209].

For this work, we chose the thiazide diuretic Indapamide. Indapamide is a commonly used first-choice thiazide in clinical practice, which allows for more clinically relevant results. In contrast, the classic thiazides such as chlorthalidone and hydrochlorothiazide typically require higher dosing, are preferably used in combination with other therapies, and generally produce more severe side effects such as hyponatraemia and hypokalemia.

Based on the literature for other diuretics, it was decided that participants should receive Indapamid for 11 days. Ideally, this duration would be sufficient to characterise the time-course of both the initial increase in sodium loss, as well as any subsequent retention. A proper understanding of this re-stabilisation time-line was essential to effectiveness of the main diuretic study (Chapter 7).

Hypothesis: Indapamide will induce a net increase in urinary excretion for up to seven days, after which there will be a net decrease in sodium excretion before a final stabilisation to pre-treatment baseline. Sodium loss will peak within the first 24 hours of treatment.
Figure 6.1: **Effect of thiazide diuretics on urinary sodium excretion.** Urinary sodium excretion was standardised to baseline output and plotted against number of days treated with diuretic. (1) Bemetizide, 25mg single dose, n=17 diseased, standardised to 12g NaCl daily intake [83]; (2) polythiazide, 8mg daily for seven days, n=5 hypertensives, standardised to 3g NaCl daily intake [75]; (3) hydrochlorothiazide, 100mg twice daily for four days, n=12 normotensives, nonstandardized NaCl intake [17]; (4) hydrochlorothiazide, 100mg twice daily for four days, n=6 normotensives, nonstandardized NaCl intake, quantitative NaCl replacement daily [17]; (5) hydrochlorothiazide, 50mg twice daily, n=13 hypertensives responding to treatment, nonstandardized NaCl intake [209].
6.2 Methodology

6.2.1 Ethical approval

This pilot study and the subsequent main study were jointly sponsored by Cambridge University Hospitals NHS Foundation Trust and University of Cambridge. It was approved by NRES Committee East of England - Cambridge Central (REC reference 15/EE/0143) on 07 October 2015.

6.2.2 Study design

Eight healthy human volunteers were recruited for this 19-day pilot study. The overall design of the study can be seen in Figure 6.2. Following an initial screening visit at the Vascular Research Clinic in Addenbrooke’s Hospital (Cambridge, United Kingdom), participants were asked to maintain a “medium salt diet” of 6g NaCl or less per day for 18 days. Guidance on NaCl consumption was provided in the form of an information leaflet. A run-in period of seven days was allowed for adjustment to the salt diet and stabilisation of background NaCl intake. For the duration of the study, participants were asked to refrain from sustained, intensive exercise which promoted excess sweating.

After the run-in period, participants were given 2.5mg Indapamide orally for 11 days, and split into two groups. Both groups were asked to collect 24-hour urine samples on the last day of the run-in period (to serve as a baseline), and the first three days on Indapamide. Group A then provided 24-hour urine collections on the fourth, sixth, eighth, and tenth days on Indapamide, while Group B provided collections on the fifth, seventh, ninth, and eleventh days on Indapamide. This staggered schedule was chosen because the past experience of this group has been that successfully getting full 24-hour urine collections from subjects is very difficult, particularly in an out-patient setting. Thus, getting a single participant to return 12 consecutive 24-hour urinary collections was not considered feasible. A treatment period of 11 days was chosen so that each group would provide eight urine collections. No follow-up visit was required after the treatment period.

Because n = 3 is common for first-in-man studies, having four participants per group was considered sufficient for this pilot.

Inclusion criteria:

- Between 18 and 50 years of age
- Generally fit and healthy

Exclusion criteria:

- Hypertensive (sustained blood pressure > 140/90 mmHg)
- Current use of antihypertensive drugs
Chapter 6. Measurement of electrolyte excretion in response to Indapamide: The GRENaDE Methods Study

Figure 6.2: GRENaDE Methods Study design. Healthy human volunteers were asked to maintain a “medium salt diet” of less than 6g NaCl per day for 18 days. After an initial seven-day run-in period, participants were treated with 2.5mg Indapamide for 11 days. 24-hour urine collections were performed on the last day of the run-in period, the first three days of the treatment period, and the fourth, sixth, eighth, and tenth days (Group A) or the fifth, seventh, ninth, and eleventh days (Group B) of treatment.
Chapter 6. Measurement of electrolyte excretion in response to Indapamide: The GRENaDE Methods Study

- Current use of diuretics or salt supplements
- Diarrhoeal illness
- Pregnancy or current breast feeding regimen
- Renal impairment
- Gout
- Heart failure
- Inability to give informed consent
- Current involvement in other research studies
- Individuals with a medical condition which would contraindicate the use of a diuretic or in which the medical assessor believes it would not be an appropriate drug to take
- Any concomitant condition or circumstance that, at the discretion of the investigator, may affect the participants ability to complete the study or preclude any of the study procedures to be carried out safely

Participants were able to resume their normal eating habits immediately upon completion of the final urine collection, and were compensated £100 for their commitment.

6.2.3 Measurements

At the initial screening visit, participant height, weight, total body water content, and seated and supine blood pressure were measured, and a medical questionnaire performed as described in Section 2.1.1. Blood samples were analysed by the Cambridge University Hospitals Pathology Partnership for sodium, potassium, urea, creatinine, renin, and aldosterone to ensure that participants were healthy. 24-hour urine samples were analysed by the Cambridge University Hospitals Pathology Partnership for sodium, potassium, and creatinine.

Excess urinary Na\(^+\) excretion in the first 48 hours ($\Sigma$ExcessNa\(_{0-48}\)) was calculated as:

$$\Sigma$$ExcessNa\(_{0-48}\) = (Na\(_{24}\) - Na\(_{baseline}\)) + (Na\(_{48}\) - Na\(_{baseline}\))

where:
- Na\(_{24}\) = total urinary Na\(^+\) excretion in 0-24 hours on Indapamide
- Na\(_{48}\) = total urinary Na\(^+\) excretion in 24-48 hours on Indapamide
- Na\(_{baseline}\) = baseline urinary Na\(^+\) excretion
Similarly, total excess urinary $K^+$ excretion in the first 48 hours ($\Sigma \text{Excess} K_{0-48}$) was calculated as:

$$\Sigma \text{Excess} K_{0-48} = (K_{24} - K_{\text{baseline}}) + (K_{48} - K_{\text{baseline}})$$

where:

- $K_{24}$ = total urinary $K^+$ excretion in 0-24 hours on Indapamide
- $K_{48}$ = total urinary $K^+$ excretion in 24-48 hours on Indapamide
- $K_{\text{baseline}}$ = baseline urinary $K^+$ excretion

### 6.2.4 Statistics

Paired Student’s $t$ tests were used to compare changes in urinary sodium and potassium excretion. Correlations were analysed using Spearman’s rank. All statistical analyses were performed using IBM SPSS (version 25). Graphing was performed in the Anaconda 5.0 Python distribution [Python 3.6]. All values are reported as mean ± SEM. A $p < 0.05$ was considered statistically significant.

### 6.3 Results

A total of nine healthy volunteers were screened for this study, however one withdrew before the first urine collection. Four males and four females were randomly allocated to either Group A or Group B. All participants reported compliance with the salt diet, Indapamide treatment, and urine collections. Two participants chose to track their salt intake during the study using the phone application MyFitnessPal. Both reported a subconscious increase in salt consumption as the study progressed.

Individual participant height, weight, total body water content, blood pressure, and blood work from the screening visit can be seen in Table 6.1. 24-hour urine sodium (Figure 6.3) and urine potassium (Figure 6.4) values at each collection were standardised to baseline for comparison. Average baseline $Na^+$ ($Na_{\text{baseline}}$) was $147 \pm 32$ mmol/L, while average baseline $K^+$ ($K_{\text{baseline}}$) was $79 \pm 14$ mmol/L.

Indapamide treatment increased urinary $Na^+$ excretion in the first 0-48 hours in all participants, although the degree of this increase varied. $Na^+$ excretion generally peaked in the first 0-24 hours. Return to baseline urinary $Na^+$ excretion was typically achieved by 72 hours. Four participants (GM001, GM002, GM003, GM004) showed distinct U-shaped refractory periods following peak $Na^+$ excretion, while the remaining participants (GM006, GM007, GM008, GM009) showed more oscillatory patterns. Average $\Sigma \text{Excess} Na_{0-48}$ was $152$ mmol/L.
Table 6.1: GRENaDE Methods Study screening results

<table>
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<th>Age</th>
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<th>TBW (%)</th>
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<th>Seated DBP (mmHg)</th>
<th>Supine SBP (mmHg)</th>
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<td>48%</td>
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(a) Demographics and blood pressure

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<th>Serum K⁺ (mmol/L)</th>
<th>Urea (mmol/L)</th>
<th>Creatinine (µmol/L)</th>
<th>Renin (mU/L)</th>
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(b) Blood sample results
Figure 6.3: GRENaDE Methods Study 24-hour urinary Na⁺. Eight healthy volunteers were standardised to 6g NaCl or less per day for seven days, followed by an initial 24-hour urine collection to serve as baseline (Day 0). Participants were then given 2.5mg Indapamide for 11 days and asked to perform seven additional 24-hour urine collections. Displayed are individual participant results for daily urine sodium output, standardised to the baseline measurement.
Figure 6.4: GRENaDE Methods Study 24-hour urinary $K^+$. Eight healthy volunteers were standardised to 6g NaCl or less per day for seven days, followed by an initial 24-hour urine collection to serve as baseline (Day 0). Participants were then given 2.5mg Indapamide for 11 days and asked to perform seven additional 24-hour urine collections. Displayed are individual participant results for daily urine potassium output, standardised to the baseline measurement.
Indapamide increased urinary $K^+$ excretion in the first 0-48 hours in some participants, but not all. Typically, this increased excretion peaked at 24-48 hours, compared to the $Na^+$ excretion peak at 0-24 hours. Where present, this increased excretion was typically corrected for by 72 hours. Average $\Sigma$ExcessK$_{0-48}$ was 58 mmol/L.

There were significant correlations between $K_{\text{baseline}}$ and percentage $Na^+$ loss in the first 24 hours (-0.738, $p=0.037$) and $K_{\text{baseline}}$ and percentage $K^+$ loss in the first 24 hours (-0.738, $p=0.037$). There was also a significant correlation between $\Sigma$ExcessNa$_{0-48}$ and $\Sigma$ExcessK$_{0-48}$ (0.714, $p=0.047$).

### 6.3.1 Discussion

These preliminary results show that treatment with 2.5mg Indapamide once daily generally increases urinary sodium excretion during the first 48 hours of treatment, with more variable effects on urinary potassium excretion. Peak sodium loss was seen in the first 0-24 hours, with peak potassium loss, where present, following at 24-48 hours. Excess sodium and potassium excretion were both typically corrected within 72 hours. Those with a higher baseline potassium excretion lost less sodium and potassium in the first 24 hours compared to those with a lower baseline potassium excretion.

Sodium in particular showed signs of compensatory retention, with urinary excretion dropping below baseline for 24-72 hours following peak loss. It may be interesting to note that over the 11-day Indapamide course, four individuals showed a clear spike in urinary sodium excretion followed by a defined U-shaped retention period, while the remaining four showed a more oscillatory pattern of urinary sodium. No similar findings have been published by other groups, but these results may suggest the presence of two separate patterns of urinary sodium control in humans. Alternatively, the oscillations may reflect an fluctuating subconscious desire to eat more salt, as reported by two participants.

Based on these findings, it was decided that participants in the main diuretic study would be given 2.5mg Indapamide daily for eight days. This time course was chosen to maximise increased urinary sodium loss, minimise time for compensatory sodium retention, and maximise time for subsequent physiological changes associated with sodium loss. It has been shown previously that changes in hyaluronan content, for example, may take up to a week to manifest [40].

### Limitations

Significant limitations of this study include the reliance on participants to maintain the salt diet in an outpatient setting, and the influence of changes in salt intake on urinary salt loss at any given time point. Furthermore, it could not be guaranteed that participants took the Indapamide as instructed. Because body weight, blood samples, and blood pressure measurements were not taken after the treatment period, no data were available on the short-term effects of Indapamide on these parameters.
Chapter 7

Osmotically inactive sodium storage in human skin: The GRENaDE Study

7.1 Background

Over the last century, substantial research has demonstrated a link between dietary salt (NaCl) consumption and essential hypertension, suggesting a role for sodium in pathogenesis. In the US and the UK, where average sodium intake far exceeds the recommended 2400 mg (100 mmol) daily, this presents an enormous medical risk. Traditionally, hypertensive models have focused on renal sodium handling as the control for body sodium homeostasis (Figure 7.1). In this view, when renal sodium excretion is unable to keep pace with dietary sodium intake, excess sodium is retained in the body. This sodium attracts water, increasing extracellular fluid volume. The increased extracellular fluid volume then increases cardiac output, leading to high blood pressure. Over time, vascular remodelling in response to elevated cardiac output can result in raised peripheral vascular resistance and sustained hypertension.

However, recent studies have shown that, in many cases, sodium retention can occur with little or no associated fluid accumulation [62, 199, 197, 200]. The general conclusion from these studies is that the additional sodium must be stored in an osmotically inactive manner. Further studies have implicated skin, the largest organ in the body, as accounting for over 70% of this osmotically inactive storage, with muscle accounting for roughly 30% [200, 197]. Increased dietary salt and accumulation of sodium in the skin is further implicated in the subsequent accretion of glycosaminoglycans (GAGs) and cardiovascular changes [64, 200, 198].

The implications of skin sodium with respect to blood pressure and hypertension have only recently been investigated, so the field is limited in scope. In 2013 Kopp et al showed that skin sodium was elevated in female hypertensives compared to normotensive controls [84], and further work by Hammon et al and Schneider et al found, respectively, that skin sodium was elevated in heart failure patients and that skin sodium was independently correlated with systolic blood pressure and left ventricular hypertrophy [58, 177].
Figure 7.1: **Guyton's critical feedback circuit for salt, water, and cardiovascular function.** (1) Relationship between arterial pressure (AP) and urinary output of salt and water (UO); (2) Summation point, salt and water intake less UO; (3) Accumulation of extracellular fluid volume (ECFV) over time (\( \frac{dE}{dt} \)); Relationship between ECFV and blood volume (BV); Relationship between mean systolic pressure (MSP) and BV; Summation point, right arterial pressure (RAP) less MSP, equal to venous return (Ven Ret) gradient; (7) Division point, Ven Ret gradient divided by resistance to venous return (R Ven Ret), equal to Ven Ret and this defining cardiac output (CO); (8) Multiplication point, CO multiplied by total peripheral resistance (TRP), equal to AP. Figure reproduced from Korner, 2007[86]
A previous study by this group, the VARSITY Study, examined the effects of skin sodium accumulation in healthy humans after salt loading [178]. This study found a male-specific increase in the skin Na\(^+\):K\(^+\) ratio after seven days of treatment with salt tablets, with no effect on haemodynamics. Women, in contrast, showed no change in skin Na\(^+\):K\(^+\) but did have increased blood pressure, stroke volume, and peripheral vascular resistance. This suggests that men are better able to buffer excessive salt loads via the skin and thus mitigate adverse haemodynamic changes, while women, who were not as capable at buffering salt, were more salt-sensitive. Although it was hypothesised that these gendered results may be related to findings of higher skin glycosaminoglycan (GAG) content in men vs women [51, 144], GAGs were not directly investigated. There was also no measurement of tissue hydration to investigate the principal concept of non-osmotic sodium accumulation.

Building on this work, the GRENaDE (Glycosaminoglycan-Remodeling Effects of Na\(^+\) Depletion and Equilibrium) Study was designed to more closely examine the relationship between water balance, skin sodium, skin GAGs, and cardiovascular health in humans, with a focus on the effects of diuretic-induced salt loss. The aim of this study was to examine how skin sodium and skin GAGs change during salt depletion, and investigate the role of non-osmotic sodium in total body sodium buffering. The duration of diuretic treatment was determined in the GRENaDE Methods Study (Chapter 6).

**Hypothesis:** Diuretic treatment will decrease sodium and potassium content in the skin, without decreasing skin water content. There will be a slight decrease in blood pressure in Indapamide-treated participants, as well as either a decrease in mRNA expression of GAG-synthesising enzymes or increase in mRNA expression of GAG-degrading enzymes.
7.2 Ethical approval

This study and its associated pilot study were jointly sponsored by Cambridge University Hospitals NHS Foundation Trust and University of Cambridge. The study was approved by NRES Committee East of England - Cambridge Central (REC reference 15/EE/0143) on 07 October 2015.

7.3 Study design

The effect of Indapamide-induced salt reduction was examined using a double blind parallel study design (Figure 7.2). Following initial screening at Visit 1, participants were asked to begin maintaining a “medium salt diet” of 6g NaCl (2400 mg Na\(^+\)) or less per day for approximately 15 days. A run-in period of seven days was allowed for adjustment to the salt diet and stabilisation of background NaCl intake. After the run-in period, participants returned to the clinic at Visit 2, for baseline skin biopsy and cardiovascular measurements. Participants were then randomised to receive either a lactose placebo or 2.5mg Indapamide tablets once daily for eight days (according to findings in Chapter 6). After the treatment period, participants returned to the clinic at the final Visit 3, for post-treatment skin biopsy and cardiovascular measurements. All study visits were conducted at the Vascular Research Clinic in Addenbrookes Hospital (Cambridge, United Kingdom).

Guidance on NaCl consumption was provided in the form of an information leaflet. A time window of ±2 days was allowed for the run-in period and associated study visits, but the treatment period was held to exactly eight days to ensure all participants underwent the same duration of treatment. Participants were initially asked to perform 24-hour urine collections and ambulatory blood pressure measurements on the days before Visits 2 and 3, to allow for comparison before and after treatment. Because the GRENaDE Methods Study suggested that sodium excretion peaks in the first 24 hours on Indapamide, a third 24-hour urine collection was added midway through the study to allow quantification of peak Na\(^+\) loss. Because no increase in Na\(^+\) loss was expected in the Placebo group, this additional 24-hour urine collection also served as a compliance test to assess whether participants took the study tablets as instructed.

For the duration of the study, participants were be asked to refrain from sustained, intensive exercise which promoted excess sweating. For six hours prior to study visits, participants were asked to refrain from caffeine ingestion, alcohol ingestion, strenuous exercise, or application of topical creams to the lower back (abstinence from topical creams was required only before Visits 2 and 3).
Figure 7.2: GRENaDE Study design. Healthy human volunteers were asked to maintain a “medium salt diet” of less than 6g NaCl per day for approximately 15 days. After an initial seven-day run-in period, participants were treated with a lactose placebo or 2.5mg Indapamide for eight days. Skin biopsies and cardiovascular measurements were performed before and after the treatment period (Visits 2 and 3). 24-hour urine collections were performed the day before Visit 2, first day of the treatment period, and the day before Visit 3. 24-hour ambulatory blood pressure measurements were performed the day before Visit 2 and the day before Visit 3.
Inclusion criteria:

- Between 18 and 50 years of age
- Generally fit and healthy

Exclusion criteria:

- Hypertensive (screening blood pressure > 140/90 mmHg)
- Current use of antihypertensive drugs
- Current use of diuretics or salt supplements
- Diarrhoeal illness
- Pregnancy or current breast feeding regimen
- Renal impairment
- Gout
- Heart failure
- Known allergy to lignocaine
- Known allergy to lactose
- Inability to give informed consent
- Current involvement in other research studies
- Any concomitant condition or circumstance that, at the discretion of the investigator, may affect participant ability to complete the study
- Individuals with a medical condition which would contraindicate the use of a diuretic or in which the medical assessor believes it would not be an appropriate drug to administer
- Any concomitant condition or circumstance that, at the discretion of the investigator, may affect the participants ability to complete the study or preclude any of the study procedures to be carried out safely

Participants were able to resume their normal eating habits immediately upon completion of the final urine collection. Participants were compensated £250 for their commitment. Recruitment was primarily accomplished through word-of-mouth and the Cambridge University Graduate Union online “Opportunities” board.
7.4 Procedures

7.4.1 Physiological measurements

All measurements were performed as described in Section 2.1.1.

At all three visits, participant height, weight, total body water (TBW) content, and seated and supine systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured, and blood samples taken. 24-hour ambulatory blood pressure monitoring (ABPM) was performed on the days preceding Visits 2 and 3. 24-hour urine collections were performed on the days preceding Visits 2 and 3. In some participants, a third 24-hour urine collection was performed during the first day of the treatment period.

Pulse pressure (PP), augmentation index (Aix), pulse wave velocity (PWV), heart rate variability (HRV), and cardiac output (CO) were measured at Visits 2 and 3 using the SphygmoCor® Cardiovascular Management Suite (CvMS) (AtCor Medical, Sydney, Australia) and Innocor® inert gas re-breathing system (Innovision, Denmark). All cardiovascular measurements were performed after skin biopsy, to minimise the effects of anticipatory stress.

7.4.2 Skin biopsies

5 mm skin biopsies were taken from the lower back/upper buttock region at Visits 2 and 3 (Figure 7.3). Biopsies were performed as described in Section 2.1.1.8. Briefly, participants were asked to lie in a prone position exposing the lower back region, near the belt-line. The site was cleaned and locally anaesthetised using custom Na-free lidocaine (Tayside Pharmaceuticals, Scotland). Biopsies were taken using a 5 mm punch and immediately snap-frozen on dry ice. Sites were closed using two sutures and dressed with water-proof plasters.

Following the biopsy procedures, participants were instructed to abstain from activities which caused excessive bending of the lower back for two days. Light jogging and commuter cycling were permitted. Participants were asked to keep plasters on the biopsy sites for one week, although many did not follow this instruction. At Visit 3, the baseline biopsy site was checked for infection and proper wound healing before performing the second biopsy. Because participants were not seen again in clinic after Visit 3, follow-up emails to check healing status were sent within four weeks.

7.4.3 Biochemical analyses

7.4.3.1 Bloods and urine

Blood sampling was performed as described in Section 2.1.1.2. Blood samples were analysed by the Cambridge University Hospitals Pathology Partnership for sodium, potassium, urea, creatinine, renin, and aldosterone. 24-hour urine samples were analysed by
Figure 7.3: **Human skin biopsies.** 5 mm skin punch biopsies were taken from the lower back after anaesthetisation with custom Na-free lidocaine. The biopsy sites were closed with two sutures and protected with waterproof plasters. (A) Skin after biopsy removal. (B) Skin after biopsy site closure with sutures. (C) Biopsy site after one week of healing. (D) Ex vivo skin biopsy prepared for division into thirds. Images are from participant GS021.
Chapter 7. Osmotically inactive sodium storage in human skin: The GRENaDE Study

the Cambridge University Hospitals Pathology Partnership for sodium, potassium, and creatinine.

Excess urinary Na$^+$ excretion in the first 24 hours (ExcessNa$_{0-24}$) was calculated as:

$$\text{ExcessNa}_{0-24} = (\text{Na}_{24} - \text{Na}_{\text{baseline}})$$

where:

- $\text{Na}_{24}$ = total urinary Na$^+$ excretion in 0-24 hours on Indapamidie
- $\text{Na}_{\text{baseline}}$ = baseline urinary Na$^+$ excretion without Indapamidie

Similarly, total excess urinary K$^+$ excretion in the first 24 hours (ExcessK$_{0-24}$) was calculated as:

$$\text{ExcessK}_{0-24} = (\text{K}_{24} - \text{K}_{\text{baseline}})$$

where:

- $\text{K}_{24}$ = total urinary K$^+$ excretion in 0-24 hours on Indapamidie
- $\text{K}_{\text{baseline}}$ = baseline urinary K$^+$ excretion without Indapamidie

Serum samples were diluted 1/2 with water and hyaluronan (HA) content assayed using the Hyaluronan DuoSet ELISA (DY3614, R&D Systems, United States of America), according to the manufacturer’s protocol.

### 7.4.3.2 Skin elemental analysis

Skin biopsies were defatted and cut into thirds, preserving the epidermis:dermis ratio. Cutting was performed on a metal platform atop a bed of dry ice, to prevent thawing (Figure 7.3D). On third was used for elemental analysis, on third for qPCR analysis, and the remainder saved.

Elemental analysis of skin Na$^+$ and K$^+$ content was performed as described in Section 2.3.1.2. Briefly, 5-10 mg of skin were rinsed with phosphate-buffered saline and blotted dry to remove residual blood, then freeze-dried for 24 hours. Dry samples were digested in 1:1 30% H$_2$O$_2$:69% HNO$_3$ for 24 hours at ambient temperature followed by 24 hours at 40°C. Digests were then diluted 1:40 by weight with ultra high purity water and Na$^+$ and K$^+$ quantified in triplicated using inductively coupled plasma-optical emission spectroscopy (ICP-OES) at the MRC Elsie Widdowson Laboratory (Cambridge, United Kingdom).

### 7.4.3.3 Skin glycosaminoglycan analysis

qPCR analysis of GAG-associated enzymes was performed as described in Section 2.3.4. Briefly, 5-10 mg of skin were stored in pre-chilled RNA$\text{later}$, a Tissue Transition Solution (AM7030, Thermo Fisher Scientific) at -20°C for at least 24 hours, according to the manufacturer’s protocol. Preserved tissues were then minced and broken down
in TRIzol® Reagent (15596026, Thermo Fisher Scientific) using 5mm steel beads and a TissueLyser LT (69980, Qiagen). RNA was extracted using the PureLink™ Pro 96 total RNA Purification Kit (12173011A, Thermo Fisher Scientific) with DNase digestion (79254, Qiagen). RNA concentration and quality were measured using a NanoDrop Spectrophotometer. 150 µg human RNA was reverse transcribed using the Promega Reverse Transcription System (A3500, Promega) with custom protocol. Resultant cDNA was diluted 1/2.5 with DNase-free water and amplified in duplicate using TaqMan® Gene Expression Assays (Thermo Fisher Scientific) in TaqMan® Gene Expression Master Mix (4360016, Thermo Fisher Scientific).

RNA expression for each target gene was analysed using the ddCt method. 18S was used as the house-keeping gene:

\[ dCt = Ct_{\text{target}} - Ct_{18S} \]
\[ \text{ddCt} = \text{dCt}_{\text{post-treatment}} - \text{dCt}_{\text{baseline}} \]

### 7.4.4 Contributions of others

Skin biopsies were performed by Dr. Lucy Yang, Dr. Michalis Kostapanos, Dr. Spoorthy Burli, Dr. James Goodman, and Dr. Douglas Maslin (Division of Experimental Medicine and Immunotherapeutics, University of Cambridge, Cambridge, United Kingdom).

### 7.4.5 Statistics

All pre-treatment data were analysed together using Pearson correlation to identify trends in the general population. Post-treatment correlations were analysed separately for the Placebo and Indapamide groups. Independent Student’s t-tests were used evaluate gender differences in pre-treatment data only. Paired Student’s t-tests were used to evaluate pre- vs post-treatment changes within each treatment group. Data were further analysed using a repeated measures ANOVA, with pre- vs post-treatment as the Within-Subjects factor and treatment type and gender as Between-Subjects factors. ANCOVA analysis was used to compare RNA expression between treatment groups, with baseline dCt values as a covariate.

All statistical analyses were performed using IBM SPSS (version 25). Graphing was performed in the Anaconda 5.0 Python distribution [Python 3.6]. All values are reported as mean ± SEM. A p < 0.05 was considered statistically significant.

### Results

A total of 59 healthy human participants (35 female, 24 male; mean age 26 years, range 20-40 years) successfully completed the study (Figure 7.4). Participants were 75% white
After random allocation, 28 participants (21 female, 7 male) were given Placebo, and 31 participants (14 female, 17 male) given Indapamide. Most participants reported no dramatic alterations to their eating habits were required to comply with the salt diet, as they already ate below 6 g salt per day.

Nine individuals passed screening but did not complete the study. Of these, seven chose to voluntarily withdraw before the first biopsy and one voluntarily withdrew before the second biopsy due to an unrelated adverse event. One participant was removed from the study analysis after the second biopsy due to an adverse reaction to treatment. This latter participant reported progressively severe nausea and dizziness after 72 hours on treatment, and was told to discontinue taking the study tablets. Blood tests indicated hypokalemia (serum K⁺ of 3.1 mmol/L), for which they were prescribed potassium supplements for 48 hours. Follow-up bloods showed a return to normal potassium levels, and the participant was monitored by email for the next two weeks to ensure recovery. Unblinding confirmed that the participant was given Indapamide treatment.

At baseline, TBW was significantly correlated with supine DBP (r = -0.421, p = 0.001), ABPM daytime SBP (r = 0.292, p = 0.026), serum Na⁺ (r = 0.368, p = 0.005), PP (r = 0.615, p < 0.001), Aix (r = -0.316, p = 0.024), SV (r = 0.470, p < 0.001), CO (r = 0.320, p = 0.020). TBW was correlated with skin water content at marginal significance (r = -0.261, p = 0.046).

Skin Na⁺ (per dry tissue weight) was correlated with ABPM daytime DBP (r = -0.472, p < 0.001), serum Ha (r = -0.340, p = 0.014), urine volume (r = -0.341, p = 0.015), and skin K⁺ (r = 0.341, p = 0.008). Serum HA was only correlated with skin Na⁺.

In Indapamide-treated participants at Visit 3, skin Na⁺ (per wet tissue weight only) was correlated with HRV Index (r = -0.382, p = 0.045), SV (r = -0.403, p = 0.027), and skin K⁺ (r = 0.883, p = 0.006).

Baseline and post-treatment values within each treatment group can be seen in Table 7.1. With the exception of seated SBP, there were no significant changes between baseline and post-treatment in the Placebo group. In the Indapamide group, TBW, serum Na⁺, serum K⁺, skin Na⁺, and skin K⁺ all significantly decreased (p < 0.05 for all), while serum urea, serum renin, serum aldosterone, urine K⁺, ABPM DBP, and PWV all significantly increased (p < 0.05 for all). Decreases in serum HA, HRV Index, and SV showed borderline significant reduction (p_{serum Ha} = 0.071, p_{HRV Index} = 0.052, p_{stroke vol} = 0.058).

Changes in water and electrolyte content can be seen in Figures 7.5 and 7.6. ExcessNa₀⁻²⁴ and ExcessK₀⁻²⁴ were higher in the Indapamide group compared to Placebo (ExcessNa₀⁻²⁴: 83.0 ± 21.6 vs 4.9 ± 8.0, p < 0.001; ExcessK₀⁻²⁴: 25.6 ± 10.3 vs 0.2 ± 5.5, p < 0.001).

The significance of the Time*Tx interaction term is reported in the last column of Table 7.1. This interaction term represents the difference in pre- vs post-treatment changes between groups. Time*Tx was significant for TBW, serum Na⁺, serum K⁺, serum urea, serum renin, serum aldosterone, urine K⁺, and HRV Index (p < 0.05 for all).
Figure 7.4: GRENaDE study CONSORT diagram. A total of 69 participants were screened for this study, of which one failed screening due to pre-existing hypertension. The remaining participants were randomly allocated to Placebo (n=35) or Indapamide (n=33) treatment. Seven participants withdrew before pre-treatment measurements were taken, citing loss of interest and scheduling conflicts. One participant withdrew after pre-treatment measurements but before post-treatment measurements, citing an unrelated adverse event (AE, ear infection). Finally, one participant was excluded from analysis because their treatment was stopped prematurely due to a related AE (hypokalemia). In total, 59 participants were analysed.
Table 7.1: Results for Placebo and Indapamide treatment groups pre-treatment (Visit 2) and post-treatment (Visit 3)

<table>
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<tr>
<th>Characteristic</th>
<th>Placebo (n = 28)</th>
<th>Indapamide (n = 31)</th>
<th>Time * Tx Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>20.0 ± 0.9</td>
<td>25.9 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Gender (f:m)</td>
<td>21:7</td>
<td>14:17</td>
<td></td>
</tr>
<tr>
<td>Serum Na⁺ (mmol/L)</td>
<td>139.5 ± 0.3</td>
<td>139.9 ± 0.2</td>
<td>0.019</td>
</tr>
<tr>
<td>Serum K⁺ (mmol/L)</td>
<td>4.1 ± 0.1</td>
<td>4.1 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum Urea (mmol/L)</td>
<td>4.6 ± 0.3</td>
<td>4.9 ± 0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum Renin (mU/L)</td>
<td>28.4 ± 3.5</td>
<td>41.3 ± 4.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum Aldosterone (pmol/L)</td>
<td>483.8 ± 80.2</td>
<td>487.1 ± 71.6</td>
<td>0.003</td>
</tr>
<tr>
<td>Urine Na⁺ (mmol/24 hr)</td>
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<td>97.0 ± 9.2</td>
<td>ns</td>
</tr>
<tr>
<td>Urine K⁺ (mmol/24 hr)</td>
<td>71.0 ± 5.1</td>
<td>82.2 ± 5.1</td>
<td>0.023</td>
</tr>
<tr>
<td>Urine volume (L)</td>
<td>2.4 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>ns</td>
</tr>
<tr>
<td>Seated SBP (mmHg)</td>
<td>106.8 ± 2.2</td>
<td>110.2 ± 3.1</td>
<td>ns</td>
</tr>
<tr>
<td>Seated DBP (mmHg)</td>
<td>70.2 ± 1.1</td>
<td>70.6 ± 1.8</td>
<td>ns</td>
</tr>
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<td>Supine SBP (mmHg)</td>
<td>105.8 ± 1.8</td>
<td>111.7 ± 2.0</td>
<td>ns</td>
</tr>
<tr>
<td>Supine DBP (mmHg)</td>
<td>64.4 ± 1.0</td>
<td>62.0 ± 1.1</td>
<td>ns</td>
</tr>
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<td>dABPMB SBP (mmHg)</td>
<td>117.0 ± 1.6</td>
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<td>dABPMB DBP (mmHg)</td>
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<td>nABPMB SBP (mmHg)</td>
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<td>nABPMB DBP (mmHg)</td>
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<tr>
<td>PP (mmHg)</td>
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</tr>
<tr>
<td>Aix</td>
<td>-3.0 ± 2.3</td>
<td>-10.6 ± 2.2</td>
<td>ns</td>
</tr>
<tr>
<td>PWV (m/s)</td>
<td>4.9 ± 0.9</td>
<td>4.8 ± 0.1</td>
<td>0.019</td>
</tr>
<tr>
<td>HRV Index</td>
<td>12.2 ± 0.7</td>
<td>13.8 ± 1.0</td>
<td>0.052</td>
</tr>
<tr>
<td>CO (L/min)</td>
<td>6.0 ± 0.2</td>
<td>7.2 ± 0.4</td>
<td>0.053</td>
</tr>
<tr>
<td>SV (mL)</td>
<td>93.7 ± 3.4</td>
<td>116.4 ± 6.9</td>
<td>0.058</td>
</tr>
<tr>
<td>Skin water content (%)</td>
<td>66.7 ± 0.8</td>
<td>66.2 ± 0.7</td>
<td>ns</td>
</tr>
<tr>
<td>Skin Na⁺ (mmol/L)</td>
<td>120.1 ± 4.8</td>
<td>122.9 ± 2.2</td>
<td>0.030</td>
</tr>
<tr>
<td>Skin K⁺ (mmol/L)</td>
<td>25.2 ± 1.1</td>
<td>28.3 ± 1.2</td>
<td>0.038</td>
</tr>
<tr>
<td>Skin Na⁺ (µg/mg dry tissue)</td>
<td>5.44 ± 0.24</td>
<td>5.33 ± 0.19</td>
<td>0.004</td>
</tr>
<tr>
<td>Skin K⁺ (µg/mg wet tissue)</td>
<td>1.94 ± 0.09</td>
<td>2.05 ± 0.10</td>
<td>0.012</td>
</tr>
<tr>
<td>Na⁺:K⁺ (µg/mg dry tissue)</td>
<td>2.80 ± 0.13</td>
<td>2.70 ± 0.12</td>
<td>ns</td>
</tr>
</tbody>
</table>

TBW = total body water, HA = hyaluronic acid, SBP = systolic blood pressure, DBP = diastolic blood pressure, dABPM = daytime ambulatory blood pressure monitor measurement, nABPM = nighttime ambulatory blood pressure monitor measurement, PP = pulse pressure, Aix = augmentation index, PWV = pulse wave velocity, HRV = heart rate variability, CO = cardiac output, SV = stroke volume, ns = not significant.
The interaction was almost significant for serum HA, CO, and SV ($p_{\text{serum HA}} = 0.064$, $p_{\text{HRV Index}} = 0.053$, $p_{\text{stroke vol.}} = 0.052$). The interaction was not significant for effects on blood pressure, skin Na$^+$, or skin K$^+$.

The effect of gender on baseline measurements and effectiveness of Indapamide can be seen in Table 7.2. There were significant differences between genders for TBW, serum Na, serum renin, seated SBP, supine SBP, ABPM daytime SBP, ABPM nighttime SBP, PP, Aix, PWV, CO, and SV ($p < 0.05$ for all). Gender significantly influenced the action of Indapamide on serum HA ($p_{\text{serum HA}} = 0.047$) and PP ($p_{\text{serum HA}} = 0.040$). This influence was almost significant for serum K$^+$, serum urea, and skin Na$^+$ ($p_{\text{serum K}} = 0.075$, $p_{\text{serum urea}} = 0.053$, $p_{\text{skin Na}} = 0.088$).

Changes in gene expression can be seen in Table 7.3. Only expression of Chpf was significantly reduced in subjects on Indapamide ($p = 0.023$) compared to Placebo, with the decreased expression of Chys1, DSE and VegfC approaching significance ($p_{\text{Chys1}} = 0.078$, $p_{\text{DSE}} = 0.088$, $p_{\text{VegfC}} = 0.059$). There was no significant influence of gender on gene expression.
Figure 7.5: Total body and skin water and electrolytes in Placebo and Indapamide subjects pre- and post-treatment. (A) Total body water content; (B) skin water content; (C) skin Na⁺ content; (D) skin K⁺ content. Pre- and post-treatment values were compared using paired t-tests. * = p < 0.05, ** = p < 0.01, *** = p < 0.001
Figure 7.6: Placebo and Indapamide skin electrolytes before and after treatment. (A) Skin Na⁺; (B) Skin K⁺. Pre- and post-treatment values were compared using paired t-tests. * = p < 0.05, ** = p < 0.01, ns = not significant
Table 7.2: Gender differences in cardiovascular and physiological measures, and their influence on the effectiveness of Indapamide

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Visit 2 (baseline)</th>
<th>Time * Tx * Gender Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female (35)</td>
<td>Male (24)</td>
</tr>
<tr>
<td></td>
<td>( \bar{x} \pm SEM )</td>
<td>( \bar{x} \pm SEM )</td>
</tr>
<tr>
<td>Age (years)</td>
<td>26.7 ± 0.8</td>
<td>25.8 ± 1.0</td>
</tr>
<tr>
<td>TBW (%)</td>
<td>49.5 ± 2.4%</td>
<td>61.8 ± 2.0%</td>
</tr>
<tr>
<td>Serum Na(^+) (mmol/L)</td>
<td>139.1 ± 0.2</td>
<td>140.5 ± 0.3</td>
</tr>
<tr>
<td>Serum K(^+) (mmol/L)</td>
<td>4.1 ± 0.0</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>Serum Urea (mmol/L)</td>
<td>4.6 ± 0.3</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>Serum Renin (mU/L)</td>
<td>28.0 ± 2.9</td>
<td>44.5 ± 4.7</td>
</tr>
<tr>
<td>Serum Aldosterone (pmol/L)</td>
<td>567.8 ± 78.1</td>
<td>372.3 ± 60.8</td>
</tr>
<tr>
<td>Serum HA (µg/mL)</td>
<td>14.4 ± 1.5</td>
<td>12.4 ± 1.5</td>
</tr>
<tr>
<td>Urine Na(^+) (mmol/24 hr)</td>
<td>88.3 ± 8.8</td>
<td>101.8 ± 8.3</td>
</tr>
<tr>
<td>Urine K(^+) (mmol/24 hr)</td>
<td>71.6 ± 4.6</td>
<td>82.2 ± 5.8</td>
</tr>
<tr>
<td>Urine volume (L)</td>
<td>2.3 ± 0.2</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Seated SBP (mmHg)</td>
<td>102.7 ± 2.1</td>
<td>119.3 ± 2.3</td>
</tr>
<tr>
<td>Seated DBP (mmHg)</td>
<td>70.0 ± 1.4</td>
<td>72.0 ± 1.4</td>
</tr>
<tr>
<td>Supine SBP (mmHg)</td>
<td>102.9 ± 1.4</td>
<td>117.1 ± 1.7</td>
</tr>
<tr>
<td>Supine DBP (mmHg)</td>
<td>63.9 ± 0.9</td>
<td>62.5 ± 1.3</td>
</tr>
<tr>
<td>dABPM SBP (mmHg)</td>
<td>114.3 ± 1.2</td>
<td>123.5 ± 1.7</td>
</tr>
<tr>
<td>dABPM DBP (mmHg)</td>
<td>70.8 ± 1.7</td>
<td>74.4 ± 2.3</td>
</tr>
<tr>
<td>nABPM SBP (mmHg)</td>
<td>105.3 ± 1.3</td>
<td>114.4 ± 1.7</td>
</tr>
<tr>
<td>nABPM DBP (mmHg)</td>
<td>62.1 ± 1.0</td>
<td>64.1 ± 1.3</td>
</tr>
<tr>
<td>PP (mmHg)</td>
<td>23.5 ± 0.6</td>
<td>33.3 ± 1.2</td>
</tr>
<tr>
<td>Aix</td>
<td>-3.6 ± 2.0</td>
<td>-11.9 ± 2.2</td>
</tr>
<tr>
<td>PWV (m/s)</td>
<td>4.7 ± 0.1</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>HRV Index</td>
<td>12.8 ± 0.8</td>
<td>13.2 ± 1.1</td>
</tr>
<tr>
<td>CO (L/min)</td>
<td>5.8 ± 0.2</td>
<td>7.7 ± 0.3</td>
</tr>
<tr>
<td>SV (mL)</td>
<td>88.9 ± 3.5</td>
<td>128.9 ± 6.4</td>
</tr>
<tr>
<td>Skin water content (%)</td>
<td>66.9 ± 0.6%</td>
<td>65.7 ± 0.9%</td>
</tr>
<tr>
<td>Skin Na(^+) (µg/mg wet tissue)</td>
<td>1.83 ± 0.06</td>
<td>1.88 ± 0.04</td>
</tr>
<tr>
<td>Skin Na(^+) (µg/mg dry tissue)</td>
<td>5.36 ± 0.22</td>
<td>5.41 ± 0.20</td>
</tr>
<tr>
<td>Skin K(^+) (µg/mg wet tissue)</td>
<td>0.67 ± 0.03</td>
<td>0.74 ± 0.04</td>
</tr>
<tr>
<td>Skin K(^+) (µg/mg dry tissue)</td>
<td>1.92 ± 0.07</td>
<td>2.11 ± 0.12</td>
</tr>
<tr>
<td>Na(^+) :K(^+) (µg/mg wet tissue)</td>
<td>2.83 ± 0.13</td>
<td>2.99 ± 0.35</td>
</tr>
</tbody>
</table>

TBW = total body water, HA = hyaluronan, SBP = systolic blood pressure, DBP = diastolic blood pressure, dABPM = daytime ambulatory blood pressure monitor measurement, nABPM = nighttime ambulatory blood pressure monitor measurement, PP = pulse pressure, Aix = augmentation index, PWV = pulse wave velocity, HRV = heart rate variability, CO = cardiac output, SV = stroke volume, ns = not significant.
### Table 7.3: Change in mRNA expression in Placebo and Indapamide subjects standardised to pretreatment levels with 18S as the housekeeping gene

<table>
<thead>
<tr>
<th>Gene code</th>
<th>Gene product</th>
<th>Function</th>
<th>ddCt Placebo $\bar{x} \pm$ SEM (n)</th>
<th>ddCt Indapamide $\bar{x} \pm$ SEM (n)</th>
<th>p value ANCOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chpf</td>
<td>chondroitin polymerising factor</td>
<td>chondroitin sulfate chain elongation</td>
<td>$0.21 \pm 0.17$ (11)</td>
<td>$-0.28 \pm 0.20$ (11)</td>
<td>0.029</td>
</tr>
<tr>
<td>Chsy1</td>
<td>chondroitin sulfate synthase 1</td>
<td>biosynthesis of chondroitin sulfate</td>
<td>$0.38 \pm 0.27$ (21)</td>
<td>$-0.42 \pm 0.37$ (25)</td>
<td>0.078</td>
</tr>
<tr>
<td>Dse</td>
<td>dermatan sulfate epimerase</td>
<td>converts chondroitin sulfate to dermatan sulfate</td>
<td>$0.26 \pm 0.22$ (21)</td>
<td>$-0.31 \pm 0.23$ (25)</td>
<td>0.088</td>
</tr>
<tr>
<td>Ext2</td>
<td>exostosin 2</td>
<td>heparin sulfate chain elongation</td>
<td>$0.08 \pm 0.13$ (21)</td>
<td>$-0.12 \pm 0.12$ (25)</td>
<td>ns</td>
</tr>
<tr>
<td>Hyal1</td>
<td>hyaluronidase</td>
<td>degradation of hyaluronan</td>
<td>$0.08 \pm 0.17$ (21)</td>
<td>$-0.08 \pm 0.14$ (25)</td>
<td>ns</td>
</tr>
<tr>
<td>Glce</td>
<td>glucuronic acid epimerase</td>
<td>heparin sulfate sulfation</td>
<td>$0.40 \pm 0.31$ (21)</td>
<td>$-0.37 \pm 0.39$ (25)</td>
<td>ns</td>
</tr>
<tr>
<td>VegfC</td>
<td>vascular endothelial growth factor C</td>
<td>promotes angiogenesis</td>
<td>$0.24 \pm 0.19$ (21)</td>
<td>$-0.27 \pm 0.17$ (25)</td>
<td>0.059</td>
</tr>
<tr>
<td>Xylt1</td>
<td>xylosyltransferase 1</td>
<td>attachment of GAG chains to proteoglycan backbone</td>
<td>$0.20 \pm 0.0.15$ (21)</td>
<td>$-0.09 \pm 0.14$ (25)</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns = not significant
7.5 Discussion

A total of 59 healthy human volunteers successfully completed the GRENaDE Study, of which 28 were randomly assigned to the Placebo group and 31 assigned to Indapamide. Similar to results from the GRENaDE Methods Study (Chapter 6), urinary sodium and potassium excretion increased in the first 24 hours on Indapamide treatment but generally returned to baseline after eight days. After Indapamide treatment, serum sodium and potassium were significantly reduced while serum urea, renin, and aldosterone were increased. These effects are consistent with the known saluretic action of Indapamide and subsequent effects on the renin–angiotensin–aldosterone system (RAAS) [35].

The blood pressure-reducing effects of thiazides can be separated into three temporal phases: 1) acute diuretic-induced reduction in fluid volume (1-2 weeks); 2) increasing influence of resistance factors with partial-to-full recovery of fluid volume (6-8 weeks); and 3) chronic reduced peripheral resistance (several months). After controlling for the effect of time generally, Indapamide treatment in this study did not alter blood pressure despite reduction in body water content. These results are not surprising, and are consistent with literature which suggests that Indapamide, like other diuretics, has no hypotensive effect in normotensive animals or healthy humans [141, 53, 135]. Therefore, any effects of Indapamide in these subjects can be considered pressure-independent.

In participants treated with Indapamide, TBW was reduced by ~1.5%, corresponding to a ~1 kg reduction in body weight and consistent with the acute action of diuretics noted above. As there was no similar reduction in skin water content, this loss was likely restricted to the extracellular fluid and decreased plasma volume. Such a reduction in plasma volume may be responsible for the noted reduction in cardiac output and stroke volume seen in these participants [35]. The significant reduction in HRV after diuretic treatment has been noted previously and is associated with reduced cardiovascular risk [151, 229].

Focusing on the skin, treatment with Indapamide was associated with a 9.4% reduction in sodium and 11.2% reduction in potassium per dry skin weight. This was found to be a significant reduction compared to pre-treatment baseline. Importantly, there was no commensurate reduction in skin water content, suggesting that the sodium, and indeed the potassium, which were lost were not “osmotically active.” These results suggest that non-osmotic storage does not discriminate between cations, similar to findings by Palatay et al in aortae [153].

Unlike in the salt-loaded VARSITY study participants, there was no change in the skin Na⁺:K⁺ ratio, and neither sodium nor potassium content were influenced by gender. The former is easily explained by noting that VARSITY only gave participants sodium, while in GRENaDE the diuretic affected both sodium and potassium.

It should be noted that the reductions in skin sodium and potassium seen in GRENaDE participants became nonsignificant when analysing all data together using a repeated-measures ANOVA with Tx as a Between-Subjects factor, although is likely an issue with
statistical power and sample size. Regardless, these results present a novel perspective on the topic of non-osmotic sodium storage in humans.

Previous studies in humans have been restricted to identifying non-osmotic sodium accumulation indirectly via dietary intervention and analysis of urinary sodium loss [197, 62]. Titze et al. first directly investigated this non-osmotic sodium accumulation in rats in 2002, and pointed to the skin as a the non-osmotic sodium reservoir in 2003 [199, 200]. They further connected this non-osmotic storage with salt-sensitive hypertension, finding reduced non-osmotic sodium storage capacity in hypertensive rat models.

Schafflhuber et al. built on this work in rats [175]. After four weeks of dietary salt restriction, total body water and skin sodium were reduced, but without concomitant reduction in skin water content. These results are remarkably similar to the human results of the GRENaDE Study, with decreased TBW and skin sodium, but no change in skin water content. Similar to Schafflhuber et al., it can thus be concluded that the skin sodium (and potassium) lost in GRENaDE participants was released from an osmotically inactive reservoir.

Based on the available data, it is difficult to accurately calculate the sodium buffering capacity of the skin, as it would require a number of substantial assumptions and approximations. However, some progress can be made if the data is analysed with respect to Nguyen-Kurtz formula:

\[
Na_{\text{serum}} = \frac{1.03 \times (Na_e^+ + K_e^+)}{TBW} - 23.8
\]

This formula, based on the Edelman equation [37, 91, 139], describes the relationship between serum sodium \( (Na_{\text{serum}}) \) and the amount of freely exchangeable (that is, osmotically active) ions \( (Na_e^+ \text{ and } K_e^+) \) per litre of water in the body. The Nguyen-Kurtz formula has previously been used to investigate osmotically inactive sodium storage in humans [145]. In 2016, Engberink et al. infused healthy volunteers with hypertonic saline and tracked the resultant changes in serum sodium and urine sodium. They found that only 55% of the sodium and potassium excretion predicted by the Nguyen-Kurtz formula could be recovered in the urine, implying that the remaining 45% was sequestered non-osmotically.

Using pre- and post-treatment \( Na_{\text{serum}} \) and \( TBW \) measurements from Indapamide-treated GRENaDE participants, the Nguyen-Kurtz formula predicts a 4.7% reduction in total body sodium and potassium. In these participants, average skin sodium concentration decreased by 9.4% and average skin potassium concentration by 11.2% - roughly double that predicted by the Nguyen-Kurtz formula if all sodium and potassium were osmotically active and freely exchangeable in the extracellular space. This supports the hypothesis of non-osmotic storage, and suggests that skin contributes disproportionally to diuretic-induced cation loss. However, an explicit estimation of the skin buffering capacity cannot be made from this data as there is no reliable way to relate changes in total body...
sodium to changes in skin sodium concentration. In future works, measurements of dietary sodium intake and urinary excretion would be useful when estimating the buffering capacity of non-osmotic storage.

As previously discussed, the mechanism of this osmotically inactive sodium storage is hypothesised to be an extensive network of GAGs in the extracellular matrix of the skin, where the large negative charge density of the GAGs attracts positively charged sodium ions. This ionic sequestering of the sodium inhibits its ability to attract water via osmosis, effectively creating a water-independent, osmotically-inactive storage mechanism. In this way, the skin is able to buffer body sodium. The prevailing hypothesis is that when dietary sodium increases, the skin produces more GAGs to accommodate the increase in sodium and minimise the negative effects of salt such as rises in blood pressure. Previous work has shown an increase in mRNA expression of GAG elongation enzymes during salt loading in rats [198] and humans [64].

A similar pattern was found in GRENaDE participants with Indapamide-induced salt loss. In these subjects, mRNA expression of the GAG elongation enzymes Chpf (chondroitin polymerising factor), Chsy1 (chondroitin sulfate synthase 1), and DSE (dermatan sulfate epimerase) were reduced, although only the reduction of Chpf reached statistical significance. It is possible that with a longer time-course or higher Indapamide dose, the reduction in Chsy1 and DSE would also reach significance, or this blunted reduction could reflect low baseline expression due to the generally healthy diets of GRENaDE participants. The lack of change in mRNA expression of other GAG-related enzymes suggests that sodium influences elongation of chondroitin sulfate and dermatan sulfate chains, but not heparan sulfate chains (Ext2), heparan sulfate sulfation (Glce), hyaluronan degradation (Hyal1), or attachment of GAG chains to proteoglycan backbones (Xylt1).

These results are supported by findings in rats. Rats fed a high salt diet has increased Chsy1 expression with no change in Xylt1 compared to rats on a low salt diet [198], as well as higher dermatan sulfate content and charge density [175]. Work in salt-loaded humans also showed no change in Xylt1 [64]. VegfC expression was likewise decreased, though no significantly, mirroring the increase in VEGF-C seen during salt-loading [178, 111, 201]. This is not surprising, as VEGF-C has been previously implicated in buffering of the haemodynamic response to salt loading through increased interstitial lymphatic drainage and expression of endothelial nitric oxide (NO) synthase.

Findings relating to hyaluronan and heparan sulfate were more complex. While Indapamide-treated GRENaDE participants saw no change in skin HYAL1 or Ext2 expression, work in salt-loaded humans induced an increase for both [64]. There are a number of possible explanations for this, including blood pressure-dependent expression rather than salt-dependent expression or low baseline expression in the generally healthy GRENaDE participants. Whatever the reason, these results can be considered complementary rather than contradictory.

Overall, the results of this study have shown that short-term treatment with 2.5 mg
of Indapamide can induce non-osmotic sodium loss from the skin. In turn, mRNA results suggest that this decrease in skin sodium curtails elongation of GAG chains in the skin in a pressure-independent fashion.

**Limitations**

The major limitations of outpatient studies such as this is the reliance on participants to accurately perform at-home measurements, stick to the prescribed diet, and take the study tablets as directed. Unfortunately it is prohibitively expensive to perform these studies in a completely in-patient setting, so this limitation is difficult to overcome.

A second limitation of this study was its restricted age range. Despite the protocol specifying an acceptable age range of 18 to 50 years, the majoring of volunteers were under the age of 35. This is a general consequence of the young student population of Cambridge, and use of the Graduate Union online board for advertising. Because of this tight range, the effects of aging could not be adequately studied. In the future, further recruitment could be targeted at older individuals to correct for this weighting towards the young.

A third limitation was the gender ratio. Females are generally more likely to volunteer for studies, and this study was skewed to 60% female. It may be worth aiming recruitment at men in the future, to try to balance this ratio, or at least prevent further skew.

**Future directions**

Continued recruitment into this study would be beneficial to improve the statistical power of the results. Furthermore, because only two of the participants in this study were black, it may be interesting to target future recruitment at black individuals given the known racial differences in salt handling and incidence of cardiovascular risk. This would be difficult in Cambridge, however, given the majority Caucasian population.

With respect to samples already collected, some skin remains for most participants. It would be interesting to measure sulphated GAG content in these skin samples to see how they compare to mRNA data. It would also be useful to analyse mRNA expression of GAG degradation enzymes and GAG sulfation enzymes to create a more complete picture of GAG remodelling in the skin.
Chapter 8

Quantification of skin sodium using $^{23}\text{Na}$ MRI: a pilot study

8.1 Background

Essential hypertension and cardiovascular risk have long been linked to disregulation of body sodium handling and the excessive sodium content in Western diets. However, recent estimations suggest that only 25-35% of people exhibit “salt-sensitive” blood pressure changes. Identification of salt-sensitive individuals and those with salt-sensitive hypertension would be a useful tool in targeting clinical counseling and antihypertensive therapy. With 40-50% of hypertensive patients considered salt-sensitive, sodium homeostasis is an obvious target for blood pressure control in this subpopulation.

Unfortunately, determining which patients are salt-sensitive is not straightforward. There is no clinical definition of salt-sensitivity, and the designation has largely been left up to individual research groups. Furthermore, determining whether an individual is salt-sensitive has thus-far required direct intervention, by tracking changes in blood pressure after salt-challenge. This makes identifying salt-sensitive individuals in a clinical setting difficult, and hinders administration of targeted therapies to these patients. It would be immensely useful if a biomarker of salt-sensitivity could be devised and implemented in clinical practice.

Developing a biomarker of salt-sensitivity requires understanding the mechanisms by which salt affects blood pressure, a topic that remains poorly understood. Historically, studies on salt and blood pressure relied on dietary intake or urinary excretion as indirect markers of total body sodium, but these metrics each have their own technical challenges and only provide information on sodium flux rather than sodium handling and pathophysiology. Use of direct analytical methods in sodium balance studies may be more useful.

In the GRENaDE Study, 5 mm skin biopsies from the lower back were used to directly quantify sodium changes before and after Indapamide-induced salt loss in humans (Chapter 7). Though direct analysis of skin biopsies was capable of detecting changes in
sodium content, its invasive nature limits its use in large-scale clinical trials. Development of a non-invasive technique would be ideal.

Recently, Titze et al. validated $^{23}$Na magnetic resonance imaging (MRI) as a non-invasive method for determining skin sodium content in vivo in man [32, 85]. $^{23}$Na MRI spectroscopy is a technique that has the ability to advance MR imaging beyond looking at protons ($^1$H) by incorporating tissue sodium ($^{23}$Na) signals. However, is complicated by the relatively low concentrations of $^{23}$Na nuclei in the body (as compared to H$_2$O used in $^1$H MRI) and the low gyromagnetic ratio of the $^{23}$Na nucleus, both of which substantially inhibit sensitivity and resolution. Modern technological advances have aimed at overcoming these limitations, and $^{23}$Na MRI is now being used to study disease states in living humans[58, 84, 85].

Despite this growing popularity, validation of $^{23}$Na MRI values against traditional biochemical sodium quantification techniques is lacking, and no studies currently exist which compare in vivo $^{23}$Na MRI to ex vivo biochemical analysis for the same tissue. Moreover, the sensitivity of $^{23}$Na MRI to physiologically modest changes in sodium content, common in pre-disease and modest disease states, is largely untested. To address these gaps in the literature, skin sodium in a subset of GRENaDE Study participants was assessed in vivo using $^{23}$Na MRI for comparison against ex vivo assessment of skin biopsies using ICP-OES.

Ultimately, we hope that the development of a non-invasive measurement of skin sodium will allow skin sodium to be incorporated into larger-scale clinical trials to investigate its viability as a biomarker of salt-sensitivity, as a way to study the mechanisms by which salt affects blood pressure, and as a means to create more personalised antihypertensive treatments for patients.

Hypothesis: There will be good correlation between in vivo $^{23}$Na MRI values and ex vivo ICP-OES analysis, and $^{23}$Na MRI will be able to detect Indapamide-induced reduction in skin sodium content in healthy human volunteers.
8.2 Methodology

8.2.1 GRENaDE Study

The GRENaDE Study has been discussed in detail previously (Chapter 7) and the study design is reproduced in Figure 8.1. Briefly, healthy volunteers 18-50 years old with no history of cardiovascular disease were asked to maintain a salt intake of 6 g or lower for two weeks. After an initial run-in period, participants were randomised to receive either the salt-depleting diuretic Indapamide or a placebo for eight days. Participants made a total of three visits to the Vascular Research Clinic at Addenbrooke’s Hospital (Cambridge, United Kingdom). At Visits 2 and 3, cardiovascular measurements and skin biopsies were performed. In a subset of GRENaDE Study participants, $^{23}$Na MRI imaging was also performed at Visits 2 and 3.

8.2.2 Skin $^{23}$Na MRI

MRI scanning was performed in Addenbrooke’s Hospital Department of Radiology (Cambridge, United Kingdom) by Dr. Frank Riemer and Dr. Mary McLean, with assistance from Dr. Josh Kaggi and Dr. Viknesh Selvarajah. Participants lay in a prone position for imaging using a clinical 3T system (GE MR750, GE Healthcare, Waukesha, WI) with a bespoke $^{23}$Na T/R coil of 2 cm diameter placed under the lower back on either the right or left side. The location of the coil corresponded to the location of subsequent skin biopsy.

Sodium imaging was performed using a 3D cones [54, 165] sequence with the following imaging parameters: echo time (TE) = 0.46 ms, repetition time (TR) = 100 ms, field of view (FOV) = 16 cm, nominal resolution 1 x 1 x 10 mm, 10 NEX, 14730 total readouts, 250,000 Hz full receiver bandwidth, 24.5 minutes acquisition duration. The imaging slab was positioned sagittally on the lower back. Sodium images were corrected for receive sensitivity [24]. A phantom replacement method was used to create a calibration curve against five NaCl in 4% agar phantoms varying between 7 and 160 mmol/L NaCl and this was applied to the in vivo data to create quantitative sodium maps [4]. Sodium images were reconstructed in Matlab 2016b (MathWorks, Natick, MA) using the Image Reconstruction Toolbox (IRT) and iterative density compensation [231]. Regions of interest were drawn in Matlab, care was taken to avoid partial volume with the air surface and deeper layers of tissue such as fat.

A total of two MRI scans were performed on each participant, one pre-treatment at Visit 2 and one post-treatment at Visit 3.

8.2.3 Skin biopsy

5 mm skin biopsies were taken after MRI scanning at Visits 2 and 3. Biopsies were taken from the same site as the MRI and performed post-MRI to prevent contamination from
Healthy human volunteers were asked to maintain a "medium salt diet" of less than 6g NaCl per day for 15 days.

After an initial seven-day run-in period, participants were treated with a lactose placebo or 2.5mg Indapamide for eight days. 24Na MRI, skin biopsies, Na MRI, skin biopsies, and cardiovascular measurements were performed before and after the treatment period (Visits 2 and 3). 24-hour urine collections were performed the day before Visit 2, first day of the treatment period, and the day before Visit 3. 24-hour ambulatory blood pressure measurements were performed the day before Visit 2 and the day before Visit 3.

Figure 8.1: GRENADE Study design.
healing-associated sodium accumulation. Skin biopsies were taken within 60 minutes following completion of the MRI.

Biopsies were performed as described in Section 2.1.1.8. Briefly, participants were asked to lie in a prone position, exposing the lower back. The site was cleaned and locally anaesthetised using custom Na-free lidocaine (Tayside Pharmaceuticals, Australia). Biopsies were taken using a 5 mm punch and immediately snap-frozen on dry ice. Sites were closed using two sutures and dressed with water-proof plasters.

A total of two biopsies were taken, one pre-treatment at Visit 2 and one post-treatment at Visit 3.

8.2.4 Skin Na\(^+\) quantification using ICP-OES

Biochemical analysis of skin Na\(^+\) content was performed as described in Section 2.3.1.2. Briefly, 5-10 mg of skin biopsy were rinsed with phosphate-buffered saline and blotted dry to remove residual blood, then freeze-dried for 24 hours. Dry samples were digested in 1:1 30\% H\(_2\)O\(_2\):69\% HNO\(_3\) for 24 hours at ambient temperature followed by 24 hours at 40\(^\circ\)C. Digests were then diluted 1:40 by weight with ultra high purity water and Na\(^+\) quantified in triplicated using inductively coupled plasma-optical emission spectroscopy (ICP-OES) at the MRC Elsie Widdowson Laboratory (Cambridge, United Kingdom).

8.2.5 Contributions of others

\(^{23}\)Na MRI scanning was performed in Addenbrooke’s Hospital Department of Radiology (Cambridge, United Kingdom) by Dr. Frank Riemer and Dr. Mary McLean, with assistance from Dr. Josh Kaggi and Dr. Viknesh Selvarajah. \(^{23}\)Na MRI image analysis and sodium quantification were performed by Dr. Frank Reimer.

8.2.6 Statistics

Sodium quantification techniques were compared using Spearman’s rank correlation. Values are presented as mean ± SD for repeat measurements on the same participant, and mean ± SEM for analysis of all participants together. A p < 0.05 was considered statically significant.
8.3 Results

Nine participants consented to MRI scanning, with pre- and post-treatment MRI scans and biopsies taken from each. Two MRI scans were corrupted and could not be analysed. ICP-OES and $^{23}$Na MRI skin sodium values for each participant can be seen in Table 8.1.

Coil positioning and MRI imaging can be seen in Figure 8.2. $^{23}$Na MRI quantification ($n=16$ scans) showed an average $\text{Na}^{+}$ concentration of $25.9 \pm 0.2 \text{ mmol/L}$ while ICP-OES quantification ($n=18$ biopsies) showed an average $\text{Na}^{+}$ concentration of $120.9 \pm 0.9 \mu g/\text{mg}$ wet tissue. When quantifying Na with respect to biopsy water content, $\text{Na}^{+}$ concentrations of $1.51 \pm 0.22 \text{ mmol/L}$ were found. There was poor correlation between $^{23}$Na MRI and either ICP-OES quantification ($r = 0.17$, $p > 0.05$ for both) (Figure 8.3A, B). However, when comparing the change in skin $\text{Na}^{+}$ content between Visit 2 and Visit 3 ($n=7$ pairs), there was significant correlation between $\Delta \text{Na}$ determined by $^{23}$Na MRI and $\Delta \text{Na}$ determined by ICP-OES ($r = 0.85$, $p = 0.018$) (Figure 8.3C).
Table 8.1: **ICP-OES** and **23Na MRI** quantification of skin sodium in nine healthy human volunteers

<table>
<thead>
<tr>
<th>ID</th>
<th>Treatment</th>
<th>Time point</th>
<th>ICP-OES (µg Na/mg wet tissue)</th>
<th>ICP-OES (mmol Na/L)</th>
<th>23Na MRI (mmol Na/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS021</td>
<td>Indapamide</td>
<td>pre-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>1.83 ± 0.05</td>
<td>122.5 ± 0.3</td>
<td>26.1</td>
</tr>
<tr>
<td>(M, 27)</td>
<td></td>
<td>post-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>1.76 ± 0.03</td>
<td>114.3 ± 0.2</td>
<td>20.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Difference (%)</strong></td>
<td>-3.8 ± 0.2%</td>
<td>-6.7 ± 0.3%</td>
<td>-21.1%</td>
</tr>
<tr>
<td>GS024</td>
<td>Indapamide</td>
<td>pre-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>1.93 ± 0.01</td>
<td>123.5 ± 0.5</td>
<td>26.9</td>
</tr>
<tr>
<td>(M, 25)</td>
<td></td>
<td>post-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>1.80 ± 0.04</td>
<td>116.9 ± 0.7</td>
<td>19.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Difference (%)</strong></td>
<td>-6.7 ± 0.3%</td>
<td>-5.3 ± 0.6%</td>
<td>-27.9%</td>
</tr>
<tr>
<td>GS034</td>
<td>Indapamide</td>
<td>pre-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>2.04 ± 0.08</td>
<td>126.8 ± 0.6</td>
<td>20.9</td>
</tr>
<tr>
<td>(M, 28)</td>
<td></td>
<td>post-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>1.88 ± 0.05</td>
<td>120.3 ± 0.6</td>
<td>corrupted</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Difference (%)</strong></td>
<td>-7.8 ± 0.4%</td>
<td>-5.1 ± 0.2%</td>
<td></td>
</tr>
<tr>
<td>GS041</td>
<td>Placebo</td>
<td>pre-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>2.23 ± 0.01</td>
<td>142.6 ± 0.6</td>
<td>25.8</td>
</tr>
<tr>
<td>(F, 21)</td>
<td></td>
<td>post-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>2.45 ± 0.02</td>
<td>166.5 ± 0.4</td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Difference (%)</strong></td>
<td>9.9 ± 0.2%</td>
<td>16.7 ± 0.3%</td>
<td>4.7%</td>
</tr>
<tr>
<td>GS044</td>
<td>Indapamide</td>
<td>pre-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>2.04 ± 0.09</td>
<td>125.0 ± 0.3</td>
<td>28.6</td>
</tr>
<tr>
<td>(M, 26)</td>
<td></td>
<td>post-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>1.91 ± 0.02</td>
<td>120.4 ± 0.5</td>
<td>corrupted</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Difference (%)</strong></td>
<td>-6.4 ± 0.4%</td>
<td>-3.7 ± 0.3%</td>
<td></td>
</tr>
<tr>
<td>GS051</td>
<td>Indapamide</td>
<td>pre-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>1.74 ± 0.03</td>
<td>111.3 ± 0.2</td>
<td>25.2</td>
</tr>
<tr>
<td>(M, 24)</td>
<td></td>
<td>post-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>1.93 ± 0.04</td>
<td>121.7 ± 0.07</td>
<td>29.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Difference (%)</strong></td>
<td>10.9 ± 0.2%</td>
<td>9.3 ± 0.4%</td>
<td>15.1%</td>
</tr>
<tr>
<td>GS053</td>
<td>Placebo</td>
<td>pre-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>1.81 ± 0.02</td>
<td>114.1 ± 0.3</td>
<td>29.3</td>
</tr>
<tr>
<td>(F, 31)</td>
<td></td>
<td>post-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>1.82 ± 0.02</td>
<td>119.9 ± 0.5</td>
<td>29.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Difference (%)</strong></td>
<td>0.6 ± 0.1%</td>
<td>5.1 ± 0.3%</td>
<td>0.7%</td>
</tr>
<tr>
<td>GS055</td>
<td>Indapamide</td>
<td>pre-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>1.66 ± 0.05</td>
<td>101.7 ± 0.8</td>
<td>26.4</td>
</tr>
<tr>
<td>(F, 23)</td>
<td></td>
<td>post-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>2.06 ± 0.06</td>
<td>129.9 ± 1.1</td>
<td>28.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Difference (%)</strong></td>
<td>24.1 ± 0.3%</td>
<td>27.7 ± 0.9%</td>
<td>7.6%</td>
</tr>
<tr>
<td>GS066</td>
<td>Indapamide</td>
<td>pre-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>1.59 ± 0.04</td>
<td>104.8 ± 0.7</td>
<td>29.7</td>
</tr>
<tr>
<td>(F, 40)</td>
<td></td>
<td>post-T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>1.51 ± 0.02</td>
<td>93.8 ± 0.8</td>
<td>21.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Difference (%)</strong></td>
<td>-5.0 ± 0.4%</td>
<td>-10.5 ± 0.7%</td>
<td>-27.3%</td>
</tr>
</tbody>
</table>

F = female, M = male, T<sub>x</sub> = treatment
Figure 8.2: $^{23}$Na MRI scanning in GREnaDE study participants. (A) Participants lay in a supine position, with the $^{23}$Na MRI coil placed under the lower back on either the right or left side. A fiducial marker was used for MRI localisation. (B) Overlay of traditional proton MRI and $^{23}$Na MRI in participant GS021. (C) High resolution close-up of $^{23}$Na MRI signal density in participant TS021.
Figure 8.3: Correlation of skin Na\(^+\) content determined by \(^{23}\textrm{Na}\) MRI and ICP-OES. (A) Absolute skin Na\(^+\) quantification in the lower back as determined by \(^{23}\textrm{Na}\) MRI in living study participants and ICP-OES analysis of biopsies. (B) Change in skin Na\(^+\) in the same participant before and after Indapamide or placebo treatment using \(^{23}\textrm{Na}\) MRI or ICP-OES. Spearman’s rank correlation coefficients are presented for each.
8.4 Discussion

In this pilot study, the feasibility of measuring skin sodium content using non-invasive \(^{23}\)Na MRI was evaluated by comparison with ICP-OES quantification. It was found that \(^{23}\)Na MRI could successfully detect changes in skin sodium in a similar fashion to direct quantification using ICP-OES. However, there was a large discrepancy in absolute sodium content as determined by MRI vs ICP-OES, with MRI values fully 80% lower than ICP-OES values. This suggests that while both techniques are internally consistent, but they cannot be directly compared to each other.

In 2012, Kopp et al. published similar comparisons [85]. Crucially, however, MRI scanning was performed \textit{ex vivo} on amputated leg slices rather than \textit{in vivo} in living subjects. After scanning, skin from each leg slice was ashed and directly quantified using atomic absorption spectroscopy (AAS). As in this work, they found lower in \(^{23}\)Na MRI values (15–65 mmol/L) compared to AAS quantification (50–105 mmol/L). Unlike in this study, however, they found good correlation between methods (\(r = 0.90\)).

When comparing this study to the Kopp et al. (2012) work, of primary note is the use of amputated limb slices for MRI rather than living humans. Because blood is generally drained from a limb prior to amputation, there is a risk of changes in fluid balance and hydration state of the tissue. This is particularly important because the group calculated sodium with respect to tissue water content (mmol/L). This may at least partially explain the difference in \(^{23}\)Na MRI values between our \textit{in vivo} and their \textit{ex vivo} measurements (19.4–29.7 mmol/L vs 15–65 mmol/L). Indeed, later in the same paper Kopp et al. present \textit{in vivo} skin \(^{23}\)Na MRI values in the leg (14–33 mmol/L) which are more in line with our study. This lower range of \textit{in vivo} values was subsequently reproduced by the same group in a later publication [84]. While some allowance must be made for differences in patient demographics, these results suggest that \(^{23}\)Na MRI produces higher quantification in \textit{ex vivo} tissue (amputated limbs) compared to \textit{in vivo} tissue.

Building on this, it is suggested that while MRI of \textit{ex vivo} skin and biochemical analysis of \textit{ex vivo} skin may be comparable, perhaps MRI of \textit{in vivo} skin and biochemical analysis of \textit{ex vivo} skin is not. MRI produces lower sodium values than biochemical analysis even when controlling for \textit{in vivo} tissue hydration. The reasons for these lower values are not immediately clear. However, it may be related to the concept of “bound” and “unbound” sodium, which has previously been discussed in the context of sodium storage in the skin (Chapter 1). In this context, sodium accumulates in the skin without commensurate water via non-osmotic “binding” to anionic glycosaminoglycans (GAGs). This “binding” may disrupt MRI quantification by partially shielding the signal. Some work from the 1970s supports this hypothesis. Cornelis and Laszlo described and demonstrated that interaction of sodium with \textit{ex vivo} biological molecules causes partial desolvation of \(\text{Na}^+\), which can be detected using \(^{23}\)Na MRI [30, 95]. Scaled up to an actual tissue, it is possible that these interactions could account for the reduced sodium values found when assessed using MRI compared to biochemical methods. Further, removal of a tissue from the body
may disrupt some of these interactions, and account for the higher $^{23}\text{Na}$ MRI content of \textit{ex vivo} skin compared to skin measured \textit{in vivo}.

It is vital to understand the limitations of sodium quantification using $^{23}\text{Na}$ MRI as it becomes more popular for scientific and clinical use. Because its primary appeal is non-invasive evaluation of disease states, such limitations may obfuscate differences between groups and limit the value of one-off measurements in individuals. However, measuring changes in an individual’s response to treatment or as a disease progresses may be a more viable use.

**Future work**

Expansion of $^{23}\text{Na}$ MRI data in GRENaDE participants and the sensitivity of $^{23}\text{Na}$ MRI sodium binding in \textit{in vivo} and \textit{ex vivo} skin must be pursued both to obtain a better understanding of the limitations of $^{23}\text{Na}$ MRI and as a potential tool for investigating non-osmotic sodium storage and its relationship with GAGs. Further works by this group aim to increase the sample size of GRENaDE participants studied, as well as investigate the influence of Na-specific ionophores on $^{23}\text{Na}$ MRI quantification, including GAGs. In this way, the capabilities of $^{23}\text{Na}$ MRI can be further explored as well as the ability of GAGs to bind sodium and influence the progression of hypertension and cardiovascular disease.

Once validated, we ultimately hope to include $^{23}\text{Na}$ MRI in large-scale clinical trials as a means to study salt-sensitive hypertension and as a tool for improving patient treatment and prognosis.
Chapter 9

Thesis conclusions

The goal of this thesis was to investigate the interplay between sodium, glycosaminoglycans (GAGs), vascular stiffness, and hypertension. More specifically, we wanted to explore 1) the ability of GAGs in the skin to bind sodium and store it non-osmotically, and how this was regulated by dietary salt intake; and 2) to what extent and by what mechanism do GAGs in the walls of large elastic arteries such as the aorta influence blood pressure (or are influenced by blood pressure), and whether arterial GAG remodelling can also be regulated by dietary salt.

The influence of dietary salt on blood pressure (BP) has been well-documented, but the mechanism of this influence is poorly understood. The traditional model, wherein additional body sodium attracts commensurate water and subsequently increases extracellular fluid volume, has been summarily challenged by studies showing non-osmotic sodium accumulation [197, 62, 55]. Furthermore, growing evidence in the last century has suggested that this salt-BP relationship is not ubiquitous, but rather affects only one third of the human population [55, 210, 209, 143]. This salt-sensitivity is higher in certain racial and family groups, indicating a genetic basis, but the exact cause remains unknown [183, 25, 108, 29].

Work in rat models has begun to suggest that, to some extent, such salt-sensitive BP changes relate to an individual’s ability to buffer free body sodium content [199, 164]. In this model, poly-anionic GAG molecules are responsible for sequestering positively-charged sodium ions without commensurate water accumulation, preventing salt-dependent changes in vascular stiffness and BP. When dietary salt intake is increased, GAG content in the skin increases to compensate. Or, in the reverse scenario, decreased salt intake will cause a fall in skin GAG content. In this way, a deficiency in this skin GAG remodelling process could cause someone to be salt-sensitive as their buffering mechanism is impaired.

A recent study by this group, the VARSITY Study, found an increase in the skin Na\(^+\):K\(^+\) ratio of humans who were salt-loaded for one week [178]. Unfortunately, the contributions of non-osmotic sodium storage and GAGs to this process were not investigated. A similar study by Heer et al found increased expression of mRNA coding for GAG synthetic enzymes in the skin after salt-loading, implicating skin GAGs in salt-handling in
humans [64]. However, they also did not directly investigate non-osmotic sodium storage.

Our work in the GRENaDE Study has filled this gap. We showed that when healthy humans are treated with a diuretic, sodium is lost from the skin. More importantly, there was no commensurate water loss, suggesting that this sodium was released from an osmotically inactive reservoir. We further showed a concomitant decrease in mRNA expression for GAG synthetic enzymes, as predicted if GAGs are responsible for managing this non-osmotic reservoir. In particular, chondroitin sulfate and dermatan sulphate seem to be more directly involved than hyaluronic or heparan sulfate. Interestingly, pilot work in this study suggests that the exact nature of the “binding” of sodium to GAGs could be probed using $^{23}$Na MRI, and illuminated the limitations of $^{23}$Na MRI for use as a non-invasive measure of skin sodium content. Finally, because the diuretic did not alter BP in our participants, we can conclude that these changes are salt-dependent rather than being driven by a diuretic-induced decrease in BP.

A similar salt-dependence of skin GAG synthesis was seen in salt-loaded rats, although the specific nature of these changes was unexpected. Nevertheless, this work in rats suggested that there may also be an additional pressure-dependent component to skin GAG regulation. It also brought to the forefront a major limitation of existing work, which is a neglect to study GAG degradation in addition to synthesis. The influence of salt and pressure on GAG degradation should be a topic of future work.

This rat work also probed the nature of salt-induced vascular stiffening and provided a look at whether dietary salt can induce arterial GAG remodelling as it does skin GAG remodelling. We found that PWV in rats was salt-dependent but pressure-independent, supporting existing literature which suggests that salt-induced increases in PWV precede changes in BP [228, 134, 65]. PPA, meanwhile, had both a salt-dependence and a pressure-dependence, again in agreement with work by other groups [140, 149, 173, 174, 203]. However, these changes in in vivo measurements of stiffness did not carry over to ex vivo tensile testing, suggesting that no physical remodelling had occurred. This is not to suggest that chronically high salt exposure does not increase stiffness through vascular remodelling, either directly or indirectly, but that none was seen in the scope of this study. This study further suggested that, in contrast to skin GAGs, arterial GAG content was not influenced by dietary salt intake or BP. Finally, it was found that aortic GAG content was not correlated with ex vivo aortic stiffness.

This GAG-independent ex vivo stiffness was further seen in human aortae. In this collection of 199 aortae from organ donors, there was no correlation between aortic GAG content and ex vivo aortic stiffness. However, hypertension was correlated with GAG redistribution. In aortae from hypertensive donors, there was a clearly delineated build-up of GAGs along the media-intima border, although the consequences of this are unclear.

In conclusion, we have shown in this thesis evidence that GAGs mediate osmotically-inactive sodium storage in human skin, and that the quantity of skin GAGs is regulated by dietary salt intake. We have also provided evidence that there may be an additional
pressure-dependent component to skin GAG synthesis. In contrast, arterial GAG content is salt-independent and pressure-independent, although chronic hypertension may cause redistribution of arterial GAGs. The effect of the distribution are unknown, but absolute GAG content does not appear to affect vascular stiffness.
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