Sodium-induced changes of the endothelial surface layer and microcirculation

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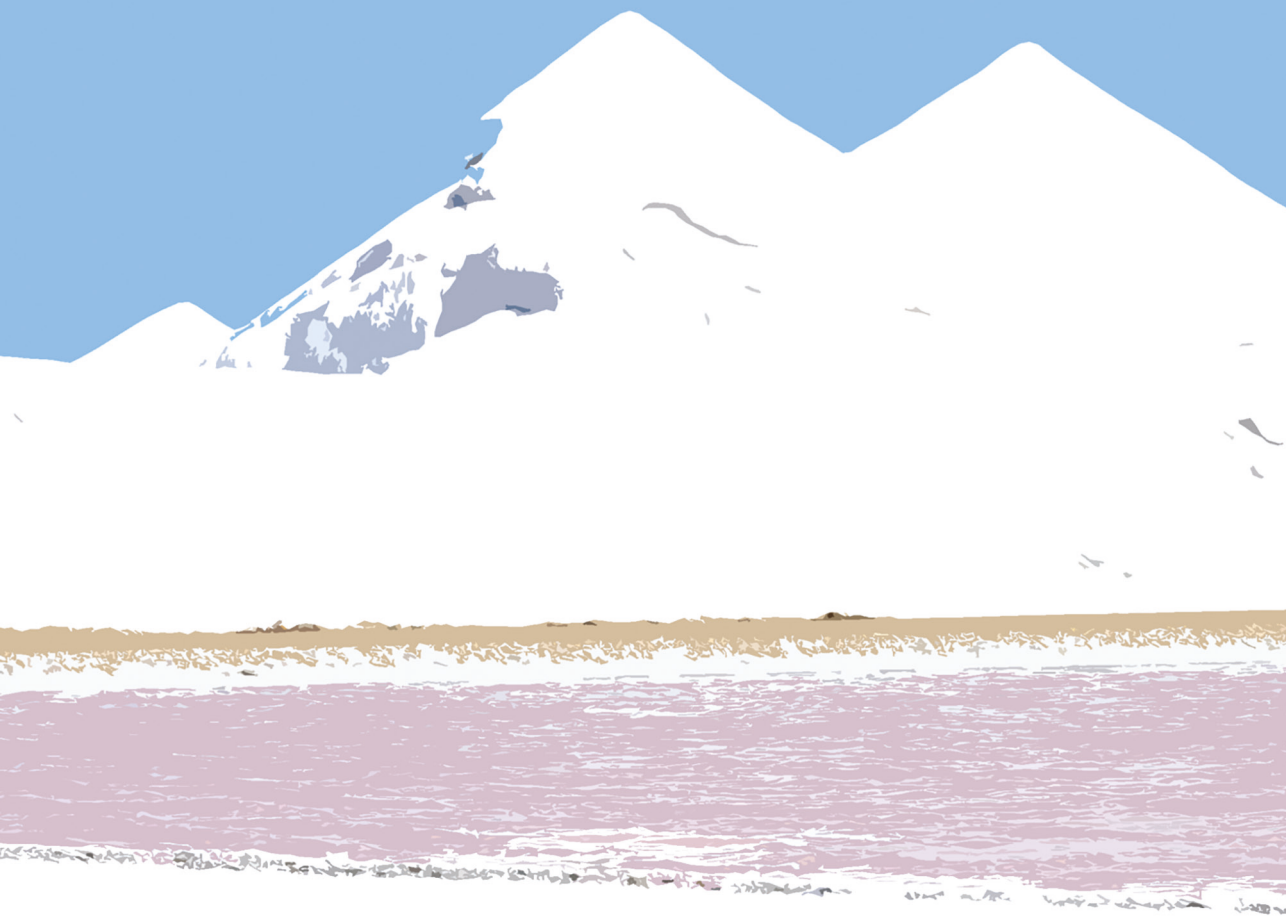

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Sodium-induced changes of the endothelial surface layer and microcirculation

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COLOFON

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Voor mijn ouders

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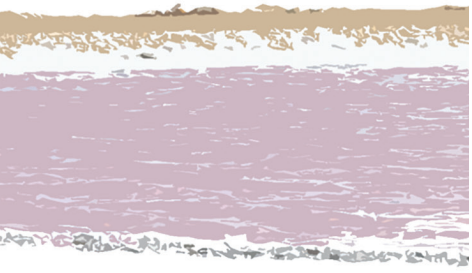

1 General Introduction and Outline

GENERAL INTRODUCTION

Sodium

Sodium is an alkali metal that was isolated by Sir Humphry Davy in 1807 using electrolysis of caustic soda (NaOH)[1]. Due to its highly reactive state, sodium is always found as a compound. Its combination with chlorine as sodium chloride (NaCl, commonly known as salt) is the most abundant form, which is usually extracted from the sea, salt lakes, brine springs or present in mineral form as rock salt.

Salt has influenced human history in many ways and has affected various domains including the arts, religion, culture, economics and also medicine. Both positive and negative associations regarding salt have been captured. Salt was once considered to be so valuable that it served as currency and it is one of the first products that was used for taxing purposes. The latin words for health (salus) and salary (salarium) are derived from the word for salt (sal). Many cities are named after their main source of income (e.g. Salzburg, Austria)[2]. Salt has also been a cause of conflict: wars have been fought over salt and dissatisfaction regarding salt taxes was one of the contributing factors to the French Revolution[3]. Throughout history, salt has been thought to have positive medicinal effects; it was found to be effective in reducing swelling and preventing decay. It was used as an antiseptic substance and thought to be beneficial for fertility and reproduction[2]. Nowadays, this view has largely been abandoned and more harmful health influences of sodium are topics of interest.

Today, dietary sodium comes from sources as table salt, sodium bicarbonate (soda) or monosodium glutamate (MSG). Back in the days of hunter-gatherers the main source of dietary sodium was meat and daily salt intake was estimated <1g/day. In our current highly industrialized society, the majority of sodium in our diet originates from processed foods[4] and worldwide mean daily sodium intake is ≈ 4 g/day, equivalent to ≈10 g/day of salt[5]. Another source of sodium for humans is intravenously admitted fluids. Sodium chloride solution has different applications. It can be used as a resuscitation fluid in order to replace or replenish fluid deficits due to for example blood loss or diarrhea, as a carrier solution for drugs, or as a flushing liquid.

The role of sodium in health and disease is still not fully clarified and continues to be an interesting and intriguing topic of research.

Sodium, hypertension and cardiovascular disease

Sodium is the principal cation in the extracellular volume and therefore predominantly determines the regulation of plasma osmolality and effective circulating volume[6]. In the classical, two compartment view of sodium and volume homeostasis, sodium intake leads to increase in blood pressure due to a renal inability to excrete a surplus of sodium. This contributes to an expansion of the extracellular fluid volume leading to increased cardiac output and subsequently higher blood pressure[7,8].

In epidemiological studies high sodium intake is associated with high blood pressure[9] and increased risk of cardiovascular events[10]. A modeling study estimated that globally 1.65 million deaths from cardiovascular causes (about 10% of all cardiovascular mortality) could be attributed to sodium consumption of more than 2 g/day[11]. Vice versa, reduction of sodium intake is associated with reduced blood pressure in both normotensive and hypertensive individuals, resulting in a decreased risk of cardiovascular disease[12,13]. These observations have led to lifestyle recommendations by the World Health Organization (WHO) to limit dietary sodium intake to a maximum of 2 g per day[14]. Reduction of sodium intake has been recognized as a target for improvement of global health and lowering of medical costs[15]. However, this target has not been achieved by any country[16]. There is a vivid scientific debate going on whether this maximum of 2 g/day is the optimal sodium intake with regards to reduction in renal and cardiovascular events. Data from current available studies[7,13-30], mainly prospective cohort studies and meta analyses, provide conflicting evidence. Some scientists have even challenged the rationale for sodium intake reduction all together[31].

So far, however, pathophysiological mechanisms of the association between sodium and high blood pressure have not been fully clarified. The microcirculation is important for blood pressure regulation[32]. Microcirculatory abnormalities are associated with high blood pressure and end organ damage[33]. As sodium is also known to cause microcirculatory alterations[34], these sodium associated microcirculatory changes might explain the link between sodium and high blood pressure, besides currently known factors such as kidney function. Still, limited data is available on the interaction between microcirculation, sodium and blood pressure in humans. This thesis aims to provide more insight into this interaction.

Microcirculation

Definition and function

The microcirculation is often defined morphologically and is therefore considered to consist of vessels with a diameter <150 μm, including arterioles, capillaries and venules. Taking physiological aspects into account, arterial vessels with a diameter of 150-300 μm that respond to increasing pressure by myogenic reduction in lumen diameter are also included in definition of the microcirculation[32]. A major function of the microcirculation is transportation and exchange of essential fluids, solutes and gasses to tissue cells. The capillaries (< 10 μm in diameter) are the principal site of this exchange. In order to maintain sufficient blood pressure levels for this capillary exchange, the microcirculation is also important in blood pressure regulation as peripheral vascular resistance is mainly determined by small arteries (150-300 μm in diameter) and arterioles (10-150 μm in diameter)[35].

Endothelial surface layer

The luminal side of the microcirculation is covered with the endothelial surface layer (ESL), a dynamic layer consisting of membrane bound proteoglycans, glycosaminoglycans and plasma proteins. The main glycosaminoglycan components of the ESL are heparan sulfate, chondroitin sulfate and hyaluronan.[36]. This layer has an estimated thickness between 0.4 – 1.5 μm and is in continuous interaction with the flowing blood, influencing both its composition and thickness[36,37]. The major functions of the ESL are regulation of vascular permeability, control of coagulation, fibrinolysis and inflammatory responses[36,37]. Furthermore the ESL operates as a regulator of vascular tone due to its mechano-shear sensing capacities and response to shear stress, through nitric oxide (NO) release and cytoskeletal adjustments[38].

Microvascular and endothelial surface layer changes and blood pressure

Microvascular alterations, such as rarefaction, remodeling and endothelial dysfunction, are known to be associated with high blood pressure[32]. Microvascular rarefaction is defined as an abnormal reduction of spacial density of microvessels[32,39] and can either be functional, structural, or both. Structural rarefaction refers to anatomic absence of microvessels, whereas in functional rarefaction microvessels are present but without perfusion. Microvascular rarefaction can either be the result of hypertension, but may in turn also cause hypertension by increasing peripheral vascular resistance and reduction of blood flow[35]. Structural modifications of small resistance arteries and arterioles, resulting in a decrease of lumen diameter and thus leading to increased systemic vascular resistance and elevated blood pressure[40], are known as remodeling.

Endothelial dysfunction, characterized by abnormalities in endothelium-derived factors such as NO, is also present in hypertension[41]. The etiology of the association between impaired NO bioactivity and hypertension is complex, and has not been fully elucidated[42]. A damaged ESL has been found in different disease states that are characterized by blood pressure alterations and increased vascular permeability[43], including kidney disease[44,45], diabetes[46,47], atherosclerosis[48], inflammation[49] and hypervolemia[50].

Assessment of microcirculation and ESL in humans

Non-invasive assessment of human microcirculation is limited to epi-illumination of superficial tissues, including skin, sublingual mucosa, bulbar conjunctiva and retina[51]. Different techniques have been developed to visualize microcirculation, including nailfold videocapillaroscopy (NVC)[52], orthogonal polarization spectral (OPS)[53] and sidestream dark field imaging (SDF)[54], laser doppler flowmetry[55], and retinal vascular imaging[56]. Most of these techniques have proven to be useful, but have their limitations in assessing microvascular function and technological constraints.

Assessment of microvascular function and the ESL in humans is even more challenging. Transcapillary escape rate of ^{125}I-labeled albumin is a method to quantify microvascular permeability[47,57,58]. In vivo visualization and functional measurement of the ESL are difficult to perform in humans due to its highly dynamic and delicate structure[59]. Indirect measurements, such as measurement of shedding products, urinary and plasma glycosaminoglycans (constituents of the ESL), are used to quantify ESL damage [50,60,61]. With the use of SDF imaging with integrated software, one can measure perfused boundary region (PBR), considered to reflect ESL thickness. The perfused boundary region includes the accessible part of the ESL for erythrocytes, and an increase in perfused boundary region is thought to reflect a decrease in ESL dimension[44,62,63].

However, interpretation of these results is complicated, as location, timing and mechanism of shedding are generally unknown[59] and the automated measurement of PBR with SDF imaging has not been validated against other methods of ESL dimension assessment[62].

New emerging concepts in sodium homeostasis

Nonosmotic sodium storage

In the beginning of the 21st century the classical two compartment view of sodium and volume homeostasis has been challenged by long-term sodium balance studies. A new concept of nonosmotic sodium storage was introduced. Heer *et al* demonstrated that increased sodium intake results in increased plasma volume but not in weight gain or expansion of the extracellular volume[64]. A consecutive long-term (135 days) space simulation study revealed that healthy volunteers were able to gain more sodium than weight, suggesting their possibility to store sodium in an osmotically inactive form (i.e. without water retention)[65]. Animal studies confirmed this mechanism of nonosmotic sodium storage[66,67]. Glycosaminoglycans, mainly present in the skin, were identified to be capable of storing sodium in an osmotically inactive form[68]. Following high dietary sodium intake in rats excessive high skin sodium concentrations were found, which concurred with increased interstitial glycosaminoglycan content, polymerization and sulfation[68]. This new concept of nonosmotic sodium storage does not only challenge traditional views on sodium and volume handling but may also provide new insights regarding diagnostics and therapeutics involving sodium homeostasis.

Sodium-sensitive hypertension

Blood pressure responses to sodium are heterogeneous; some humans respond to increased sodium intake with higher blood pressure, while others may not, traits also known as salt-sensitivity and salt-resistance. Conditions associated with sodium-sensitive hypertension include African-American ethnicity, low-renin hypertension, obesity, older age and the metabolic syndrome[69,70]. Mechanisms of sodium-sensitive hypertension are not well understood and long-established views have also been questioned by recent studies. In contrast to the autoregulation theory[7,8], a vasodysfunction mechanism has been proposed as the underlying mechanism of sodium-induced hypertension[71]. An abnormal response following sodium loading with failure to normally decrease systemic vascular resistance initiates the series of events resulting in sodium-sensitive hypertension. Laffer *et al* have demonstrated that sodium-sensitive subjects are unable to adjust vascular tone in response to either salt loading or salt depletion and link this inability to possible differences in interstitial sodium and water handling[72].

And a whole new salt shaker full of research questions has been opened…

OUTLINE OF THIS THESIS

The main focus of this thesis is to evaluate the influence of sodium loading (either by diet or infusion) on the microcirculation and endothelial surface layer to improve our understanding of sodium induced microcirculatory changes in relation to sodium-mediated blood pressure changes.

In **CHAPTER 2** the concept of nonosmotic sodium storage and its implications on sodium homeostasis are discussed, with a special focus on the role of the endothelial surface layer. We propose that the endothelial surface layer can serve as an intravascular buffer for sodium storage without osmotic effects on extracellular volume. Due to its presumed function in sodium homeostasis the endothelial surface layer may serve as a target for therapy in treatment of high blood pressure and in conditions with expanded extracellular volume.
CHAPTER 3 includes the results of our study that researches the contribution of nonosmotic sodium storage in healthy volunteers following intravenous sodium loading. We compare the observed changes in plasma sodium and urinary cation excretion with the expected values as calculated with the Adrogue-Madias and Nguyen-Kurtz formulas.
CHAPTER 4 describes our in vivo study of the sublingual microcirculation in healthy volunteers following an increase of dietary sodium. We assess whether high salt intake decreases sublingual microvascular density in normotensives and determine the influence of body weight on microvascular density changes.
The effects of acute intravenous sodium loading and chronic dietary sodium loading on microvascular permeability and the endothelial surface layer are discussed in **CHAPTER 5.** We hypothesize that an acute intravenous sodium load and chronic dietary sodium load differently impact the endothelial surface layer, microcirculation and blood pressure.
Furthermore we assess whether restoration of the endothelial surface layer with sulodexide, a highly purified mixture of glycosaminoglycans, decreases blood pressure.
CHAPTER 6 describes a systematic review and meta-analysis of eight studies including 3019 subjects concerning the antihypertensive effects of sulodexide.

Finally, **CHAPTER 7** summarizes the results of the studies described in this thesis and puts them into perspective. **CHAPTER 8** includes a Dutch Summary.

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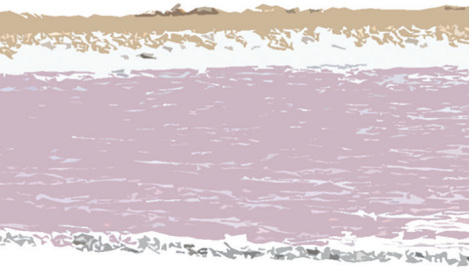

2 Role of the Vascular Wall in Sodium Homeostasis and Salt Sensitivity

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ABSTRACT

Excessive sodium intake is associated with both hypertension and an increased risk of cardiovascular events, presumably because of an increase in extracellular volume. The extent to which sodium intake affects extracellular volume and BP varies considerably among individuals, discriminating subjects who are salt-sensitive from those who are salt-resistant. Recent experiments have shown that, other than regulation by the kidney, sodium homeostasis is also regulated by negatively charged glycosaminoglycans in the skin interstitium, where sodium is bound to glycosaminoglycans without commensurate effects on extracellular volume. The endothelial surface layer is a dynamic layer on the luminal side of the endothelium that is in continuous exchange with flowing blood. Because negatively charged glycosaminoglycans are abundantly present in this layer, it may act as an intravascular buffer compartment that allows sodium to be transiently stored. This review focuses on the putative role of the endothelial surface layer as a contributor to salt sensitivity, the consequences of a perturbed endothelial surface layer on sodium homeostasis, and the endothelial surface layer as a possible target for the treatment of hypertension and an expanded extracellular volume.

In Western society, average daily intake of salt is 8–12 g, thereby greatly exceeding the recommended amount by the World Health Organization of 5 g daily[1,2]. This recommendation is on the basis of the observation that dietary salt intake exceeding 5 g/d, which is equivalent to 2 g or 85 mmol sodium, is associated with hypertension and increased cardiovascular risk in many cohort studies[3, 4]. Other than negative effects on cardiovascular morbidity and mortality, high salt intake has also been related to intermediate end points for kidney damage, such as proteinuria, in both patients with CKD and the general population[5,6]. Dietary salt restriction is, therefore, regarded as an important target for improvement of global health[4]. For example, in the United States, it has been estimated that a reduction of dietary salt intake by 3 g/d would reduce annual health costs by $10–$24 billion[7].

Generally, detrimental effects of excessive sodium intake have been linked to expansion of extracellular volume (ECV) and hypertension, which is evidenced by various observations that low sodium reduces BP in both normotensive individuals and individuals with hypertension[4,8]. The increase in BP after dietary sodium excess is highly variable, with some individuals showing a relatively small increase, whereas large BP increases can be observed in others[9, 10]. It is likely that these individual variations in salt sensitivity differentially affect cardiovascular and renal risk and may also explain the inconsistent results from population studies investigating the relation between sodium intake and cardiovascular risk[11].

According to Guyton's pressure–natriuresis curve, the kidney regulates long-term BP by altering renal sodium excretion in response to variations in sodium intake. When renal sodium excretion capacity is limited by intrarenal or extrarenal factors, this will result in an increase in BP, which in turn, increases renal sodium excretion at the expense of a higher BP (i.e., salt sensitivity)[12]. The pathophysiology of the differential BP response to a salt load has not yet been fully elucidated[13]. Recent studies have shown that, other than mechanisms that directly or indirectly influence renal sodium excretion, an extrarenal compartment exists in the skin interstitium, where glycosaminoglycans (GAGs) bind and inactivate sodium (i.e., nonosmotic sodium storage)[14]. The endothelial surface layer (ESL), also containing these GAGs, may be another compartment involved in nonosmotic storage of sodium with important implications for ECV and BP regulation.

Nonosmotic sodium storage

On the basis of the intracellular and extracellular sodium concentrations and the distribution of total body water (42 L in an average 70-kg man), it is classically thought that about 1960 mmol sodium (140 mmol/L; 14 L) is present in the extracellular compartment and 336 mmol sodium (12 mmol/L; 28 L) is present in the intracellular

compartment. The intravascular compartment representing one third of the ECV contains approximately 653 mmol sodium in 4.7 L. The notion that a significant proportion of sodium is nonosmotically stored has dramatically changed this view. The evidence for nonosmotic sodium storage has come from space simulation programs that have monitored electrolyte intake and excretion in healthy subjects for extended periods of time. In the first study, the difference between net sodium intake and excretion increased to 2973–7324 mmol after 135 days of normal dietary sodium intake diet (160 mmol sodium/d) without any change in total body water[15]. A second study in individuals on a long-term stable sodium diet showed changes in total body sodium (−200 to +200 mmol) that were not related to changes in ECV or body weight, advocating the presence of a clinically relevant buffer where sodium can be nonosmotically stored[16]. The capacity for nonosmotic sodium storage has been shown for various tissues, such as skin, cartilage, bone, and muscle[14, 17-20]. Studies using ^{23}Na magnetic resonance imaging and conventional ^{1}H magnetic resonance imaging have revealed that, in both patients with primary hyperaldosteronism and patients with essential hypertension, considerable amounts of sodium are stored in muscle and skin without commensurate water retention[19, 20].

Experiments in both mice and rats have identified that GAGs in the skin interstitium are responsible for sodium storage[14, 17]. GAGs are large, negatively charged linear polymers consisting of disaccharide unit repeats. Specific combinations of these repeating units result in different types of GAGs, such as heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate, and hyaluronan, which have reviewed elsewhere[21]. The negative charge density of GAGs is determined by their sulfation grade[22]. In rats, high dietary sodium intake has been shown to coincide with increased interstitial GAG content as well as increased polymerization and sulfation of these GAGs, with skin sodium concentrations (180–190 mmol/L) increasing to values far exceeding plasma sodium concentrations under these conditions[14, 17]. Because these high extracellular sodium concentrations were not accompanied by extracellular water retention, it was proven that a substantial part of the measured sodium has to be stored osmotically inactive. The concept that GAGs have the capacity to bind and osmotically inactivate sodium is not new. In 1957, Farber et al. [23] dialyzed sodium chondroitin sulfate against sodium chloride at various concentrations in an in vitro experiment. After 24 hours, higher sodium concentrations were consistently found in the chondroitin sulfate compartment compared with sodium concentrations outside this compartment, whereas chloride was equally distributed among both compartments[23]. These results indicate that a part of the sodium present in the chondroitin sulfate solution was not ionized but inactivated by this GAG, because it did not exert the expected osmotic effect[23].

From an evolutionary point of view, genes involved in GAG sulfation are highly conserved[24]. GAG sulfation degree does not increase in higher animals. In general, aquatic species contain a more structural variety in their GAGs than terrestrial animals, and the degree of sulfation increases as a function of the salt content of the environment of the organism[24]. Because interactions between components of the extracellular matrix seem to occur at higher salt concentrations in marine invertebrates than vertebrates, GAGs with increased charge density may be needed for keeping the intracellular environment stable, indicating that the more a subject is exposed to sodium, the more sulfated GAGs may be required[24, 25]. In humans, highly sulfated heparan sulfates are mainly found in tissues having a barrier function, including skin, lung, intestine, and endothelium.

ESL function

The ESL is a dynamic layer on the luminal side of the endothelial cell that is in continuous exchange with flowing blood. It comprises a network of glycoproteins, adsorbed plasma proteins, and proteoglycans to which GAG chains are attached. Heparan sulfate GAGs are most prominent on endothelial cells followed by chondroitin sulfate and hyaluronan GAGs. ESL composition and volume depend on the local microenvironment and are actively regulated by endothelial cells[21]. The ESL is instrumental in regulating vascular permeability and hemostasis and possesses anti-atherogenic and anti-inflammatory properties[21]. Moreover, the ESL is an important mediator in shear-induced nitric oxide (NO) production[21].

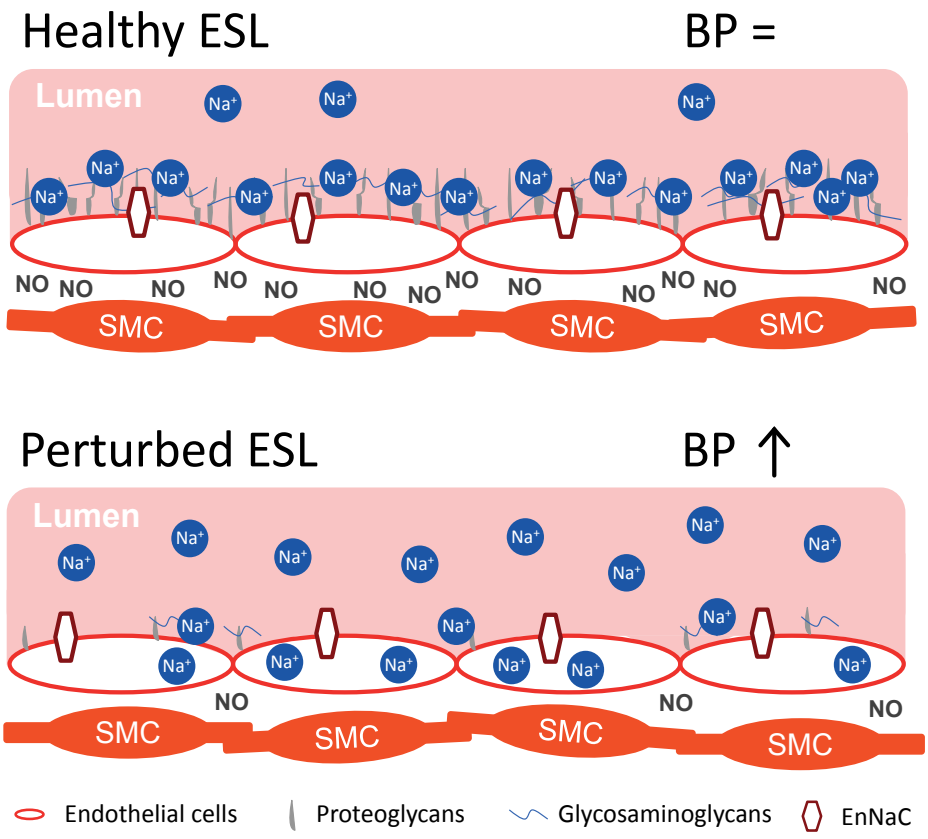
Other than these features, the highly sulfated negatively charged GAGs within the ESL may have sodium-binding properties representing a potential buffering zone for circulating sodium. In contrast to the skin interstitium, the ESL is in direct contact with plasma sodium and therefore, could function as a first sodium buffer before sodium enters the interstitium. The negative charges of the endothelial cell and ESL automatically attract ions of the opposite charge when they are located within an electrolyte solution, such as blood[26]. Because sodium is the most abundant cation in circulating blood, sodium forms a so-called ion atmosphere around the endothelial cell and ESL. Considering the sodium-binding properties of GAGs, it is conceivable that the attracted sodium ions are bound and inactivated by GAGs in the ESL[26]. This has been shown in vitro by ^{23}Na nuclear magnetic resonance experiments that have shown that sodium reversibly binds to GAGs in the ESL under flow[26]. Because of abundant presence of highly sulfated, negatively charged GAGs, the ESL may have a significant role in sodium homeostasis. At present, the sodium-binding capacity of the ESL is not known. The negative charge of the entire vascular ESL in humans has been estimated to be able to inactivate about 30 mmol sodium[27]. These calculations are on the basis of

in vitro experiments and most likely underestimate ESL dimensions, because 7–30 times larger ESL volumes have been reported in vivo, suggesting that, under normal physiologic conditions, the ESL may be able to store significant amounts of sodium[28, 29].

Current methods to measure ESL dimension in humans comprise measurement of shedding products in plasma, noninvasive side stream dark-field or orthogonal polarization spectral imaging of the sublingual microcirculation, in which the erythrocyte–endothelium gap is estimated, and a tracer dilution technique, in which circulating blood volume is compared with the distribution volume of an ESL-permeable tracer[30-32]. Great variability in systemic ESL volume has been reported between different medical conditions, regardless of how ESL volume was estimated[33-35]. Mean systemic ESL volume in healthy individuals was shown to be 1.5–1.7 L, whereas much lower systemic ESL volumes were found in treated (1.1 L) and untreated (0.8 L) patients with heterozygous familial hypercholesterolemia and normoalbuminuric (0.8 L) and microalbuminuric (0.2 L) patients with type 1 diabetes[33, 34]. Elevated shedding products, reflecting ESL breakdown, have been found in patients with severe sepsis, CKD, or ESRD, patients on dialysis, patients after major vascular surgery, and patients during acute or chronic hyperglycemia[31, 33, 35-40]. Most of these conditions associated with a perturbed ESL are also characterized by an expanded ECV, higher BP, or both, suggesting that variability in sodium homeostasis and salt sensitivity may be related to the quality of the ESL, in which endothelial GAGs act as an intravascular buffer compartment for sodium.

Considering the large ESL volume, it seems likely that, next to the skin interstitium, nonosmotic sodium storage by endothelial GAGs is a clinically relevant sodium buffer. In patients on hemodialysis, for example, increased plasma syndecan-1 levels, reflecting ESL breakdown, have been associated with an increased need for ultrafiltration, advocating that loss of ESL sodium buffer capacity is clinically relevant in patients prone to volume overload[41]. In addition, in patients with type 1 diabetes characterized by a decreased ESL volume, BP was inversely associated with ESL volume, which suggests a possible association between ESL volume and BP regulation[33].

Figure 1. The putative effects of the ESL on sodium homeostasis.



Negatively charged GAGs have been shown to be able to osmotically inactivate circulating sodium ions[14, 23]. These GAGs are abundantly present in the ESL, where they may function as a first buffer that inactivates consumed sodium. In addition, an intact ESL has been shown to control EnNaC-mediated sodium transport into the endothelial cell and be crucial for shear stress mediated NO production[44-46, 51]. When the ESL is perturbed and its buffer and barrier functions are lost, an increased amount of osmotically active sodium is located in the vascular lumen, which can lead to water retention. Moreover, shear stress-mediated NO production will be diminished, and EnNaC-mediated sodium transport into the endothelial cell will increase, subsequently reducing NO production[49]. An increase in dietary sodium intake may, therefore, result in a BP increase when the ESL is perturbed, whereas an intact ESL prevents the BP from rising. SMC, smooth muscle cell.

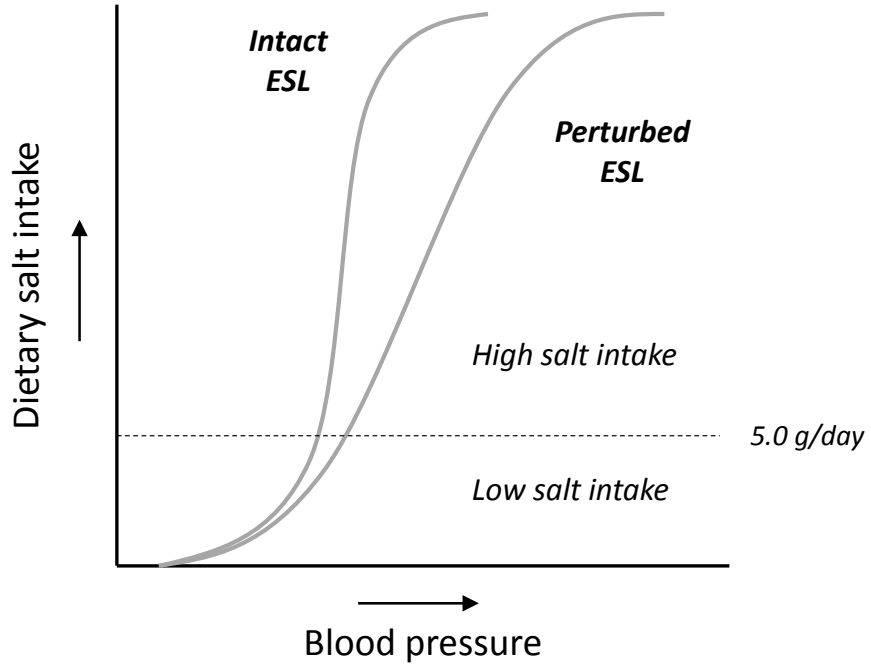
Although GAGs in the ESL seem to relate to sodium homeostasis, so far, few studies have investigated the direct effects of sodium on ESL volume and function. Most of the observations have come from experiments that have studied the interaction between resuscitation fluids and vascular barrier function. After acute volume loading, ESL volume has been shown to decrease considerably independent of the colloidal resuscitation fluid used (5% albumin or 6% hydroxyethylstarch; both containing 0.9% NaCl)[42]. For example, after 5% albumin infusion (20 ml/kg; 220 mmol sodium in a 70-kg person), absolute ESL volume decreased by 47%[42]. These data may suggest a direct detrimental effect of acute sodium loading on ESL volume, which has been supported by *in vitro* data reporting that high sodium concentrations decrease ESL volume as measured by atomic force microscopy[43]. However, expansion of intravascular volume and subsequent ESL compaction may serve as an alternative explanation for the reduction in ESL volume. Because these observations are on the basis of acute sodium loading by using colloids, they may not reflect ESL changes that might occur during chronic high dietary sodium intake.

Changes in ESL characteristics may lead to changes in endothelial cell function. Enzymatic removal of GAGs in the ESL, for example, has been shown to significantly decrease shear-induced NO production[44-46]. Apart from regulating mechanotransduction, the ESL has been shown to determine NO availability by mediating sodium transport into the endothelial cell. After discovery of the epithelial sodium channel on the endothelial luminal surface (EnNaC) next to its known presence on the apical plasma membrane of epithelia, it was shown that this EnNaC regulates endothelial nanomechanics and subsequently affects NO production[47-50]. By enhancing sodium influx, the EnNaC increases mechanical stiffness of the endothelial cellular cortex[48, 49]. The stiffness of this 50- to 100-nm layer, which mainly consists of actin filaments, subsequently modulates endothelial NO synthase activity and NO production, where an increasing stiffness attenuates NO production[48]. The density of EnNaCs on the endothelial surface is regulated by aldosterone and plasma sodium concentration. A rise in plasma sodium concentration increases EnNaC density, which in turn, increases sodium uptake, stiffens the endothelial cellular cortex, and subsequently, leads to diminished NO production[50]. An increase in sodium delivery to the endothelial cell as a result of an increase in sodium intake could, therefore, lead to an increase in vascular tone. These experimental findings are consistent with studies in humans, in which dietary sodium restriction has been shown to improve macrovascular and microvascular endothelial function by an enhanced bioavailability of NO directly induced by the low sodium diet[51, 52]. By covering the endothelial cells, an intact ESL may be pivotal to control EnNaC-mediated sodium transport into endothelial cells. Enzymatic removal of the ESL has been shown to facilitate EnNaC-mediated sodium

transport into the endothelial cells, which led to increased endothelial stiffness[48, 50]. These results indicate that an intact ESL functions as a barrier to control EnNaC-mediated sodium transport.

On the basis of the data as discussed above, the ESL seems to affect sodium homeostasis by functioning as an intravascular buffer for sodium as well as a barrier protecting the endothelial cell against EnNaC-mediated sodium uptake (Figure 1). An intact ESL having sufficient buffering function would, therefore, be able to transiently store osmotically inactive sodium, whereas a perturbed ESL cannot. In the latter condition, a sodium load would result in water retention and a rise in BP because of an increase in osmotically active sodium (Figure 2). A decrease in shear-mediated NO production will presumably allow an additional BP increase[44,45].

Figure 2 Proposed influence of a perturbed ESL on the relation between dietary salt intake and BP.



According to Guyton's pressure–natriuresis curve, the kidneys regulate long-term BP by altering renal sodium excretion. When renal sodium excretion capacity is limited by intrarenal or extrarenal factors, this will result in an increase in BP, which in turn, increases renal sodium excretion capacity at the expense of an increase in BP (i.e., salt sensitivity). Other than factors that limit renal sodium excretion, the ESL may also determine salt sensitivity. An intact ESL, containing many GAGs, may provide a first intravascular buffer that osmotically inactivates sodium before it results in water retention and increases BP. When the ESL is perturbed, sodium cannot be buffered in the ESL, and the curve between dietary salt intake and BP is expected to shift to the right.

ESL modulation

Irrespective of the presence of salt sensitivity, dietary sodium reduction represents the cornerstone in the treatment of hypertension and expanded ECV[53]. Sodium restriction has been shown to reduce BP and potentiate antihypertensive treatment, including renin-angiotensin system blockers and diuretics, and it has even shown similar BP reduction as a single-drug treatment[5, 54]. Moreover, because a small increase in plasma sodium (1.5–3.0 mmol) already can be achieved with an increased dietary sodium intake for 4–14 days, dietary sodium restriction is a simple way to prevent sodium-induced changes of endothelial function[55].

Beyond the beneficial effects of dietary sodium restriction and diuretic therapy, preservation and restoration of the ESL seem to be an interesting new target for treatment in cardiovascular and renal diseases. In this respect, sulodexide, a highly purified mixture of ESL constituents containing 20% dermatan sulfate and 80% heparan sulfate GAGs, is of interest. The relative bioavailability of oral sulodexide (40%-60%) and its relatively long elimination half-life (19-26 hours) make it suitable for oral administration[56]. In patients with diabetes, sulodexide has been shown to restore ESL dimension[57]. In addition, sulodexide treatment has been shown to improve albuminuria in patients with types 1 and 2 diabetes in several small studies[58-60]. Various mechanisms that could be responsible for an increase in ESL volume and a decrease in albuminuria have been described[61-64]. For instance, it has been shown that sulodexide increases synthesis and sulfation of heparan sulfates[63]. Both mechanisms are thought to attenuate glomerular capillary permeability for plasma proteins. However, in the randomized, double-blind, placebo-controlled, sulodexide macroalbuminuria trial that included 1248 patients, no additional renoprotective effect of sulodexide was seen when added to maximal renin-angiotensin system blocking therapy in patients with stages 3 and 4 CKD after a mean follow-up of 10 months[65]. Interestingly, this trial showed a small but significant reduction in systolic BP after sulodexide treatment compared with placebo[65]. BP-reducing effects have also been observed in a number of other randomized, controlled trials investigating long-term sulodexide administration (Table 1)[58-60]. In addition, a trial investigating effects of sulodexide on ESL reported that the observed drop in BP after sulodexide treatment (systolic/diastolic BP, 2/3 mmHg) coincided with increased retinal and sublingual ESL dimension in patients with diabetes, whereas no BP reduction or ESL restoration was observed in control subjects[57]. It is conceivable that the observed reduction in BP in these studies may have resulted from an increased nonosmotic sodium storage capacity after restoration of the ESL or by increased sulfation that also allows an increased amount of sodium to be buffered[57, 63]. An increase in shear-mediated NO production or preservation of endothelial function as a result of increased ESL dimensions may also have contributed to the BP changes

induced by sulodexide. The potency of sulodexide to reduce BP is still difficult to estimate. Not all trials investigating long-term sulodexide administration reported BP values or BP reduction, and none of the studies were designed to specifically examine the effect of sulodexide on BP[59, 66-68]. Moreover, BP was already on target when sulodexide was added in most of the studies, and antihypertensive drugs were not actively controlled[58, 66-68]. Because the largest BP-lowering effect (mean placebo-subtracted BP reduction = 14.6/8.3 mmHg) was observed in a crossover study in patients with diabetes and uncontrolled hypertension, sulodexide may especially lower BP in patients with hypertension and expanded ECV[60].

Table 1. Data of randomized, placebo-controlled trials that reported BP before and after treatment with sulodexide.

Study	Population	Mean age (yrs)	Sulodexide dose (mg)	Duration (months)	Baseline BP (mmHg)	BP after treatment (mmHg)	Placebo subtracted BP (mmHg)
Bang 2012[68]	77 Macroalbuminuric IgA nephropathy patients	40	150	6	121.4/73.6	120.4/71.0	-0.6/-4.0
		42	75		118.9/73.1	121.8/73.8	3.3/-0.7
Gambaro 2002[58]	223 Micro- and macro-albuminuric type 1 and 2 diabetes patients	47	200	4	139.7/82.8	136.8/81.2	-1.6/-2.5
		47	100		136.1/82.1	133.9/82.0	-0.9/-1.0
		49	50		139.6/82.6	134.6/82.1	-3.7/-1.4
Heerspink 2008[67]	149 Microalbuminuric type 2 diabetic patients treated with maximally dosed RAS inhibition	61	400	6	129/75	129/73	-1/0
		64	200		130/73	131/71	0/0
Packham 2012[65]	1248 Macroalbuminuric type 2 diabetic patients treated with maximally dosed RAS inhibition	62	200	10[a]	138.0/73.6	137.1/72.8	-2.4[b]/NA
Solini 1997[60]	12 Hypertensive micro- and macroalbuminuric diabetic patients	52	100	4	155/81	143/75	-14.6[c]/-8.3

[a]BP measurements after 3 months. [b]P=0.04 compared with placebo treatment. [c]P=0.002 compared with placebo treatment. NA, not available

CONCLUSION

In conclusion, new pathophysiologic concepts have arisen from the notion that sodium homeostasis and salt sensitivity seem to relate to not only the kidney, but also extrarenal factors—most intriguingly, the endothelium. This novel concept may provide alternative therapeutic targets in treatment of expanded ECV and hypertension in the future, but first, additional studies need to assess the extent to which changes in the sodium-buffering capacity of the ESL translate into changes in ECV, BP, and ultimately, cardiovascular and renal risk.

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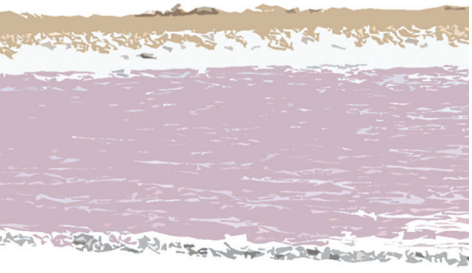

3 Quantification of Nonosmotic Sodium Storage Capacity Following Acute Hypertonic Saline Infusion in Healthy Individuals

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ABSTRACT

The assumption that sodium accumulation in the human body is always accompanied by water retention has been challenged by data showing that sodium can be stored nonosmotically. Here we investigated the contribution of nonosmotic sodium storage to short-term sodium homeostasis after hypertonic saline infusion in healthy individuals on a low-sodium diet. During four hours after infusion, we compared the observed changes in plasma sodium concentration and urinary cation excretion with changes that were calculated with the Adrogue-Madias and Nguyen-Kurtz formula, formulas widely implemented to guide the treatment of dysnatremias. We included 12 healthy nonsmoking male individuals with normal blood pressure, body mass index, and kidney function. Right after infusion, the average observed plasma sodium change from baseline (3.5 mmol/L) was similar to the predicted changes by the Adrogue-Madias (3.3 mmol/L) and Nguyen-Kurtz formula (3.1 mmol/L). However, the observed plasma sodium concentration change after four hours (–1.8 mmol/L) was very different from the changes as predicted by the Adrogue-Madias (0.4 mmol/L) and the Nguyen-Kurtz formula (–0.9 mmol/L). Moreover, only 47% and 55%, respectively, of the expected sodium and potassium excretion were retrieved in the urine. Thus, healthy individuals are able to osmotically inactivate significant amounts of sodium after hypertonic saline infusion. Further research is needed to uncover factors that determine nonosmotic sodium storage.

INTRODUCTION

Being the principal cation in the extracellular compartment, sodium (Na^+) is the most important determinant of plasma osmolality and is essential to maintain the effective circulating volume. Generally, the kidney is believed to be mainly responsible for matching Na^+ excretion with Na^+ intake, resulting in an almost perfect equilibrium during constant Na^+ intake[1]. However, recent well-controlled Na^+ balance studies have demonstrated that Na^+ can accumulate in the human body without concurrent water retention[2,3]. In these studies, total body Na^+ varied as much as 200 mmol during fixed Na^+ intake[2]. Surprisingly, the observed variation in total body Na^+ did not induce any changes in body weight or blood pressure (BP)[2]. An additional compartment that is able to temporarily store excessive Na^+ without volume effects seems therefore to be uncovered. Experimental studies have identified glycosaminoglycans as the principal Na^+-binding site[4]. Glycosaminoglycans are large, negatively charged polysaccharides that are abundantly present in the skin, endothelial surface layer, bone and cartilage. Early studies dating from the 1950s have already acknowledged Na^+ binding to glycosaminoglycans in bone and cartilage[5]. However, the majority of this Na^+ pool was considered to be constant, thus having marginal influence on osmoregulation. This is in contrast with data showing that glycosaminoglycans, present under the skin and at the luminal side of the endothelium, are able to store and release significant amounts of Na^+[6-10]. Considering the amount of these skin and endothelial glycosaminglycans, the ability to vary glycosaminoglycan concentration and sulfation may affect osmoregulation and BP in response to changes in Na^+ balance. Yet, the exact volume of this variable Na^+ buffer has not been quantified, and its importance in clinical practice remains to be determined.

To examine whether nonosmotic Na^+ storage is clinically relevant for osmoregulation, one can apply the Adrogue-Madias or Nguyen-Kurtz's formula, which are both widely used in the clinic for estimating the effect of saline infusion on plasma [Na^+] in dysnatremic patients[11,12]. These formulas are based on the Edelman equation, which describes the correlation between serum [Na^+] and the amount of exchangeable cations (i.e., Na^+ and K^+) per liter of body water[13]. Edelman et al. postulated that osmotically inactive bone and cartilage Na^+ were taken into account in this formula, as represented by the y-intercept[13]. However, because this equation was based on steady state observations, it may not account for any residual capacity for nonosmotic Na^+ storage capacity or variations in nonosmotic Na^+ storage capacity. Estimations of treatment effects based on this equation may therefore be inaccurate. As the presence of dysnatremia is associated with increased morbidity and mortality, inadequate treatment may lead to worse outcomes[14-16]. To study the contribution of the

nonosmotic Na^+ storage capacity to Na^+ homeostasis on the short term, we compared the observed changes in Na^+ and water balance after hypertonic NaCl infusion in healthy subjects with the changes that were expected according to the classic two-compartment model as described by the Adrogue-Madias and Nguyen-Kurtz formulas.

METHODS

Participants

We carried out an experimental intervention study in healthy, nonsmoking male subjects between 18 and 40 years of age who were able to provide written informed consent. We excluded overweight subjects (body mass index >30 kg/m^2) as well as subjects with BP >140/90 mmHg or with a history of renal or cardiovascular disease. The study was conducted in the Academic Medical Center in Amsterdam, the Netherlands, after approval of the local ethics committee. All subjects provided written informed consent and our study was in accordance with the Declaration of Helsinki.

Study design

All subjects pursued an 8-day low Na^+ diet (target <50 mmol Na^+/day). We checked dietary compliance by collecting 24-hour urine samples on day 3, 6 and 8. On day 8, after an overnight fast, subjects visited our research department for baseline blood sampling and BP measurements. After a standardized low-salt breaktfast and lunch, we intravenously infused 2.4% NaCl in 30 minutes. We corrected the infused amount of Na^+ for differences in total body water (TBW) among subjects (5 mmol Na^+/L TBW). By adding 20% NaCl solution (range, 29 - 50 mL) to 500 mL of 0.9% NaCl, we were able to infuse 5 mmol Na^+/L TBW in every subject with only minor differences in infused volume. Before infusion, subjects were requested to fully empty their bladder. After infusion we measured hemodynamic parameters, and collected blood and urine samples at timed intervals during a 4-hour period. During this period, water intake was standardized to 400 mL in all subjects.

Laboratory analyses

Blood was collected in 4.5 mL lithium heparin tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) for analysis of plasma [Na^+], [K^+], osmolality and creatinine. Three-milliliter tubes with 5.4 mg spray-dried K_2 ethylenediamine tetraacetic acid (BD Vacutainer) were used for hematocrit determination. All blood samples were centrifuged at 2000*g* for 10 minutes at 18°C and analysed within 60 minutes of collection. We used the indirect ion selective electrode method to measure plasma [Na^+]

and [K^+], and urine [Na^+] and [K^+]. Plasma and urinary osmolality was determined by freezing point depression.

Hemodynamic measurements

BP and heart rate were measured at the right upper arm with a semi-automated device (Omron 705 IT, Omron Healthcare Europe B.V., Hoofddorp, the Netherlands) in supine position after resting for at least 10 minutes in a quiet and temperature-controlled room. The mean of the last two measurements was used for analysis.

Calculations

For calculation of the expected increments of plasma [Na^+] after hypertonic NaCl infusion, we used the Adrogue-Madias formula[11]. To calculate the expected urinary Na^+ excretion as a result of an observed decrease in plasma [Na^+] and vice versa, we used the Adrogue-Madias fluid-loss formula[17]. We used the Nguyen-Kurtz formula, a more recent and extensive formula, to test the robustness of our results[12]. To calculate the expected urinary Na^+ excretion, we rearranged this formula (Supplemental Table 2)[12]. The expected increase of plasma osmolality was calculated by dividing the sum of total baseline osmoles (baseline plasma osmolality x TBW) and infused osmoles by the sum of baseline TBW and infused volume. For these analyses, we estimated baseline TBW as 60% of body weight. Extracellular and intracellular volume at baseline were estimated as 33% and 67% of TBW, respectively[18]. We calculated the extracellular volume after infusion by dividing the sum of baseline extracellular osmoles and infused osmoles by the observed osmolality[19]. Intracellular volume after infusion was estimated by subtracting extracellular volume after infusion from TBW after infusion. Subsequently, we estimated changes in extra- and intracellular volume induced by infusion. By using the expected osmolality in this calculation, we were able to estimate changes of intra- and extracellular volume that should have taken place, considering full equillibration of water among the intra- and extracellular compartment. To investigate renal Na^+ handling, we calculated fractional Na^+ excretion. To test the robustness of our results, we performed a sensitivity analysis in which the maximum plasma [Na^+] increments after infusion was used for calculations.

MRI Measurements

We estimated the renal pelvis volume in T2-weighted coronal MRI scans of three healthy male volunteers. Image acquisition was performed using a Ingenia 3.0-Tesla MRI scanner (Philips, Best, The Netherlands) with the following acquisition parameters: field of view, 400 × 400 mm^2; voxel size, 0.875 × 0.875 mm^2; slice thickness, 4 mm;

repetition time, 807 ms; flip angle, 90°; echo time, 70 ms. In the scans, the urine-containing pelvis was easily delineated, after which the volume was calculated.

Statistical analyses

Continuous data are shown as mean and standard error of the mean (SEM) when data followed normal distribution and median plus interquartile range (IQR) when skewed distribution was apparent, unless otherwise specified. We used paired t-tests to test whether changes in laboratory and hemodynamic parameters were significant when compared to baseline values. To assess correlation between variables, we calculated Pearson's correlation coefficient (SPSS, Version 21.0, SPSS, Inc., Chicago, IL).

RESULTS

We included 12 male healthy subjects with an average age of 23 ± 1 years with normal BP, kidney function and body mass index in our comparisons (Table 1). One of the 12 subjects had to be excluded because blood sampling within the first 10 minutes after NaCl infusion was problematic. All subjects adequately followed an 8-day low NaCl diet that resulted in a mean 24-hour urinary Na^+ excretion of 19 ± 3 mmol.

Table 1. Baseline characteristics of 11 healthy volunteers included in the analysis.

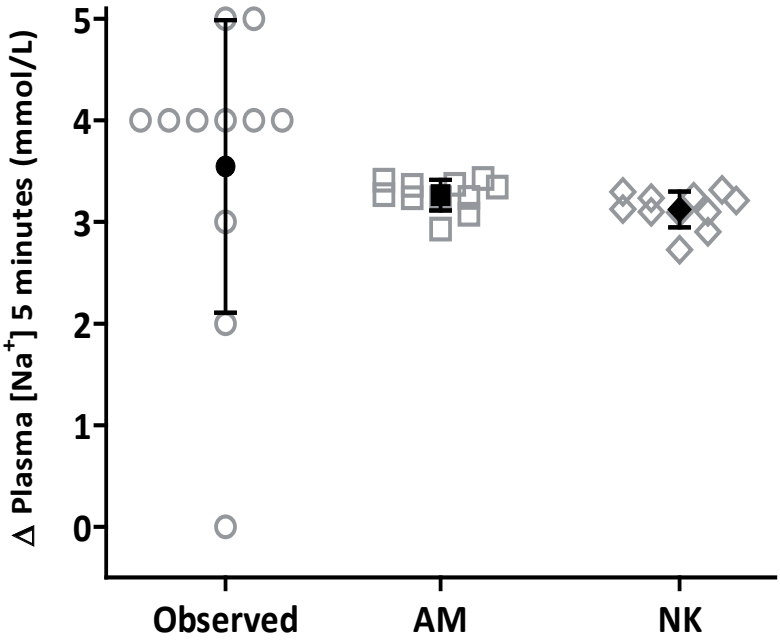
Patient characteristics	Mean (SEM)
Age (years)	22.8 (1.3)
BMI (kg/m^2)	21.4 (0.7)
TBW (L)	44.4 (1.3)
Hematocrit (L/L)	0.42 (0.01)
Plasma Na^+ (mmol/L)	137.5 (0.5)
Plasma K^+ (mmol/L)	3.9 (0.1)
Plasma Osmolality (mOsm/kg)	285 (1)
Plasma Creatinine (µmol/L)	85 (3)
Supine Systolic BP (mmHg)	117 (2)
Supine Diastolic BP (mmHg)	59 (2)
Supine heart rate (bpm)	55 (2)

Average acute plasma [Na^+] increments after NaCl infusion can be accurately estimated

To investigate whether nonosmotic Na^+ storage affected plasma [Na^+] increments after infusion of NaCl, we measured Na^+ balance after acute infusion of 543 mL of 2.4% NaCl in Na^+-depleted subjects. We used the Adrogue-Madias and Nguyen-Kurtz formulas to calculate the expected increase in plasma [Na^+] after infusion (see

supplementary data for both formulas and detailed calculations)[11, 12]. Five minutes after infusion, the average plasma [Na^+] increased 3.5 ± 0.4 mmol/L. This was in accordance with the expected 3.3 ± 0.1 mmol/L increase, as calculated by the Adrogue-Madias formula, or the 3.1 ± 0.1 mmol/L increase, as calculated by the Nguyen-Kurtz formula (Figure 1).

Figure 1. Variation in the observed and expected changes in plasma [Na^+] 5 minutes after infusion.



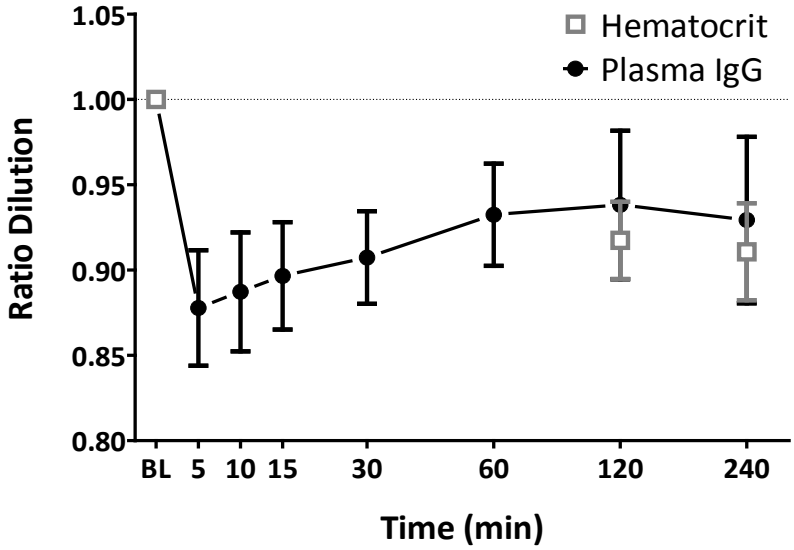
Five minutes after hypertonic saline infusion, plasma [Na^+] increased 3.5 ± 0.4 mmol/L (P<0.001). This was equal to the expected 3.3 ± 0.1 mmol/L increase as estimated by the Adrogue-Madias (AM) formula or the 3.1 ± 0.1 mmol/L increase as calculated by the Nguyen-Kurtz (NK) formula. Large heterogeneity was present among in the observed values, which was not expected according to the Adrogue-Madias and Nguyen-Kurtz formula. Data are given as the mean and SD.

Although the observed changes in plasma [Na^+] showed more heterogeneity than the calculated values, we observed a significant association between the observed and calculated values, both when using the Adrogue-Madias formula (R^2=0.41, P=0.034) and the Nguyen-Kurtz formula (R^2=0.41, P=0.034). Ten subjects showed a maximum plasma [Na+] increase 5 minutes after infusion, whereas one of the subjects had no

change of plasma [Na^+] after 5 minutes but showed a maximum 3-mmol/L increase after 10 minutes. A sensitivity analysis, based on all maximum increments of plasma [Na^+], demonstrated that the mean plasma [Na+] increase after 5 minutes was 3.8 ± 0.3 mmol/L, which was 0.5 mmol/L and 0.7 mmol/L different from estimates based on the Adrogue-Madias and Nguyen-Kurtz formula, respectively.

Plasma potassium concentration ([K^+]) levels did not show significant changes after hypertonic NaCl infusion. The mean plasma osmolality increased with 6.9 ± 0.9 mosm/kg (P<0.001) to 293.3 ± 0.9 mosm/kg directly after infusion, which was higher than the expected osmolality of 292.8 ± 1.1 mosm/kg. Based on the observed osmolality, the extracellular volume had increased with 1.17 ± 0.05 L after 5 minutes (Supplemental Figure 1). This was somewhat lower than the increase of 1.20 ± 0.03 L that was expected following infusion according to full equilibration between the intracellular and extracellular compartment. Plasma IgG concentrations decreased significantly by 12 ± 1% (P<0.001) after infusion reflecting hemodilution. The lowest IgG values were present 5 minutes after infusion, after which IgG concentrations increased steadily (Figure 2).

Figure 2. Effects of saline infusion on plasma IgG concentration and hematocrit.



*Five minutes after infusion, plasma IgG concentrations were decreased significantly by 12 ± 1% (P<0.001). Two and four hours after infusion, both plasma IgG concentration (-6 ± 2%, P=0.005; and -8 ± 2%, P=0.005) and hematocrit (-8 ± 1%, P<0.001; and -9 ± 1%, P<0.001) were decreased. Compared with 5-minute IgG levels, IgG levels after 15 to 240 minutes were significantly higher. *p≤0.01 compared to baseline levels, **p≤0.01 compared to 5-minute levels.*

Only half of the Na^+ that is cleared from the body water can be retraced in the urine

To examine whether nonosmotic Na^+ storage contributes to the clearance of an acute Na^+ load, we analysed plasma [Na^+] and urinary Na^+ and K^+ excretion up to four hours after infusion of hypertonic NaCl. Plasma [Na^+] gradually decreased after the initial increase (Figure 3). Four hours after infusion, plasma [Na^+] had decreased with 1.8 ± 0.5 mmol/L (P=0.002) compared with the initial rise after 5 minutes, but was still significantly increased with 1.7 ± 0.4 mmol/L (P=0.002) compared with values before infusion. Plasma [K^+] did not show any significant change during follow-up. After four hours, plasma osmolality was 5.4 ± 1.0 mosm/kg higher than baseline (P<0.001). Plasma IgG concentration (-8 ± 2%, P=0.004) and hematocrit (-9 ± 1%, P<0.001) were decreased.

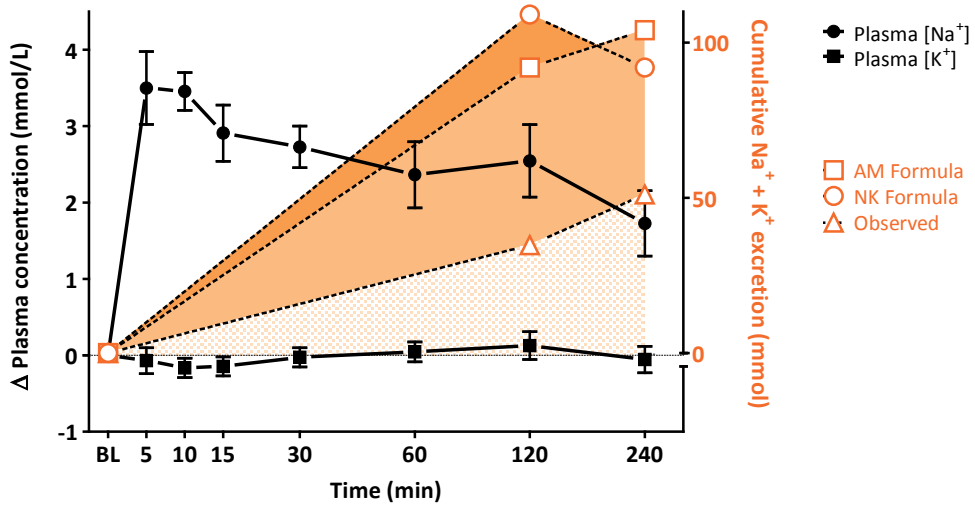
Four hours after hypertonic NaCl infusion, an average of 472 ± 51 mL urine was collected with a mean [Na^+] of 57 ± 7 mmol/L accounting for 27 ± 6 mmol Na^+. Urinary K^+ excretion accounted for 24 ± 3 mmol. According to the Adrogue-Madias fluid loss formula, the observed total urinary loss of both Na^+ and K^+ should have accounted for a 0.4 ± 0.1 mmol/L increase in plasma [Na^+], which is considerably different than the 1.8 ± 0.5 mmol/L decrease in plasma [Na^+] that was observed (Figure 4; Supplementary Table S1). Conversely, an average plasma [Na^+] decrease of 1.8 mmol/L in healthy subjects with constant K^+ levels and a mean TBW of 45.0 L after infusion should have resulted in excretion of 108 ± 15 mmol of Na^+ or K^+ in the urine according to the Adrogue-Madias formula. However, we were only able to retrieve 27 mmol Na^+ (24%) and 24 mmol K^+ (22%) from all urine collections, leaving 57 mmol of cations undetected (Figure 3; Supplementary Table S1). We found similar results when using the Nguyen-Kurtz formula. According to this formula, urinary Na^+ and K^+ loss should have resulted in a 0.9 ± 0.1 mmol/L decrease of plasma [Na^+] instead of the observed 1.8-mmol/L decrease (Figure 4; Supplementary Table S2). The other way around, 92 ± 26 mmol of Na^+ or K^+ should have been excreted, which is in contrast with the 51 mmol that was actually excreted (Figure 3; Supplementary Table S2). In a sensitivity analysis based on the maximally achieved plasma [Na^+] increments, the discrepancy between plasma [Na^+] changes and urinary Na^+ excretion increased. Only 45% and 50% of the Na^+ or K^+ that should be in the urine according to the Adrogue-Madias or Nguyen-Kurtz formula, respectively, could be retraced.

No correlation was present between the plasma [Na^+] changes that were observed after two hours and the values that were calculated using the Adrogue-Madias formula (R^2=0.07, P=0.42) and Nguyen-Kurtz formula (R^2=0.12, P=0.30) (Supplementary Figure S2). Also, the Adrogue-Madias (R^2=0.01, P=0.72) and Nguyen-

Kurtz formula (R^2=0.04, P=0.58) were not correlated with observed changes after 4 hours.

Urinary osmolality increased from 291 ± 16 mOsm/kg at baseline to 666 ± 30 mOsm/kg (P<0.001) 4 hours after infusion. Fractional Na^+ excretion at baseline showed that subjects were actively retaining Na^+ (0.08 ± 0.01%). Fractional Na^+ excretion increased 2 hours (0.37 ± 0.09%, P=0.004) and 4 hours after infusion (0.45 ± 0.10%, P=0.004).

Figure 3. Mismatch of plasma and urinary cation changes after hypertonic saline infusion.



Hypertonic NaCl infusion in healthy subjects leads to a predictable initial rise in plasma [Na^+] while plasma [K^+] remained stable. However, the observed urinary output of cations could only explain half of the gradual decrease in plasma cations that was observed during the subsequent 4 hours. We calculated the expected urinary output of cations as a result of plasma changes with the Adrogue-Madias (AM) and Nguyen-Kurtz (NK) formulas. Data are presented as mean and standard error. BL, baseline.

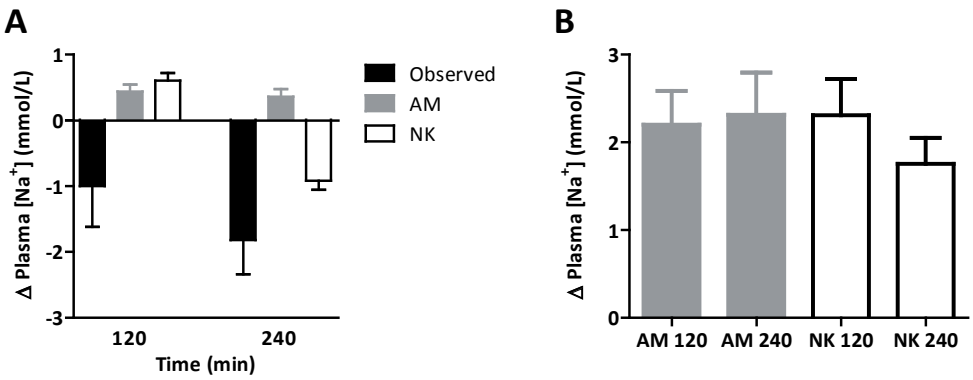
Hypertonic saline had a minimal effect on hemodynamics

No changes in systolic BP were observed during hypertonic NaCl infusion or in the following 4 hours. Diastolic BP decreased significantly with 4 ± 2 mmHg during hypertonic NaCl infusion (P=0.04) and turned back to baseline values after the infusion. The mean heart rate increased with 2 ± 1 beats per minute (P=0.001) during infusion but showed an average decrease of 3 ± 1 beats per minute from 90 to 240 minutes after infusion (P=0.004).

Postvoid urine retention cannot explain the missing cations

Three-dimensional reconstruction of the renal pelvis using magnetic resonance imaging (MRI) demonstrated that the volume of a single renal pelvis was 2.89 ± 0.01 mL. The potential intra-luminal volume of a ureter was estimated to be 7 - 8 mL (π x 250 - 300 mm [length] x 3 mm^2 [$radius^2$])[20]. Together with the estimated postvoid urine bladder retention of ~14 mL, the potential maximum postvoid urine retention may be ~36 mL[21]. This is much lower than the 324 mL that would explain the missing cations based on the 4-hour urine cation concentration.

Figure 4. Mismatch of the observed and expected plasma [Na^+] changes after hypertonic NaCl infusion.



After the initial increase, plasma [Na^+] slowly decreased during the four hour follow-up.
(A) Two and 4 hours after infusion, plasma [Na^+] decreased by 1.0 ± 0.6 mmol/L and 1.8 ± 0.5 mmol/L, respectively. The Adrogue-Madias (AM) formula calculated that plasma [Na^+] would increase by 0.4 ± 0.1 mmol/L, both after 2 and 4 hours. According to the Nguyen-Kurtz (NK) formula, plasma [Na^+] should have increased by 0.6 ± 0.1 mmol/L after 2 hours and decreased by 0.9 ± 0.1 mmol/L after 4 hours.
(B) The absolute difference between the observed and calculated values represented by the square root of the squared difference. Calculations with the AM formula were 2.2 mmol/L (95% confidence interval [CI] 1.3 - 3.1, P<0.001) and 2.3 mmol/L (95% CI 1.2 - 3.4, P<0.001) different from the observed values after 2 and 4 hours. Calculation with the NK formula were 2.3 mmol/L (95% CI 1.4 - 3.2, P<0.001) and 1.8 mmol/L (95% CI 1.1 - 2.4, P<0.001) different from the observed values after 2 and 4 hours. Data are represented as mean and standard error.

DISCUSSION

This is the first balance study that aimed to quantify the amount of nonosmotic Na^+ storage in healthy subjects after an acute intravenous saline load. We observed that half of the osmotically active cations that were cleared from the body water after hypertonic saline infusion could not be retrieved in the urine.

Considering the ability of glycosaminoglycans to osmotically inactivate Na^+ and the fact that the Adrogue-Madias and Nguyen-Kurtz formulas take into account changes in total body K^+ and water, our data indicate that healthy volunteers are able to osmotically inactivate a significant amount of Na^+ after hypertonic saline infusion.

The Adrogue-Madias and Nguyen-Kurtz formulas are both based on the Edelman equation and widely used to estimate the effect of infusion of saline solutions on plasma [Na^+] to establish infusion strategies for dysnatremic patients. The Edelman equation was based on the relation between serum [Na^+] and exchangeable cations under steady-state low-salt conditions[5]. It therefore does not consider a possible residual capacity for nonosmotic Na^+ storage that may help to counterbalance changes in total body Na^+. Such a residual volume may be particularly present after a low Na^+ diet, as suggested by our experiment. In keeping with this, skin Na^+ content has shown to decrease after a low Na^+ diet in an earlier study[4]. Moreover, nonosmotic Na^+ storage capacity may increase in response to stimuli that may potentially disturb Na^+ homeostasis, such as high dietary Na^+ intake or saline infusion. In aquatic species, for example, the quantity of glycosaminoglycans and their sulfation degree increases with higher environmental NaCl concentrations, which allows Na^+ inactivation and thereby prevents osmotic stress[22]. The results of our study indicate that healthy subjects on a low NaCl diet have a compensation mechanism similar to that in high NaCl conditions. This may be either caused by a large residual capacity for nonosmotic Na^+ storage that was present after a low Na^+ diet or by a rapid increase in nonosmotic Na^+ storage capacity as a result of increased glycosaminoglycan concentration and/or sulfation that can be achieved within 4 hours. Interestingly, abnormalities in nonosmotic Na^+ storage have been recently linked to impaired vasorelaxation and salt sensitivity and may therefore have implications for cardiovascular and renal disease[23].

To test the robustness of our results, we used both the Adrogue-Madias and the Nguyen-Kurtz formulas. The latter more extensive formula incorporates the y-intercept and slope of the Edelman equation that were left out of the Adrogue-Madias formula and accounts for ongoing changes in Na^+ and K^+ balance. Nguyen and Kurtz[24] showed that the values of the y-intercept and slope represent the effects of the osmotic coefficient of Na+ at physiological concentrations and the Gibbs-Donan equilibrium and should therefore be taken into account when estimating plasma [Na^+]. However, this formula resulted in an almost similar discrepancy between the amount of exchangeable cations that were cleared from the body water and the amount of cations that could be retrieved from the urine. Because plasma [K^+] remained stable, indicating that there were no shifts of potassium accounting for this discrepancy, the only explanation for the disappearance of over 50 mmols of cations is nonosmotic Na^+

storage. As our findings are based on a mismatch between changes in plasma cation concentration and urinary cation excretion, these results are not influenced by time.

The Adrogue-Madias and Nguyen-Kurtz formulas were able to predict the average plasma [Na^+] increment directly following infusion of hypertonic NaCl. However, in a sensitivity analysis based on all maximum increments of plasma [Na^+], we observed larger differences between the observed and predicted values. Moreover, the heterogeneity of the observed plasma [Na^+] changes was far greater than that of the calculated values in both analyses. Because we analysed plasma [Na^+] right after infusion, the observed change in plasma [Na^+] was mainly the result of an instant redistribution of body water, which can be predicted using the 2-compartment model. The observed discrepancy and heterogeneity of the 5-minute estimations may therefore be caused by inadequate prediction of water redistribution. Within 5 minutes after infusion, plasma [Na^+] and osmolality increased to their highest levels. The maximum plasma [Na^+] and osmolality were accompanied by a nadir in IgG concentration, suggesting that the infused Na^+ had rapidly distributed over the extracellular volume and that the majority of water had shifted from the intracellular to the extracellular compartment. However, the observed increase in osmolality was larger than expected, suggesting incomplete equilibration of water between the intra- and extracellular compartment. This may be the result of slow equilibration of water between the intra- and extracellular compartment, which has been demonstrated after large volumes of water intake[25].

During the 4 - hour follow-up, we observed an even larger difference between the observed plasma [Na^+] changes and the values that were predicted by the Adrogue-Madias or Nguyen-Kurtz formula. The average discrepancy between the observed and expected changes was ~2 mmol/L. In addition, the heterogeneity of the observed changes was far greater than expected. These findings suggest that, in addition to variables that are derived from Na^+, K^+ and water balance, there are more, so far unknown or incompletely understood variables that cause the large discrepancy and heterogeneity of plasma [Na^+] changes after infusion. The fact that we observed no correlation between the observed plasma [Na^+] changes 2 and 4 hours after infusion and the values that were estimated with both formulas supports the notion that this discrepancy is not due to a structural error in application of variables that are included in these formulas, but seems merely caused by thus far missing variables. Nonosmotic Na^+ storage capacity, via binding to glycosaminoglycans, may be one of the additional variables that may significantly vary among individuals with different diseases and diets. For example, the endothelial surface layer volume (mainly consisting of glycosaminoglycans) of macroalbuminuric diabetic patients has been shown to be reduced by 85% compared with healthy subjects, and patients with chronic kidney

disease are known to have a 5-fold higher plasma concentration of glycosaminoglycan breakdown products[26,27]. In addition, male sex, older age, hypertension, hyperaldosteronism, heart failure, end-stage kidney failure and infection have been shown to be associated with an increased skin Na^+ concentration[10,28-31]. Next to these demographic variables, nonosmotic Na^+ storage is affected by interventions that are used to treat hypertension or dysnatremia. In a patient with hypernatremia, for instance, muscle Na^+ showed a 20 mmol/L decrease after treatment with water and desmopressin. It goes without saying that recruitment of such large amount of Na^+ from a third compartment will complicate prediction of plasma [Na^+] in these patients. The heterogeneity in nonosmotic Na^+ storage capacity among individuals may therefore explain the large standard deviation of the slope and y-intercept in the Edelman equation as well as the inability to estimate individual plasma [Na^+] changes after hypertonic NaCl infusion[13,32].

Relative to healthy subjects, prediction of plasma [Na^+] changes in unbalanced, ill, dysnatremic patients with often multiple comorbidities may be an even greater challenge. In line with our results, previous retrospective studies that attempted to estimate plasma [Na^+] in dysnatremic subjects using several formulas demonstrated the difficulty of predicting plasma [Na^+] over time. In hypo- and hypernatremic patients, Liamis et al.[33] showed that the observed plasma [Na^+] was higher than the calculated plasma [Na^+]. In a group of 15 volume depleted hyponatremic subjects, plasma [Na^+] was even 5.6 mmol/L higher than expected after 24 hours[33]. This is similar to the observation by Mohmand et al.[34] who showed that the observed plasma [Na^+] changes after hypertonic NaCl infusion in hyponatremic patients were 66% greater than the estimated values. In both studies, the Adrogue-Madias formula was used to estimate changes in plasma [Na^+][33,34]. A comparative analysis of the Adrogue-Madias and Nguyen-Kurtz formulas in intensive care unit patients showed that the predictive power of these formulas was equally poor in hypo- and hypernatremic patients[35]. Only 50% of the variability of the observed values could be explained by the variability of the predicted values[35]. The average observed values were 3.4 - 4.5 mmol/L higher in hyponatremia patients and even 5.0 - 6.7 mmol/L higher in hypernatremia patients[35]. Considering the hazardous consequences of hypo- and hypernatremia itself as well as undertreatment or overcorrection of these conditions, these formulas should be carefully applied[14-16, 36, 37]. Dysnatremic patients should be closely monitored, including frequent laborato-ry follow-up. Meanwhile, further investigation of nonosmotic Na^+ storage and its contribution to Na^+ homeostasis in dysnatremic patients is warranted.

In addition to the variability in nonosmotic Na^+ storage capacity, previously discussed limitations of the Edelman equation may contribute to the structural inconsistencies between our findings and the formulas that are based on this equation[32].

Intraindividual measurements were not performed simultaneously in the Edelman study[32]. Also, the population was very heterogeneous and covered the entire range of plasma [Na^+] and may therefore not represent normal physiology. Moreover, the included population largely consisted of patients with significant comorbidities that is clearly different from the healthy population that was included in the current study.

A potential limitation of this study is that we have not directly measured the amount of nonosmotic Na^+ stored in the tissues. ^{23}Na-MRI is, for example, an imaging technique that is able to measure changes in skin Na^+. Another potential limitation is that we have not measured Na^+ that was still present in the bladder, ureters, and renal pelvis after voiding and Na^+ sweat loss. However, the observed discrepancy greatly exceeds the possible Na^+ content of postvoid residual urine present in the bladder, which is maximally ~36 mL and may have, therefore, accounte for 6 mmol of Na^+ or K^+, or sweat Na^+ loss that has been shown to be only 3 mmol/day[21, 38]. Third, we estimated TBW using a commonly used formula, which may be inaccurate. Considering that our main results were based on changes in total body water which were measured accurately, this will not influence our results. Last, it is important to emphasize that this study was performed in Na^+-depleted subjects. Whether these findings are also true for subjects with high dietary salt intake remains to be determined.

CONCLUSION

Together, these data challenge the traditionalists' vision upon physiology in which water and solutes are simply divided over intra- and extracellular compartments. Further research is needed to uncover factors that determine nonosmotic Na^+ storage capacity as well as the exact contribution to Na^+ homeostasis, both in the short and long term. With regard to the detrimental effects of both osmoregulatory disturbances and body Na^+ accumulation (potentially inducing extracellular volume expansion), full understanding of nonosmotic Na^+ storage may significantly affect health outcomes of various patient categories, such as dysnatremic patients, but also salt-sensitive hypertensive patients and heart failure and kidney failure patients. Future studies should therefore investigate infusion of solutions with different NaCl amounts, either controlled for osmolality or water content, along with imaging techniques that demonstrate the sites where Na^+ is stored. Until now ^{23}Na-MRI is the only available technique to image changes in Na^+ storage. For now, current treatment recommendations for dysnatremias are adequate but should always include frequent measurements of plasma [Na^+] after initiation of treatment because of the difficulties of predicting plasma [Na^+] changes over time in clinical practice.

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Supplemental Table 1. Adrogue Madias Formula.

1A. Comparison of observed and estimated plasma [Na^+] changes 5 minutes after infusion.

Adrogue-Madias formula[11]:

$$\Delta[\mathrm{Na}]plasma\ (\text{per 1L of infusate}) = \frac{[\mathrm{Na}+\mathrm{K}]infusate - [\mathrm{Na}]plasma_{pre}}{\mathrm{TBW} + 1}$$

Observed parameters 5 minutes after infusion		
[$Na^+ + K^+$]*infusate*	409.3 ± 34.1 mmol/L	
[Na^+]*plasma* $_{baseline}$	137.9 ± 1.2 mmol/L	
Total body water (TBW)	44.4 ± 4.2 L	
Infused volume	0.543 ± 0.006 L	
Estimated Plasma [Na^+] changes 5 minutes after infusion		**Observed change**
Δ[Na^+]*plasma* (per 1L of infusate)	6.0 ± 0.2 mmol/L	
Δ[Na^+]*plasma* (per 0.543 L)	3.2 ± 0.2 mmol/L	3.5 ± 1.4 mmol/L

1B. Comparison of observed and estimated plasma [Na^+] changes between 5 and 240 min after infusion.

Adrogue-Madias fluid loss formula[17]:

$$\Delta[\mathrm{Na}]plasma = \frac{[\mathrm{Na}]plasma_{pre} - [\mathrm{Na}+\mathrm{K}]fluid\ loss}{\mathrm{TBW} - 1}$$

Observed parameters 5-240 minutes after infusion		
[Na^+]plasma$_{pre\ (5\ min\ after\ infusion)}$	141.5 ± 2.0 mmol/L	
[$Na^+ + K^+$]fluid loss	109.5 ± 33.0 mmol/L	
TBW $_{including\ infusion}$	45.0 ± 4.2 L	
Fluid volume lost	0.472 ± 0.168 L	
Estimated Plasma [Na^+] changes 5-240 minutes after infusion		**Observed change**
Δ[Na^+]plasma (per 1L fluid loss)	0.7 ± 0.8 mmol/L	
Δ[Na^+]plasma (per 0.543 L)	0.4 ± 0.4 mmol/L	-1.8 ± 1.7 mmol/L

1C. Comparison of observed and estimated urinary Na^+/K^+ loss between 5 and 240 minutes after infusion.

Rearranged Adrogue-Madias formula:

$$[\mathrm{Na}+\mathrm{K}]fluid\ loss = [\mathrm{Na}]plasma_{pre} - (\Delta[\mathrm{Na}]plasma * (\mathrm{TBW} - 1))$$

Observed parameters 5-240 minutes after infusion		
[Na^+]*plasma*$_{pre\ (5\ min\ after\ infusion)}$	141.5 ± 2.0 mmol/L	
Δ [Na^+]*plasma*	-1.8 ± 1.9 mmol/L	
[$Na^+ + K^+$]*fluid loss*	109.5 ± 33.0 mmol/L	
TBW$_{including\ infusion}$	45.0 ± 4.2 L	
Fluid volume lost	0.472 ± 0.168 L	
Estimated urinary [$Na^+ + K^+$] loss 5-240 minutes after infusion		**Observed loss**
[$Na^+ + K^+$] (per 1L fluid loss)	223.7 mmol/L	
[$Na^+ + K^+$] (per 0.472 L fluid loss)	108.2 mmol	51.1 ± 25.8 mmol

Supplemental Table 2. Nguyen-Kurtz formula.

2A. Comparison of observed and estimated plasma [Na+] changes 5 minutes after infusion.

Nguyen-Kurtz formula[12]:

$$[\mathrm{Na}]plasma_{post} = \frac{(([\mathrm{Na}]plasma_{pre} + 23.8) * \mathrm{TBW}) + (1.03 * (\mathrm{Na} + \mathrm{K\ input}) - (\mathrm{Na} + \mathrm{K\ output}))}{\mathrm{TBW} + \Delta\mathrm{Volume}} - 23.8$$

Observed parameters 5 minutes after infusion		
$[Na^+]plasma_{pre}$	137.9 ± 1.2 mmol/L	
TBW	44.4 ± 4.2 L	
$(Na^+ + K^+)input$	222.2 ± 20.8 mmol	
$(Na^+ + K^+)output$	0 mmol	
ΔVolume	0.543 ± 0.006 L	
Estimated plasma [Na^+] changes *5 minutes after infusion*		**Observed change**
$[Na^+]plasma_{post}$	141.0 ± 1.3 mmol/L	141.5 ± 2.0 mmol/L
$\Delta[Na^+]plasma$	3.1 ± 0.2 mmol/L	3.5 ± 1.4 mmol/L

2B. Comparison of observed and estimated plasma [Na^+] changes between 5 and 240 min after infusion.

Nguyen-Kurtz formula[12]:

$$[\mathrm{Na}]plasma_{post} = \frac{(([\mathrm{Na}]plasma_{pre} + 23.8) * \mathrm{TBW}) + (1.03 * (\mathrm{Na} + \mathrm{K\ input}) - (\mathrm{Na} + \mathrm{K\ output}))}{\mathrm{TBW} + \Delta\mathrm{Volume}} - 23.8$$

Observed parameters 5-240 minutes after infusion		
$[Na^+]plasma_{pre\ (5\ min\ after\ infusion)}$	141.5 ± 2.0 mmol/L	
$TBW_{\ with\ infusion}$	45.0 ± 4.2 L	
$(Na^+ + K^+)input$	0 mmol	
$(Na^+ + K^+)output$	51.1 ± 25.8 mmol	
Volume intake (standardized, water)	0.400 ± 0.000 L	
Volume output	0.472 ± 0.168 L	
Estimated plasma [Na^+] changes 5-240 minutes after infusion		**Observed change**
$[Na^+]plasma_{post}$	140.5 ± 2.2 mmol/L	139.6 ± 1.3 mmol/L
$\Delta[Na^+]plasma$	-0.9 ± 0.4 mmol/L	-1.8 ± 1.7 mmol/L

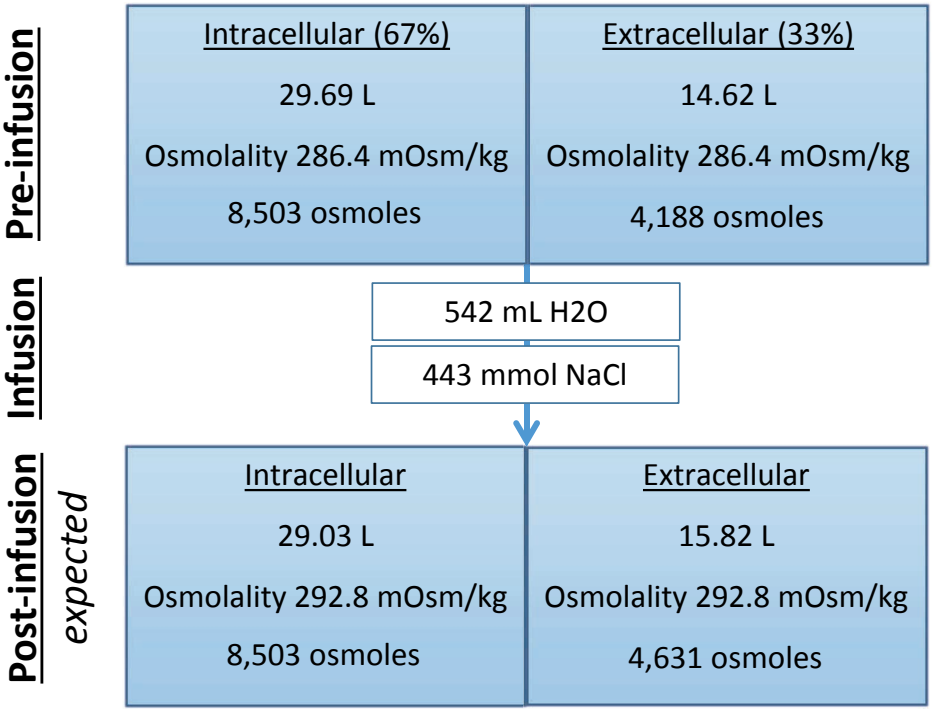
2C. Comparison of observed and estimated urinary Na^+/K^+ loss between 5 and 240 minutes after infusion.

Rearranged Nguyen-Kurtz formula:

$$(\mathrm{Na} + \mathrm{K\ input}) - (\mathrm{Na} + \mathrm{K\ output}) = \frac{(([\mathrm{Na}]\mathrm{plasma}_{\mathrm{pre}} + 23.8) * \mathrm{TBW}) - ((23.8 + [\mathrm{Na}]\mathrm{plasma}_{\mathrm{post}}) * (\mathrm{TBW} + \Delta\mathrm{Volume}))}{-1.03}$$

Observed parameters 5 - 240 minutes after infusion			
$[Na^+]plasma_{pre\ (5\ min\ after\ infusion)}$		141.5 ± 2.0 mmol/L	
$TBW_{\ with\ infusion}$		45.0 ± 4.2 L	
$[Na^+]plasma_{post}$		139.6 ± 1.3 mmol/L	
Volume intake	(standardized, water)	0.400 L	
Volume output		0.472 ± 0.168 L	
$(Na^+ + K^+)input$		0 mmol	
Estimated urine [$Na^+ + K^+$] loss 5-240 minutes after infusion			**Observed urinary loss**
$(Na^+ + K^+)output$		92.4 ± 85.4 mmol	51.1 ± 25.8 mmol

Supplemental Figure 1.



TBW: 44.31 + 0.54 = 44.85 L

TBW osmoles: 12,691 + 443 = 13,134 osmoles

Expected values at 5 minutes

TBW osmolality (TBW osmoles/TBW): 13,134 / 44.85 = 292.8 mOsm/kg

ECV osmoles (ECV osmoles pre-infusion + infused osmoles) 4,188 + 443 = 4,631 osmoles

ECV: (ECV osmoles/expected ECV osmolality): 4,631 / 292.8 = 15.82 L

ICV: (TBW after infusion – expected ECV after infusion) 44.85 – 15.82 = 29.03 L

Δ ECV: 15.82 - 14.62 = 1.20 L

Δ ICV: 29.03 - 29.69 = -0.66 L

Supplemental Figure 1 continued.

Post-infusion *observed*

Intracellular	Extracellular
29.06 L	15.79 L
Osmolality 293.3 mOsm/kg	Osmolality 293.3 mOsm/kg
8,503 osmoles	4,631 osmoles

Observed values at 5 minutes *based on observed osmolality of 293.3 mOsm/kg*

ECV: (ECV osmoles/observed ECV osmolality): 4,631 / 293.3 = 15.79 L

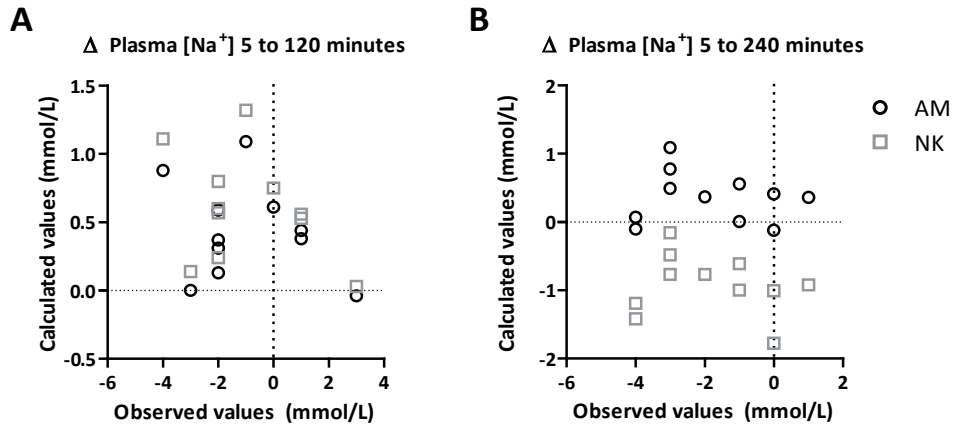
ICV: (TBW after infusion - observed ECV after infusion): 44.85 – 15.79 = 29.06 L

Δ ECV: 15.79 - 14.62 = 1.17 L

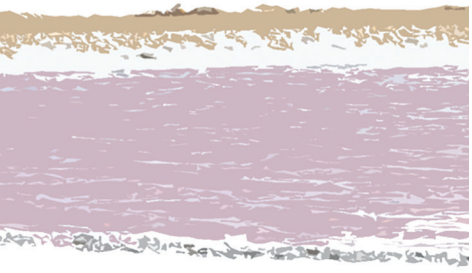
Δ ICV: 29.06 - 29.69 = -0.63 L

ECV, extracellular volume; ICV, intracellular volume; TBW, total body water

Supplemental Figure 2.



(A) No correlation was present between the observed plasma [Na^+] changes after two hours, from 5 minutes after infusion, and the values that were calculated using the Adrogue-Madias (AM) (R^2=0.07, P=0.42) and Nguyen-Kurtz formula (NK) (R^2=0.12, P=0.30). (B) The Adrogue-Madias (R^2=0.01, P=0.72) and Nguyen-Kurtz formula (R^2=0.04, P=0.58) were not correlated with observed changes after four hours.



4 High Salt Intake Affects Sublingual Microcirculation and Is Linked to Body Weight Change In Healthy Volunteers: a Randomized Cross-Over Trial

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ABSTRACT

Background: The pathophysiology of salt-sensitive hypertension remains uncertain, but may involve microvascular alterations. High-salt intake decreases microvascular density in hypertensive patients, but due to lack of studies in normotensive patients the causal pathway remains unclear. We studied whether high-salt intake decreases sublingual microvascular density in normotensive individuals and assessed the influence of body weight on changes in microvascular density.

Methods: In an open label randomized cross-over trial 18 healthy men were included to study the effect of a 2 week high-salt (>12 g/day) and low-salt (<3 g/day) diet on microvascular (diameter <20μm) density with sublingual sidestream darkfield imaging. We used sublingual nitroglycerin (NTG) to recruit microvessels.

Results: There was no significant difference in microvascular density between diets (0.96±3.88mm/mm^2; P=0.31, following NTG; and -0.03±1.64mm/mm^2; P=0.95, without NTG). Increased salt intake was correlated with a decrease in microvascular density following NTG (r=-0.47; P=0.047), but not without NTG (r=0.06; P=0.800). The decrease in microvascular density following high-salt intake was significantly larger for those with a large change in body weight as compared with those with a small changer in body weight (-0.79±1.35 and 0.84±1.56mm/mm^2 respectively, P=0.031).

Conclusion: We demonstrate in healthy volunteers that higher salt intake is correlated with decreased sublingual microvascular density following administration of NTG and; larger changes in body weight following high-salt intake coincide with a larger decrease in microvascular density. Changes in microvascular density occurred without blood pressure effects, indicating that high-salt load as such contributes to microvascular changes, and may precede hypertension development.

INTRODUCTION

Daily dietary salt intake exceeds recommended maximum levels[1], and has been linked to hypertension and increased cardiovascular risk[2]. However, not everyone responds to high salt intake with a blood pressure (BP) increase[3]. The trait characterized by a BP increase following high-salt intake is known as salt-sensitivity, as opposed to salt-resistance[4]. The pathophysiology of salt-sensitivity remains to be elucidated. Until recently it was thought that in salt-sensitive subjects hypertension is caused via renal salt retention, leading to an increase in extracellular fluid volume (ECFV) and cardiac output (CO). Contrarily, recent studies have demonstrated that an increase in CO is also seen in salt-resistant subjects, but that a compensatory reduction in systemic vascular resistance (SVR) is impaired in salt-sensitive subjects[5, 6]. Nitric oxide (NO) mediated pathways might explain this differential response in SVR, as activity of this endogenous vasodilator is generally decreased after high-salt intake, but is more extensively reduced in salt-sensitive subjects[7,8]. Theoretically this NO response could lead to a decrease in microvascular density that is demonstrated both in hypertensive patients[9-12] and following high-salt intake in both hypertensive and normotensive subjects[13-16]. This decrease in microvascular density can either be structural (anatomical absence of vessels) or functional (vessels are anatomically present yet not perfused), or both[9]. As reduction of microvascular density increases SVR[17], this phenomenon could be the missing link in understanding the mechanism of salt-sensitivity. It is yet unknown whether high salt intake causes reduction of microvascular density in normotensive patients, and therefore the causal relationship with hypertension remains unclear. An alternative explanation for the differential response in SVR could involve recent findings of sodium compartmentalization. Sodium can be stored in various tissues without commensurate water retention and subsequent expansion of the ECFV[18]. Whereas salt-resistant individuals have this capacity for nonosmotic sodium storage, this seems perturbed in those who are salt-sensitive[6], resulting in weight gain and high SVR following salt loading via mechanisms not well understood[6]. So far, it has not been studied whether ECFV changes and microvascular alterations are related.

Improvements of imaging modalities grand the opportunity to have a closer look into the relationship between high-salt intake and the microcirculatory changes. The aim of this study was to determine whether high-salt intake causes a reduction of sublingual microvascular density in normotensive subjects. Though not the primary aim of this study, in light of newly emerged evidence regarding nonosmotic sodium storage, we also assessed the effect of sodium-induced body weight changes on the relationship between salt intake and microvascular density.

METHODS

We studied healthy male volunteers between 18 and 40 years old in an open label randomized crossover trial. The study was performed at the Academic Medical Center Amsterdam between October 2016 and April 2017 according to the principles of the Declaration of Helsinki[19]. Eligibility and exclusion criteria are presented in the supplemental material (S1). Volunteers were recruited via local advertisement and provided informed written consent. The protocol was approved by the local ethics committee and registered at the Netherlands trial registry (NTR4785;http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=4785).

All subjects were asked to subsequently adhere to a high-salt diet (>12 g/d) and low-salt diet (<3 g/d) for 14 days each in randomized order. Randomization was performed by the research coordinator via sealed, opaque envelopes in blocks of four after assessment of eligibility and signing informed consent. There was no washout period between diets. Dietary compliance was verified at day 7 and day 11 with collection of 24-hour urine. On day 15 after an overnight fast subjects visited our research department for measurement of microvascular density, hemodynamic parameters and laboratory testing.

The primary aim of this study was to assess a difference in sublingual microvascular density after a high-salt diet compared to a low-salt diet, with participants serving as their own controls. Microvascular densities were measured sublingually with Sidestream Dark-Field (SDF) videomicroscopy (Microscan; Microvision Medical B.V. Amsterdam) that captures hemoglobin in passing red blood cells with green light emitting diodes (540 nm). Therefore, vessels filled with red blood cells are captured, but non-perfused vessels are not. In order to visualize and maximize the residual capacity of the sublingual microcirculation (structural vessel density), vasodilation was induced via one dose of 0.4 mg sublingual nitroglycerin (NTG)[20].

Videos were assessed for sufficient quality (supplemental methods S3)[21].Video-image analysis was performed with the operator blinded for the characteristics of the participants using a semi-automated analysis program (Automated Vessel Analysis (AVA) 3.2) and in concordance with the 2007 consensus statement[22]. With AVA 3.2 densities are measured as vessel-length per surface (mm/mm^2) for vessels with diameters less than 20 μm (TVD_{small}) and all vessels with diameters less than 150 μm ($TVD_{all\ vessels}$). Microvessels with diameters less than 20 μm are mostly capillaries, therefore we used TVD_{small} to answer our hypothesis. $TVD_{all\ vessels}$ mostly consists of venules and is considered a quality check[22]. The video analyst ranked the flow of erythrocytes through the vessels from no flow to continuous flow. The proportion of vessels with flow is expressed as PPV_{small} (% of vessels with diameters <20 μm with

flow) and $PPV_{all\ vessels}$ (% of all vessels with flow). This proportion is multiplied with TVD to assess density of vessels with flow: perfused vessel density $(PVD)_{small}$ (density of vessels with diameters <20 μm with flow) and $PVD_{all\ vessels}$ (density of all vessels with flow). Finally microvascular flow index was measured for all vessels and for small vessels, in which flow is ranked per video quadrant. More detailed methods regarding SDF analysis are provided in the Supplement (S3).

Hemodynamic parameters were measured after both diets. Seated systolic, diastolic BP and heart rate (mean of the last 2 measurements) were done after five-minute rest at the non-dominant arm with an automated device (Omron M4 oscillometric device, OMRON Healthcare Europe B.V., Hoofddorp, The Netherlands). On day 14 of both diets 24h ambulatory systolic and diastolic BP, mean arterial pressure and heart rate were recorded at 15 minute daytime intervals and 30 minute nighttime intervals (Mobil-O-Graph 24h PWA Monitor, I.E.M. GmbH, Stolberg, Germany). The cuff was secured at the non-dominant arm. When arm circumference exceeded 32 cm a large cuff was used.

Laboratory testing included plasma sodium, potassium and creatinine, and analysis of 24h urinary sodium, potassium and creatinine levels. All biochemical tests were performed on a COBAS C8000 Modular Analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

Subjects were stratified to groups with a large and small salt induced increase in weight, to assess the effect of sodium-induced body weight changes on the relationship between salt intake and microvascular density.

Sample size calculations were based on results of a previously conducted pilot study, showing that 18 participants were needed to detect a mean difference of 8.4% (standard deviation (SD) of 12.0) in microvascular density measured with SDF-videomicroscopy, with 80% power using a 2-sided paired t-test at the 0.05 significance level. Continuous variables are reported as mean and SD, or as median and interquartile range (IQR) if the data were not normally distributed. We checked for period and carry-over effects (supplemental methods S4)[23].

To compare the outcomes between diets, paired t-tests or Wilcoxon signed ranks tests were used. Pearson correlation was used to test the correlation between the change in sodium excretion and the change in microvascular density between diets. Subjects were stratified by median-split for amount of weight change. One-sample t-tests or Wilcoxon rank sum tests were used to compare salt-induced changes in microvascular density between groups. As SDF imaging captures hemoglobin, we suspected that salt-induced differences in hematocrit levels could influence our results. Therefore, we repeated these tests with a correction for hematocrit levels in a linear mixed model. A 2-tailed P less than 0.05 was considered statistically significant.

Statistical analyses were done in Rstudio (Version 1.0.136; RStudio, Inc., Boston, Massachusetts, USA).

RESULTS

Between October 2016 and April 2017 we included 18 subjects, 10 subjects were randomized to start with the high-salt diet, and 8 to start with the low-salt diet. The mean (SD) age was 29(5) years, and baseline BP was 118(8)/73(5) mmHg. Baseline characteristics are shown in Table 1.

Table 1. Baseline characteristics.

Healthy male subjects (n=18)	
Age (yrs)	29.3 (4.5)
Weight (kg)	80.6 (8.5)
BMI (kg/m^2)	24.4 (2.6)
Waist-to-hip ratio	0.92 (0.04)*
Ethnicity	16 European descent, 2 Arabic descent
Plasma	
Sodium (mmol/L)	140 (3)*
Creatinine (umol/L)	84 (11)*
eGFR (ml/min/1.73m^2)	106 (12)
Osmolality (mmol/L)	293 (2)
Glucose (mmol/L)	5.0 (1.0)
24h Urine	
Volume (mL/24 h)	2262 (1269)*
Creatinine (umol/ 24 h)	17.0 (4.6)
Sodium (mmol/ 24 h)	150 (77)*
Potassium(mmol/ 24h)	83 (49)*
Creatinine clearance (mL/min)	141 (42)
Office BP	
Systolic BP (mmHg)	118 (8)
Diastolic BP (mmHg)	73 (5)
Mean arterial pressure (mmHg)	88 (5)
Heart rate (bpm)	65 (8)

*All values are expressed as mean (SD) unless otherwise marked. *Values are presented as median (interquartile range). BP, blood pressure. eGFR, estimated glomerular filtration rate*

Mean 24h urinary sodium and creatinine excretion indicated overall compliance to both diets and complete urine sampling. Salt excretion was 15.2 g/d following high-salt intake and 2.3 g/d after low salt intake (Table 2).

An unintended effect of salt intake reduction was decreased 24 hour potassium excretion (P=.02; Table 2). The difference in weight between diets was 1.8 (0.9) kg (P<.0001). No differences in BP were observed (Table 2), irrespective of diet sequence. We found no differences in microvascular density between diets (Supplemental file S5). Mean microvascular density following NTG was slightly higher following the low-salt diet, but the difference between diets was not significant (P=.31, S5). No period or carry-over effects were detected. Adjustment for plasma hematocrit did not affect these results (data not shown).

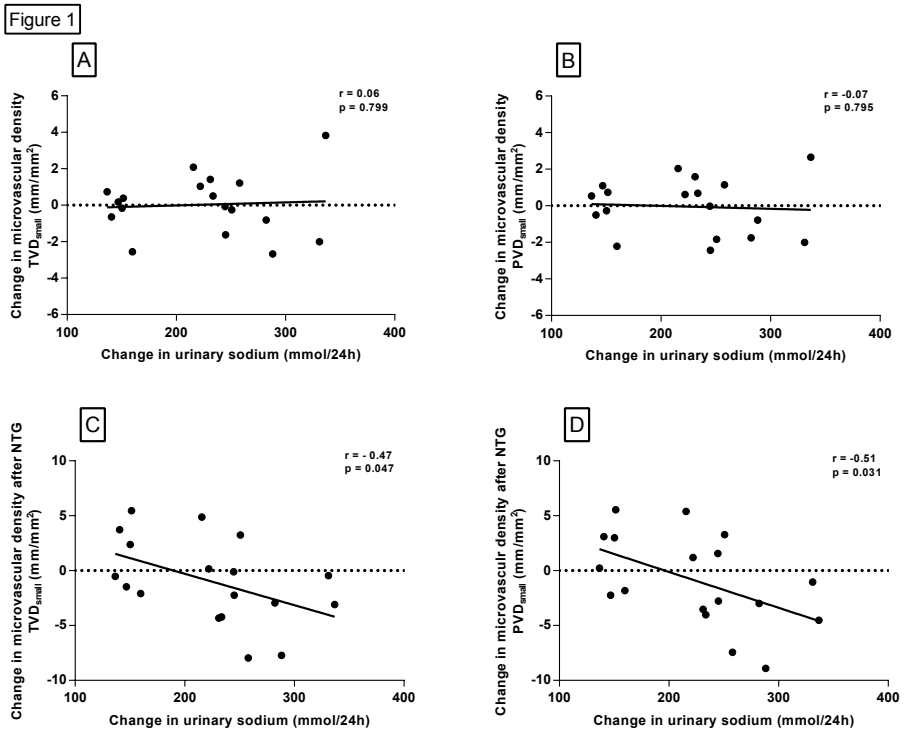
Table 2. Outcome measurements after high-salt and low-salt diet.

	High-salt diet (n=18)	Low-salt diet (n=18)	P-value
Weight (kg)	80.8 (8.1)	78.1 (8.4)	P<.0001
Plasma			
Sodium (mmol/L)	141 (1)	140 (2)	P=.13
Potassium (mmol/L)	3.9 (0.2)*	4.0 (0.4)*	P=.092
Osmolality (mOsm/kg)	293 (3)	291 (3)	P=.020
Hematocrit (L/L)	0.44 (0.03)	0.44 (0.02)	P=0.71
24h urine			
Volume (mL/24h)	2111 (1051)*	1973 (555)*	P=.40
Creatinine (umol/ 24h)	17.3 (2.8)	16.9 (3.3)	P=.80
Sodium (mmol/ 24h)	264 (65)*	40 (19)*	P<.0001
Potassium (mmol/ 24h)	114 (21)*	91 (29)*	P=.022
Creatinine clearance (mL/min)	157 (23)	128 (18)	P<.0001
Office BP			
Systolic BP (mmHg)	114 (10)	110 (8)	P=.022
Diastolic BP (mmHg)	70 (7)	71 (5)	P=.71
Mean arterial pressure (mmHg)	85 (7)	84 (5)	P=.50
Heart rate (bpm)	60 (8)	62 (10)	P=.35
Ambulatory BP measurement (24-hours)			
Daytime	*(n=17)[1]*	*(n=17)[1]*	
Systolic BP (mmHg)	124 (6)	123 (6)	P=.47
Diastolic BP (mmHg)	74 (7)	73 (5)	P=.66
Mean arterial pressure (mmHg)	95 (5)*	96 (7)*	P=.82
Heart rate (bmp)	65 (6)	64 (6)	P=.098
Nighttime	*(n=16)[2]*	*(n=15)[3]*	
Systolic BP (mmHg)	110 (10)	108 (8)	P=.91
Diastolic BP (mmHg)	61 (7)	60 (5)	P=.72
Mean arterial pressure (mmHg)	83 (7)	82 (6)	P=.64
Heart rate (bmp)	55 (6)	53 (8)	P=.22
Nightly dipping systolic (%)	12.4 (5.5)	11.7 (4.9)	P=.29
Nightly dipping diastolic (%)	16.4 (7.5)	17.7 (5.5)	P=.67

*All values are expressed as mean (SD) unless otherwise marked. *Values are presented as median (interquartile range). [1]One subject had no daytime measurement after both high and low salt diet due to device malfunction. [2]Two subjects had no nighttime measurements after high salt diet due to device malfunction. [3]Three subjects had no nighttime measurements after low salt diet due to device malfunction*

There was no correlation between the increase in 24h urine sodium excretion (i.e. salt exposure) and the change in microvascular density before administration of NTG (Figure 1A and B), but there was a significant correlation between the increase of 24h urine sodium excretion and the decrease in microvascular density following nitroglycerin administration (Figure 1C and D).

Figure 1. Scatterplots of the correlation between the change in 24h urinary sodium excretion and the change in microvascular density.



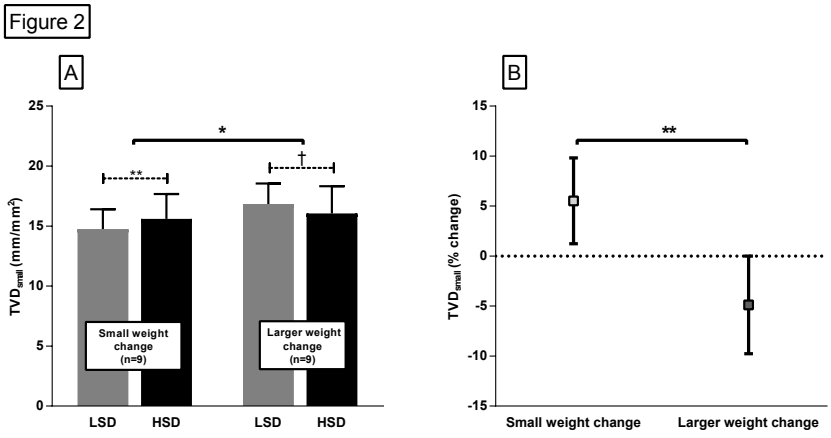
A+B) *There was no significant correlation between change in 24h urinary sodium (i.e. salt exposure) and the change in microvascular density for TVD_{small} or PVD_{small}.*

C+D) *There was a significant correlation between the increase in 24h urinary sodium and decrease of microvascular density following nitroglycerin administration, for both TVD_{small} and PVD_{small}.*

NTG, nitroglycerin; PVD_{small}, perfused vessel density: diameter less than 20μm; TVD_{small}, total vessel density: diameter less than 20μm.

When subjects with a small change in weight were compared to subjects with a larger change in weight, the change in microvascular density (TVD_{small}) before administration of NTG differed significantly between groups (Δ1.63 (1.43), P=0.031, Figure 2A and B). Whereas we found an increase in microvascular density following the high-salt diet in the group with a small salt-induced change in weight (P=0.008), a decrease in microvascular density was present in the group with a larger salt-induced change in weight (P=0.054). In line with these results, the weight change of all subjects demonstrated significant correlation with the change in microvascular density (TVD_{small}; Figure 3). There was no correlation between weight change and the change in microvascular density following NTG (Figure 3). Baseline characteristics, 24 hour urine excretion and microvascular density following NTG did not differ significantly between subjects with a small change in weight and those with larger change in weight (Table 3).

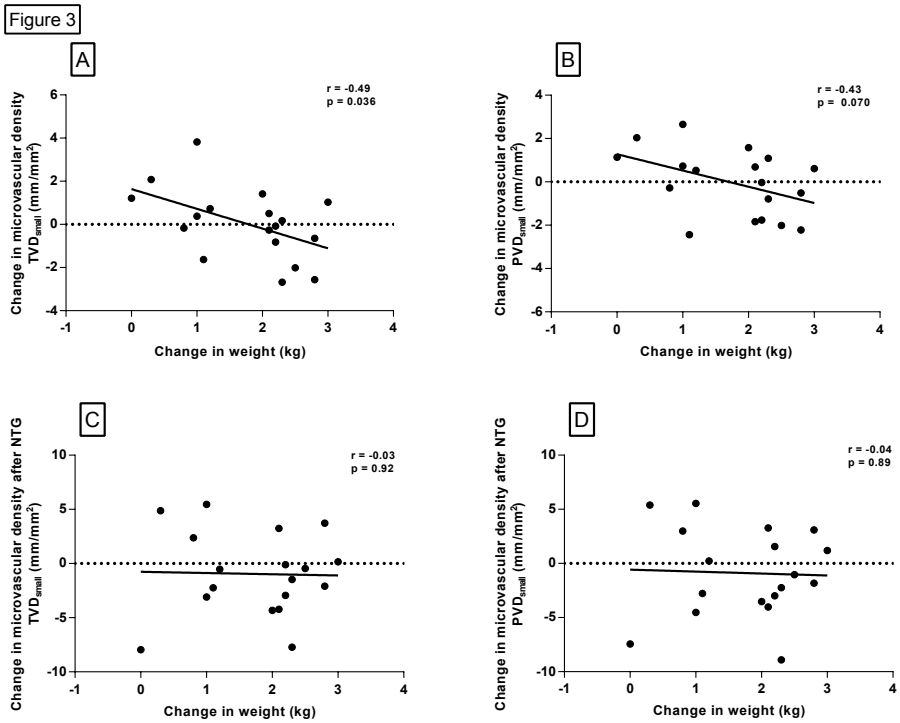
Figure 2. Results of the stratified analysis. Groups were defined via median-split for salt-induced change in weight.



A) *Salt induced change in microvascular density. Microvascular density after high-salt diet increases in the group with a small salt-induced change in weight and decreases in the group with a larger salt-induced change in weight. The change microvascular (TVD_{small}) differed significantly between groups. Bars and whiskers signify mean and SD.*

B) *Percentual change of salt induced change in microvascular density. The change in microvascular (TVD_{small}) differed significantly between groups. Data are represented as means and SD. LSD = Low-salt diet (<3 g/d), HSD = High-salt diet (>12 g/d), PVD_{small}, perfused vessel density: diameter less than 20μm; TVD_{small}, total vessel density: diameter less than 20μm. ** P < 0.01, * P < 0.05, † P< 0.10*

Figure 3. Scatterplots of the correlation between the change in weight and the change in microvascular density.



A) *There was a significant correlation between the salt induced change in weight and microvascular density (TVD_{small})*

B) *There was no significant correlation between the change in weight and microvascular density (PVD_{small})*

C+D) *There was no correlation between the change in weight and microvascular density after administering nitroglycerin spray) for TVD_{small} and PVD_{small}.*

NTG, nitroglycerin; PVDsmall, perfused vessel density: diameter less than 20µm; TVDsmall, total vessel density: diameter less than 20µm.

Table 3. Difference in outcome measurements between groups stratified for weight change.

Δ High-salt diet - Low-salt diet	Group with small change in weight (n=9)	Group with larger change in weight (n=9)	P-value
Weight (kg)	1.1 (0.7)	2.5 (0.3)	NA
Plasma			
Sodium (mmol/L)	0 (2)	2 (3)	P=.63
Potassium (mmol/L)	-0.1 (0.4)	-0.3 (0.3)	P=.53
Osmolality (mmol/L)	1 (3)	2 (3)	P=.59
Hematocrit (L/L)	-0.01 (0.02)	0.00 (0.01)	P=1.0
24h Urine			
Volume (mL/24h)	298 (977)	93 (983)	P=.66
Creatinine (umol/ 24h)	-0.4 (2.3)	0.8 (3.3)	P=.40
Sodium (mmol/ 24h)	283 (100)	253 (120)	P=.57
Potassium (mmol/ 24h)	21 (39)	22 (36)	P=.93
Creatinine clearance (mL/min)	30 (25)	29 (16)	P=.96
Office BP			
Systolic BP (mmHg)	4 (6)	2 (6)	P=.63
Diastolic BP (mmHg)	-1 (6)	-1 (4)	P=.99
Mean arterial pressure (mmHg)	1 (5)	0 (4)	P=.30
Heart rate (bpm)	-3 (6)	0 (7)	P=.82
Ambulatory BP measurement (24-hours, average of day- and nighttime measurements)			
Systolic BP (mmHg)	-1 (7)	3 (5)	P=.13
Diastolic BP (mmHg)	0 (3)	-1 (4)	P=.53
Mean arterial pressure (mmHg)	0 (4)	1 (4)	P=.84
Microvascular density			
Total vessel density small (mm/mm^2)	0.84 (1.56)	-0.79 (1.35)	P=.03
Perfused vessel density small (mm/mm^2)	0.45 (1.71)	-0.55 (1.24)	P=.17
PPV small (%)	-2.49 (4.30)	-0.98 (3.58)	P=.08
MFI small	0.0 (0.0)	0.0 (0.0)	P=.36
Total vessel density all vessels (mm/mm^2)	0.35 (1.40)	-0.97 (1.23)	P<.05
Perfused vessel density all vessels (mm/mm^2)	-0.10 (1.97)	-0.68 (1.25)	P=.46
PPV all vessels (%)	-2.37 (5.03)	1.13 (4.28)	P=.13
MFI all vessels	0.0 (0.2)	0.0 (0.2)	P=.39
Microvascular density following sublingual nitroglycerin			
Total vessel density small (mm/mm^2)	-0.24(4.56)	-1.10 (3.89)	P=.45
Perfused vessel density small (mm/mm^2)	-0.09 (4.68)	-0.81 (3.98)	P=.43
PPV small (%)	0.71 (4.08)	1.50 (5.29)	P=.70
MFI small	0.0 (0.1)	0.0 (0.1)	P=.50
Total vessel density all vessels (mm/mm^2)	-0.35 (4.00)	-0.88 (2.90)	P=.49
Perfused vessel density all vessels (mm/mm^2)	-0.28 (4.22)	-0.63 (3.03)	P=.41
PPV all vessels (%)	0.20 (5.13)	1.36 (5.99)	P=.96
MFI all vessels	0.0 (0.2)	0.0 (0.2)	P=.55

All values are presented as mean (SD). Vessel parameters marked as 'small' have diameters <20μm, representing capillary density. Vessel parameters marked as 'all vessels' include vessels of all sizes and is considered a quality check. BP, blood pressure; MFI = Microvascular flow index; PPV, Proportion of perfused vessels.

DISCUSSION

In this study we aimed to obtain insight in the effect of salt intake on the sublingual microcirculation in healthy men. Overall no microvascular differences were found between the low-salt and high-salt diets. However, the increase in salt consumption from the low to the high salt diet significantly correlated with a lower recruitment rate of sublingual capillaries after administration of NTG, which indicates lower structural microvascular density. In subjects with larger body weight increase following high-salt intake, we observe significantly lower rates of perfused capillaries reflecting impaired functional microvascular density. As both phenomena occur independently of BP effects, our study indicates that high salt load as such contributes to microvascular dysfunction, which is generally considered as an early feature of end-organ damage in various cardiovascular risk patients, including hypertensive patients.

To our knowledge this is the first study that has investigated the effect of salt on microvascular densities in normotensive patients using in vivo sublingual imaging. With the use of other in vivo techniques and in different microvascular tissues, previous studies have demonstrated reduction of capillary density in hypertensive[9,10,24], borderline hypertensive individuals[25,26] and offspring of hypertensive individuals[27,28], suggesting that reduction of microvascular density is an early feature of increased BP. However, these studies did not take sodium intake nor sodium-sensitivity into account as possible contributors to the BP associated microvascular changes. Other studies that did explore the combined effects of sodium, BP and the microcirculation, have shown that sodium-sensitive subjects with borderline hypertension or normal BP had significantly lower capillary density in the conjunctival microvasculature[14] and demonstrate an inverse association of nailfold capillary recruitment and the sodium-sensitive BP response among hypertensive and normotensive subjects[15]. As these studies had a cross-sectional design and did not report dietary sodium status at time of measurements, direct effects of sodium reduction and associated BP response on the microcirculation were not evaluated. So far, data on effects of sodium reduction on microvascular networks are therefore currently available in untreated hypertensive subjects, in either the conjunctival vascular bed[16] or skin capillaries[13]. In contrast to these studies we could not demonstrate salt induced microvascular changes when comparing low vs. high-salt diets in a cross-over fashion. Yet, there was a significant correlation between the increase in salt intake and decrease in microvascular density following administration of NTG. This suggests that salt exposure has negative effects on the recruitment of sublingual microvasculature, as they move together in a linear fashion. Our results are in line with studies in the cremaster muscle of rats in which high-salt intake led to decrease in microvascular densities[29,30]. However, we were unable to detect differences in

microvascular density between diets. The absence of blood pressure effects might be explanatory for our observation that there was no difference in microvascular density when comparing the high-salt diet and low-salt diet. He *et al* [13] have demonstrated an increase in both functional and structural microvascular density following sodium reduction among hypertensive patients. This may be related to the fact that He *et al* reported an increase in microvascular density with a decrease in BP, while we did not find a change in BP.

The data of our stratified analysis are of interest in light of the recently rediscovered concept of nonosmotic sodium storage and the effect of salt on microvascular density. In the current study, we observed that the amount of weight change between diets differed substantially between subjects that, considering the duration of the salt intervention periods, can be attributed to changes in ECFV. Although there was a difference in salt-induced fluid expansion between subjects, there was no difference in salt excretion. This is in line with previous observations of Laffer *et al* [6] who demonstrated that with the same amount of total body sodium, salt-sensitive subjects had an increase in body weight, whereas salt-resistant individuals did not. Our observations with a wide range of weight change for similar salt levels challenge traditional beliefs that salt retention induces iso-osmolar water retention. Via ^{23}Na MRI it was shown that high amounts of nonosmotic sodium storage in striated muscle were associated with hypertension[31].This was further substantiated in studies in mice where disruption of salt efflux from the nonosmotic storage compartment led to high BP[18]. One may hypothesize that saturation of the nonosmotic storage compartment leads to a subsequent smaller capacity for nonosmotic storage of added salt. The following increase in BP may be related to microcirculatory alterations.

We did not observe a difference in BP response and therefore we can only assume that those who showed a larger salt-induced fluid expansion might become more salt-sensitive in terms of BP response at an older age. Furthermore, after administration of NTG microvascular densities in the group with large salt-induced weight gain increased to similar levels of vessel densities measured in the group with small weight changes, dissolving the correlation between weight change and vessel densities. This suggests a relatively larger response to exogenous NO in the group with a larger salt-induced fluid expansion, indicating a decrease of NO activity in those subjects. These results are in line with studies among salt-sensitives. Schmidlin *et al* [8] demonstrated an increase in asymmetric dimethylarginine (ADMA), a NO inhibitor, in salt-sensitives but not in those salt-resistant. Another study rendered similar results measuring plasma NOx (NO metabolites nitrate and nitrite) concentrations[7]. Our results add to their findings and show that NO activity might play a role in the

interaction between microvascular changes and a smaller capacity for nonosmotic sodium storage.

Our study has some limitations. First, we only assessed sublingual microcirculatory parameters. It remains uncertain if our findings can be extrapolated to other microvascular beds. Also, though SDF imaging obtains high resolution images, Incident Dark Field imaging (IDF) is considered to provide images with higher resolution[32]. However, images were graded for sufficient quality, and discarded if necessary. Also, the fact that administering NTG led to significant increase in vessel density suggests that our image quality was sufficient for detecting differences between groups. Finally, our analyses generated similar results in comparison to a study using IDF imaging that also used NTG[20]. Another limitation might be that we did not precisely measure the ECFV, but used an indirect measurement (i.e. body weight). Given the short time frame of our intervention, most of the differences in body weight are likely to be attributable to changes in body water, also because 24-h creatinine excretion levels, reflecting muscle mass, remained similar. Also, we studied healthy male subjects to exclude the influence of menstrual cycle related hormonal changes, and therefore one might question whether our results are applicable to other patient categories. Finally, we found that potassium excretion was also significantly different between both intervention periods. Considering the effects of potassium intake on BP, this may explain why no BP effects were observed. Yet, potassium excretion levels were above WHO recommendations of 90 mmol/d in both interventions, and no associations between potassium excretion and BP or sodium-to-potassium ratio and BP were seen.

Perspectives

We show that increment of dietary salt intake is associated with a reduction in sublingual microvascular density following administration of NTG among healthy men. Our results suggest that the ability of our healthy male volunteers to maintain sufficient microvascular density is vital in maintaining normal BP. We demonstrate that *in vivo* a decrease of sublingual microvascular density is present in subjects with a larger change in body weight which may reflect smaller capacity for nonosmotic sodium storage. Furthermore, when exogenous NO in the form of NTG was administered, no change in microvascular density was present, suggesting that impaired activity of NO may play a role in the link between nonosmotic sodium storage and microvascular alterations. This also may imply that healthy individuals may benefit from dietary sodium reduction before hypertension or microvascular end-organ damage becomes apparent. More research is needed to further assess underlying pathophysiological mechanisms and

longitudinal consequences of the interaction between the microcirculation and salt intake.

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SUPPLEMENTAL FILES

S1. Inclusion and exclusion criteria

1.1 Inclusion criteria

- Male between 18 and 40 years of age
- Healthy, as based on a medical evaluation including medical history, physical examination (PE) and laboratory tests carried out in the screening visit
- Non-treated office BP < 130/85 mmHg
- A body mass index < 30 kg/m²
- Capable of giving written informed consent and able to comply with the requirements and restrictions listed in the informed consent form

1.2 Exclusion criteria

- An office BP > 130/85 mmHg
- A body mass index > 30 kg/m²
- A major illness in the past 3 months or any significant chronic medical illness that the investigator would deem unfavourable for enrolment, including chronic inflammatory diseases
- A history of any type of malignancy within the past 5 years with the exception of successfully treated basal cell cancer of the skin
- A history of any renal disease
- A history of any auto-immune disease
- A history of cardiovascular disease (in the past 6 months) defined as documented coronary artery disease including myocardial infarction, (un-)stable angina pectoris or acute coronary syndrome, percutaneous transluminal coronary angioplasty, coronary artery bypass grafting, cerebrovascular disease including ischemic and hemorrhagic stroke or a subarachnodial bleeding, or peripheral artery disease including aortic aneurysmata
- A history of eye-surgery, glaucoma or retinal eye disorder
- A history, within 3 years, of drug abuse (including benzodiazepines, opioids, amphetamine, cocaine, THC, methamphetamine)
- A history of alcoholism and/or drinking more than 3 units of alcohol per day. Alcoholism is defined as an average weekly intake of >21 units for males. One unit is equivalent to 8 g of alcohol: a half-pint (~240 mL) of beer, 1 glass (125 mL) of wine or 1 (25 mL) measure of spirits
- Smoking or use of tobacco products less than 30 days ago
- Any other issue that in opinion of the investigator could be harmful to the subject or compromise interpretation of data

S2. Dietary intervention

Prior to starting both diets, participants were given instructions on what products in a regular Dutch diet contain high and low concentrations of salt. Also, they were asked to refrain from heavy exercise and drinking alcohol or smoking.

S3. Sidestream Dark-Field (SDF) videomicroscopy

Nitroglycerin induces vasodilation of the sublingual microcirculation and therefore increases the density visible by SDF imaging, as shown previously by Hilty et al[1]. The density of perfused vessels after nitroglycerin was considered to approximate structural vessel density as closely as possible. As SVR is located at the arteriole level, not the venule level, only the increase in arteriole density was of interest. Therefore images of structural vessel density where obtained in a time frame between 3 to 10 minutes after administering nitroglycerin, because the increase in density observed in this time frame was thought to be primarily arteriole vasodilation. Image acquisition and analysis was performed with the 2007 consensus statement as a guideline. We are aware that these guidelines have since been updated[2]. Upon reviewing the new 2018 consensus statement we conclude that during the study we adhered to most recent notions about SDF imaging. At least three images of separate areas of the sublingual microcirculation were obtained. To ensure sufficient image quality two methods were used. Firstly during caption of the videos special caution was taken towards pressure artefacts, and distortion of results due to saliva and movement. Secondly, all videos were scored for quality by a blinded observer using Massey's scoring system and analysis was performed in videos with a score <10. A minimum of 20 subsequent steady frames was considered sufficient, since the measurement of blood cell velocity was not relevant for our study. Video-image analysis was performed blinded, with a semi-automated analysis program (Automated Vessel Analysis (AVA) 3.2). This allowed a primary automatic assessment of vessel density, which was corrected manually. It is important to note that the abbreviations PPV and PVD stand for Proportion of Perfused Vessels, and Perfused Vessel Density respectively. The flow of erythrocytes through the vessels (subjectively scored as: no flow, intermittent flow, continuous or hyperdynamic flow) is considered the measure for perfusion. This is the generally accepted interpretation in studies using SDF. However, truly non-perfused vessels are not visible with SDF, due to its capture of hemoglobin. This has been shown by the increase in visible vessels after nitroglycerin administration[1]. Since the topic of this study was the assessment of the density of perfused and non-perfused vessels via the latter method, using the interpretation generally accepted in literature does not suffice. However, not presenting this data would not be in line with the 2007 concordance statement[4]. Therefore the authors have

decided to explain PPV and PVD as respectively the proportion of vessels with flowing erythrocytes and the density of vessels with flowing erythrocytes, for matters of clarity.

S4. Assessment of period and carry-over effects

To check for period effects, the difference in outcome between diets was calculated separately for the group that started with the low-sodium diet and the group that started with the high-sodium diet. If this differs significantly between diet sequences (independent t-test or Mann Whitney U), period effects should be expected. To check for carry-over effects the average of the outcomes after both diets was calculated for both diet sequences separately. If this differs significantly between diet sequences (independent t-test or Mann Whitney U), carry-over effects should be expected[5].

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Supplemental tables

S5. Sublingual microvessel densities after high and low salt diet.

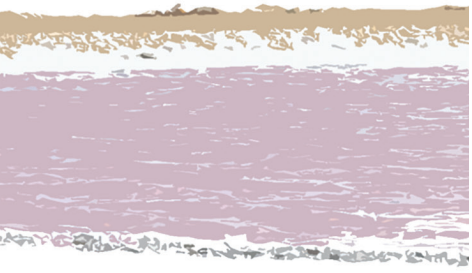
	High salt diet (n=18)	Low salt diet (n=18)	P-value
Microvascular vessel density			
Total vessel density (small) (mm/mm^2)	15.82 (2.12)	15.80 (1.95)	P=.95
Perfused vessel density (small) (mm/mm^2)	14.83 (2.10)	14.88 (2.00)	P=.86
PPV (small) (%)	95.23 (6.98)	96.29 (5.89)	P=.76
MFI (small)	2.90 (0.16)	2.94 (0.17)	P=.26
Vessel density (all vessels) (mm/mm^2)	18.03 (1.97)	18.35 (2.19)	P=.37
Perfused vessel density (all vessels) (mm/mm^2)	16.94 (2.10)	17.33 (2.31)	P=.32
PPV (all vessels) (%)	95.62 (7.09)	96.16 (5.75)	P=.60
MFI (all vessels)	2.99 (0.11)	3.00 (0.20)	P=.10
Microvascular vessel density after nitroglycerin			
Total vessel density (small) (mm/mm^2)	18.23 (2.47)	19.19 (2.84)	P=.31
Perfused vessel density (small) (mm/mm^2)	17.44 (3.06)	18.48 (2.96)	P=.27
PPV (small) (%)	96.09 (8.47)	97.07 (3.79)	P=.34
MFI (small)	3.00 (0.09)	3.00 (0.06)	P=.27
Vessel density (all vessels) (mm/mm^2)	20.99 (2.11)	21.94 (2.72)	P=.27
Perfused vessel density (all vessels) (mm/mm^2)	20.21 (2.35)	21.29 (3.01)	P=.33
PPV (all vessels) (%)	96.48 (7.49)	97.15 (5.25)	P=.27
MFI (all vessels)	3.00 (0.07)	3.00 (0.12)	P=.27

*All values are expressed as mean (SD) unless otherwise marked. *Values are presented as median (interquartile range). Differences between diets were tested with paired t-test or Wilcoxon signed ranks test. Vessel parameters marked as 'small' have diameters <20µm. Vessel parameters marked as 'all vessels' include vessels of all sizes. MFI = Microvascular flow index; PPV = Proportion of perfused vessels.*

S6. Baseline characteristics after stratification for weight change.

	Group with a small salt-induced change in weight (n=9)	Group with a larger salt-induced change in weight (n=9)	P-value
Age (yrs)	29.8 (4.1)	27.6 (3.4)	P=.28
Weight (kg)	82.2 (6.2)	77.8 (10.9)	P=.34
BMI (kg/m2)	24.17 (2.32)	24.07 (3.06)	P=.95
Waist-to-hip ratio*	0.94 (0.03)	0.91 (0.04)	P=.12
Ethnicity (white)	9	9	
Plasma			
Sodium (mmol/L)	140 (1)	141 (1)	P=.06
Creatinine (umol/L)	82 (11)	88 (9)	P=.20
eGFR (ml/min/1.73m2)	108 (13)	103 (11)	P=.51
Osmolality	293 (2)	293 (2)	P=.50
Ureum (mmol/L)	5.2 (1.5)	4.9 (0.9)	P=.61
24h Urine			
Volume (mL/24h)	2763 (1477)	2168 (804)	P=.34
Creatinine (umol/ 24h)	18.1 (4.4)	17.1 (4.5)	P=.65
Sodium (mmol/ 24h)	158 (82)	181 (68)	P=.55
Potassium(mmol/ 24h)	91 (35)	98 (39)	P=.70
Ureum (mmol/ 24h)	597 (405)	453 (126)	P=.36
Creatinine excretion (mL/min)	158 (50)	134 (30)	P=.28
Office BP			
Systolic BP (mmHg)	118 (9)	119 (8)	P=.82
Diastolic BP (mmHg)	73 (6)	73 (5)	P=.97
Mean arterial pressure (mmHg)	88 (7)	88 (5)	P=.93
Heart rate (bpm)	63 (10)	63 (7)	P=.92

All values are presented as mean (standard deviation). Differences between groups were tested by means of an independent sample t-test. BP; blood pressure, eGFR; estimated glomerular filtration rate.



5 Microvascular Permeability after an Acute and Chronic Salt Load in Healthy Subjects: A Randomized Open-label Crossover Intervention Study

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ABSTRACT

Background: Sodium-induced microcirculatory changes, endothelial surface layer alterations in particular, may play an important role in sodium-mediated blood pressure elevation. However, effects of acute and chronic sodium loading on the endothelial surface layer and microcirculation in humans have not been established. The objective of this study was to assess sodium-induced changes in blood pressure and body weight as primary outcomes and also in microvascular permeability, sublingual microcirculatory dimensions, and urinary glycosaminoglycan excretion in healthy subjects.

Methods: Twelve normotensive males followed both a low-sodium diet (less than 50 mmol/day) and a high-sodium diet (more than 200 mmol/day) for eight days in randomized order, separated by a crossover period. After the low-sodium diet, hypertonic saline (5 mmol sodium/liter body water) was administered intravenously in 30 min.

Results: Both sodium interventions did not change blood pressure. Body weight increased with 2.5 (95% CI, 1.7 to 3.2) kg ($P < 0.001$) after dietary sodium loading. Acute intravenous sodium loading resulted in increased transcapillary escape rate of ^{125}I-labeled albumin (2.7 [0.1 to 5.3] % cpm $\cdot$ g^{-1} $\cdot$ h^{-1}; $P = 0.04$), whereas chronic dietary sodium loading did not affect transcapillary escape rate of ^{125}I-labeled albumin (−0.03 [−3.3 to 3.2] % cpm $\cdot$ g^{-1} $\cdot$ h^{-1}; $P = 1.00$), despite similar increases of plasma sodium and osmolality. Acute intravenous sodium loading coincided with significantly increased plasma volume, as assessed by the distribution volume of albumin, and significantly decreased urinary excretion of heparan sulfate and chondroitin sulfate. These changes were not observed after dietary sodium loading.

Conclusions: Our results suggest that intravenous sodium loading has direct adverse effects on the endothelial surface layer, independent of blood pressure.

INTRODUCTION

High dietary sodium intake is associated with increased blood pressure,[1] a major cause of premature death worldwide. The adverse effects of sodium are partly explained by expansion of extracellular volume and direct effects of sodium on the vessel wall,[2] but the underlying mechanisms are not fully clarified yet. Microvascular alterations, such as rarefaction, remodeling, and endothelial dysfunction, are known to be associated with high blood pressure and end organ disease.[3] Because sodium is known to induce these microcirculatory changes, this might therefore explain, at least in part, the relation between sodium and high blood pressure. In humans, high sodium intake leads to a reversible decrease of skin capillary density in hypertensive subjects,[4,5] and in normotensive subjects, high-sodium diet results in impaired endothelial function.[6–8] Other studies have shown improvement of endothelial function after dietary sodium restriction, both in normotensive and hypertensive subjects.[9,10]

Several studies have demonstrated that glycosaminoglycans in tissues such as skin and cartilage influence sodium homeostasis *via* nonosmotic storage of sodium.[11,12] This could indicate an important role for the microcirculatory endothelial surface layer in sodium handling because the endothelial surface layer is known to consist of different glycosaminoglycans. It is conceivable that nonosmotic sodium storage within these endothelial surface layer glycosaminoglycans may affect extracellular volume as represented by body weight and blood pressure changes after sodium loading.[13] In addition, impairment of the endothelial surface layer decreases its vascular barrier function and induces protein extravasation and tissue edema, loss of nutritional blood flow, and an increase in platelet and leukocyte adhesion.[14] A perturbed endothelial surface layer has been demonstrated in different disease states that are characterized by blood pressure alterations and increased vascular permeability,[15] including kidney disease,[16,17] diabetes,[18,19] atherosclerosis,[20] inflammation,[21] and hypervolemia.[22] Several intravenous fluids, both colloids and crystalloids, have been shown to increase shedding of endothelial surface layer constituents and impact microvascular permeability.[22,23] Moreover, *in vitro* studies have shown that sodium overloading increases endothelial surface layer stiffness and decreases endothelial surface layer height,[24] yet the effects of sodium loading on the endothelial surface layer in humans, either by chronic dietary sodium or acute sodium loading by means of intravenous administration of saline, have not been established. Therefore we hypothesized that an acute intravenous sodium load and a chronic dietary sodium load differently affect blood pressure, the endothelial surface layer, and microcirculation.

MATERIAL AND METHODS

Study population

In this experimental nonblinded crossover intervention study, we included healthy, nonsmoking, male volunteers between 18 and 40 yr old of age. Exclusion criteria were hypertension (greater than or equal to 140/90 mmHg), obesity (body mass index greater than or equal to 30 kg/m^2), and history of primary hyperlipoproteinemia, coagulation disorders, and renal or cardiovascular diseases. The study was performed at the Academic Medical Center in Amsterdam, The Netherlands, between September 2013 and October 2014 and was conducted according to the principles of the Declaration of Helsinki (originally adopted by the 18th World Medical Association General Assembly, Helsinki, Finland, June 1964, with last amendment in Fortaleza, Brazil, October 2013). All participants provided written informed consent, and approval was obtained from the local ethics committee. The study was registered in the Netherlands Trial Register (NTR4095;http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=4095; accessed April 23, 2017). Recently our group has published a manuscript that answered an ancillary research question regarding quantification of nonosmotic sodium storage capacity, using data that were also obtained during this study.[25]

Study design

All subjects were enrolled by one of the research physicians and randomized to both a low-sodium diet (less than 50 mmol daily) and a high-sodium diet (more than 200 mmol daily) for eight days, separated by a crossover period of at least 1 week. Block randomization was performed after the screening visit and informed consent by the research physician (block size: n = 6); six subjects started with the low-sodium diet, and six others commenced the study with high-sodium diet. Dietary compliance was verified at days 3, 6, and 8 with collection of 24-h urine samples. After an overnight fast, subjects visited our research department at day 8 of both diets for blood and urine sampling, hemodynamic and microcirculatory measurements. Subjects were instructed to refrain from alcohol intake and heavy physical exercise 24 h before the study visit and to avoid caffeine intake 12 h in advance. At the study visit, two intravenous catheters were placed in the left and right antecubital veins. In the afternoon of day 8 of the low-sodium diet, hypertonic saline (≈2.4% NaCl) was administered intravenously during 30 min. We corrected the infused amount of sodium for total body water (5 mmol sodium/liter body water) by adding a calculated volume of 20% NaCl solution to 500 ml of 0.9% NaCl. Before infusion, subjects were requested to empty their bladder. Blood and urine sampling and hemodynamic and microcirculatory measurements were carried out at fixed time intervals up to 4 h after infusion.

The primary outcome of the study was the extracellular volume, as represented by body weight and blood pressure. Hemodynamic measurements were carried out with semiautomated devices for blood pressure measurement and finger arterial pulse contour analysis (see "Hemodynamic Measurements"). Secondary outcomes consisted of microcirculatory and endothelial surface layer measurements, including the transcapillary escape rate of ^{125}I-labeled albumin (TERalb), representing microvascular permeability,[19] and sublingual sidestream darkfield (SDF) imaging, measuring perfused boundary region, reflecting endothelial surface layer thickness. In addition, endothelial surface layer constituents in the urine were measured. Urinary radioactivity after ^{125}I-labeled albumin administration was indicative for the endothelial surface layer glomerular barrier function. Finally, SDF imaging was also used to quantify red blood cell (RBC) filling and microvascular density, *i.e.*, measures of microvascular perfusion.

Hemodynamic measurements

Systolic blood pressure, diastolic blood pressure, and heart rate were measured at the right upper arm with an appropriate adjusted cuff size in both seated and supine positions with a semiautomated oscillometric device (Omron 705 IT, OMRON Healthcare, The Netherlands) after resting for at least 10 min in a quiet and temperature-controlled room. The mean of the last two measurements was used for analysis. Mean arterial pressure was calculated as 1/3 × (systolic blood pressure) + 2/3 × (diastolic blood pressure). Pulse pressure was expressed as the difference between systolic blood pressure and diastolic blood pressure. Blood pressure, heart rate, mean arterial pressure, cardiac output, systemic vascular resistance, stroke volume, and an index of left ventricular contractility (dP/dt) were also measured in supine position at the intermediate phalanx of the left middle finger with finger arterial pulse contour analysis (Nexfin, BMEYE, The Netherlands). These hemodynamic parameters were calculated from the average of a 30-s stable recording period after at least 15 min of supine rest.[26]

Transcapillary escape rate of ^{125}I-labeled albumin

Microvascular permeability was determined after both diets and 1 h after intravenous saline infusion by TERalb. We administered an intravenous bolus of saline solution with 100 kBq ^{125}I-labeled albumin.[19] Blood samples were drawn from the contralateral arm at baseline and after 3, 4, 5, 10, 15, 20, 30, 45, and 60 min. Urine was collected before and after the measurement. Radioactivity in plasma and urine was measured in duplicate with a Wizard^2 2480 Automatic Gamma Counter (PerkinElmer, USA). TERalb was calculated with regression analysis and expressed as percentage decline in plasma radioactivity per hour (% cpm $\cdot$ g^{-1} $\cdot$ h^{-1}). Plasma volume was determined by calculating the y-intercept of the disappearance curve of ^{125}I-albumin, corrected for the injected dose of tracer.

Sublingual microvascular imaging

Imaging of sublingual microvasculature was performed with a SDF MicroScan Video Microscope (MicroVision Medical, The Netherlands). The SDF camera captures the hemoglobin of passing red blood cells with green light emitting diodes (540 nm), using a 5× objective resulting in a field of view of 0.95 mm × 0.70 mm (0.665 mm^2) at a resolution of 720 × 576 pixels. The images were automatically captured with integrated Glycocheck software (version 1.2.7.7394, Glycocheck, The Netherlands) that identified all visible microvessels with a diameter between 5 and 25 μm. At every 10 μm along each microvessel, measurement sites were selected. Vessels with sufficient contrast in more than 60% of measurement sites were considered as valid vascular segments. Data acquisition automatically started when image quality was within acceptable range and automatically stopped when a data of minimum number of 3,000 measurement sites had been reached. Measurements were performed after low-sodium diet and high-sodium diet and 120 min after saline infusion. Three sequential measurement cycles were carried out in each participant, and the average values of these measurements were used for analysis. Glycocheck software assessed perfused boundary region, RBC filling, and microvascular density. The methods for the automatic analysis of endothelial surface layer dimensions have been described elsewhere.[27] Because the perfused boundary region is dependent on erythrocyte,[27,28] we corrected the perfused boundary region measurements for erythrocyte.

Urinary glycosaminoglycan measurement

Glycosaminoglycans were enzymatically digested into disaccharides, and the results were reported as previously described.[29] Disaccharide concentrations were adjusted for creatinine concentration of the urine samples. A validated high performance liquid chromatography with mass spectrometry/mass spectrometry method was used to quantify urinary excretion of heparan sulfate, dermatan sulfate, and chondroitin sulfate. Values below the lower limit of quantification were assigned values of half the lower limit of quantification.

Other laboratory testing

Laboratory testing included plasma hematocrit, sodium, potassium, chloride, osmolality, creatinine, glucose, and N-terminal pro b-type natriuretic peptide (NTproBNP). Urine analysis consisted of sodium, potassium, chloride, creatinine, and osmolality. All biochemical tests (plasma and urine) were performed on a COBAS C8000 modular analyzer (Roche Diagnostics, Germany) except for osmolality, which was performed on the Osmo station OM-6060 (Menarini Diagnostics, Italy). We used ion-selective electrode methods to measure sodium, potassium, and chloride in plasma and urine.

Plasma and urinary osmolality were quantified by freezing point depression. Creatinine in plasma was measured with an enzymatic spectrophotometric method and creatinine in urine with a spectrophotometric method. We measured plasma glucose with an enzymatic (ultraviolet) spectrophotometric method and NTproBNP with a two–monoclonal antibody sandwich method. Plasma hematocrit was calculated with the formula: hematocrit (l/l) = (mean corpuscular volume (fl) × erythrocyte (10^12/l))/1,000. We calculated estimated glomerular filtration rate with the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) creatinine equation.

Statistical analysis

Continuous variables are presented as means and 95% CI and as median plus interquartile range when data were not normally distributed. Characteristics were compared using a paired *t* test or Wilcoxon signed rank test where appropriate. To compare the results of the three conditions, a general linear model with repeated measurements was used. For variables with a skewed distribution, log transformed values were used for repeated measurement analysis. We performed Bonferroni *post hoc* adjustment for multiple comparisons. Data was analyzed with IBM SPSS Statistics (version 22.0, 2013, IBM, USA). *P* values less than 0.05 were considered statistically significant. Sample size was calculated using primary endpoints. Our pilot data showed a 1.7 (SD 1.0) kg body weight difference between subjects on a low-sodium diet and a high-sodium diet. Thus, at least six subjects were needed for each group (based on a two-sided *t* test; power 80%, α-error 5%). We demonstrated a 5 mmHg (SD 5) systolic blood pressure difference between subjects on low-sodium diet and high-sodium diet, indicating that at least ten subjects were needed (two-sided *t* test; power 80%, α-error 5%). To provide enough power for both primary outcomes and taking into account possible drop-out of subjects once they were included in the study, we decided to include at least 12 subjects.

RESULTS

After screening of 19 healthy males, three subjects were excluded: one due to high systolic blood pressure and two others because of difficulties with blood drawing. Four subjects withdrew their consent after inclusion before randomization and did not participate in the sodium interventions. Therefore there are no data available on these subjects regarding the primary and secondary outcomes of this study. The trial was conducted in accordance to the original protocol. Twelve subjects with a mean age of 23 (range, 18 to 31) yr completed the study.

All subjects successfully adhered to both diets, resulting in urinary sodium excretion of 19 (95% CI, 13 to 25) mmol/24 h after low-sodium diet, and 341 (275 to 407) mmol/24 h after high-sodium diet ($P < 0.001$), and urinary chloride excretion of 23 (21 to 26) mmol/24 h after low-sodium diet and 342 (282 to 402) mmol/24 h after high-sodium diet ($P < 0.001$). Urinary potassium excretion was comparable ($P = 0.93$) after low-sodium diet (88 [71 to 104] mmol/24 h) and high-sodium diet (92 [79 to 105] mmol/24 h). Saline infusion contained 542 (539 to 546) ml of 2.4 (2.3 to 2.5) % NaCl. In comparison with low-sodium diet, the body weight increased to 76.5 (72.2 to 80.7) kg (+ 2.5 [1.7 to 3.2] kg, $P < 0.001$) after high-sodium diet. There were no adverse events reported during the study—not during the diets but also not after infusion.

Increase of plasma volume after saline infusion without hemodynamic changes

In comparison to the low-sodium diet, acute saline infusion induced a significant increase ($P = 0.01$) in plasma volume of 287 (113 to 462) ml, resulting in a volume of 3,727 (3,377 to 4,077) ml. Plasma volume after high-sodium diet (3,568 [3,166 to 3,971] ml) and after low-sodium diet (3,440 [3,030 to 3,850] ml) were comparable ($P = 0.99$), also when corrected for body weight (low-sodium diet: 46 [42 to 51] ml/kg; high-sodium diet: 47 [42 to 51] ml/kg, $P = 1.00$). We detected no distinct peripheral and central hemodynamic changes, as measured with the semiautomatic oscillometric device and finger arterial pulse contour analysis respectively, despite a small increase of cardiac output (0.6 [0.2 to 1.0] l/min; $P = 0.03$) after high-sodium diet compared to saline infusion. Table 1 summarizes the hemodynamic characteristics of the different sodium conditions.

Endothelial surface layer changes after saline infusion, but not after high-sodium diet

TERalb did not differ between low-sodium diet (7.0 [5.5 to 8.6] %) and high-sodium diet (7.0 [4.5 to 9.5] %) but increased significantly after saline infusion to 9.7 (7.8 to 11.6) % ($P = 0.04$). Correction for hematocrit, to eliminate the influence of plasma volume changes during the sampling period, demonstrated similar results of TERalb (data not shown). Urinary radioactivity significantly increased after saline infusion in comparison to both the low-sodium diet and high-sodium diet ($P = 0.006$ and $P = 0.006$, respectively). Figure 1 gives an overview of TERalb and changes in urinary radioactivity.

Perfused boundary region as measured by SDF imaging did not change after high-sodium diet (1.95 [1.88 to 2.03] μm) or saline infusion (1.92 [1.81 to 2.03] μm) in comparison to low-sodium diet (2.02 [1.93 to 2.12] μm).

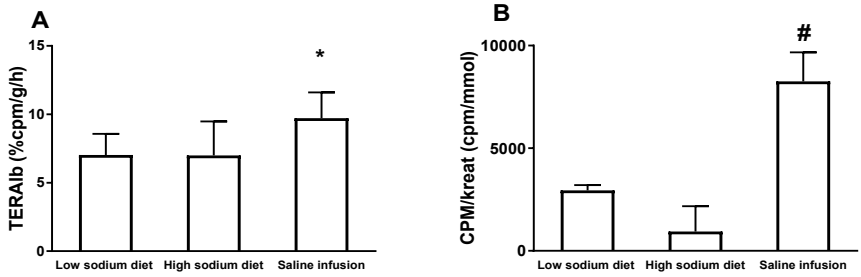
Table 1. Hemodynamic characteristics.

Characteristics (N=12)	Low-sodium Diet Mean (95%CI)	High-sodium Diet Mean (95%CI)	Saline infusion Mean (95%CI)	P value
Plasma volume (mL)	3440 (3030-3850)	3568 (3166-3971)	3727 (3377-4077)	0.03 *
Plasma volume/body weight (mL/kg)	46 (42-51)	46 (42-51)	50 (47-54)	0.004 #
Blood pressure (Omron)				
Systolic BP (mmHg)	117 (112-122)	118 (115-122)	116 (111-120)	0.25
Diastolic BP (mmHg)	58 (55-62)	58 (54-61)	58 (56-61)	0.76
Heart Rate (bpm)	56 (51-61)	57 (50-63)	54 (49-60)	0.45
MAP (mmHg)	78 (75-81)	78 (75-81)	78 (75-80)	0.84
Pulse Pressure (mmHg)	59 (54-64)	61 (57-65)	57 (54-61)	0.18
Finger arterial pulse contour analysis (Nexfin)				
Systolic BP (mmHg)	127 (122-133)	130 (125-136)	127 (121-134)	0.45
Diastolic BP (mmHg)	72 (69-75)	73 (70-77)	73 (69-77)	0.83
Heart Rate (bpm)	56 (51-61)	59 (52-66)	55 (50-60)	0.09
MAP (mmHg)	91 (87-94)	93 (88-98)	91 (86-96)	0.41
Cardiac Output (L/min)	6.6 (5.9-7.3)	7.2 (6.3-8.0)	6.5 (5.9-7.2)	0.02 ‡
Systemic Vascular Resistance (dyn*s/cm^5)	1134 (986-1282)	1072 (944-1200)	1137 (991-1283)	0.25
Stroke Volume (mL)	118 (112-125)	122 (116-128)	119 (112-125)	0.21

Bonferroni post-hoc tests
** low sodium diet vs. saline infusion p<0.05*
 # low sodium diet vs. saline infusion p<0.05; high sodium diet vs. saline infusion p<0.005
‡ high sodium diet vs. saline infusion p<0.05;
BP; blood pressure, MAP; Mean Arterial Pressure

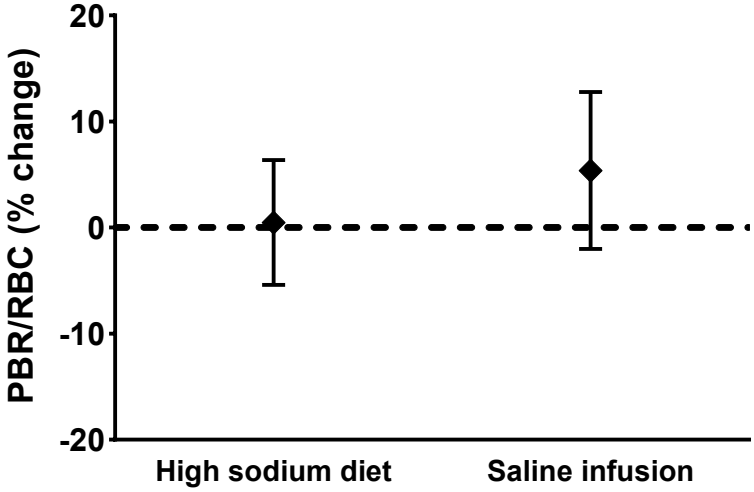
The correction of the perfused boundary region for erythrocyte showed no changes in the levels of erythrocyte-corrected perfused boundary region after saline infusion (P = 0.14) (fig. 2). After saline infusion, decreases in heparan sulfate and chondroitin sulfate disaccharide concentrations were detected in urine, whereas the urinary amount of dermatan sulfate disaccharides did not change after saline infusion. Figure 3A shows the percentage change of concentrations of the urinary glycosaminoglycans (corrected for urinary creatinine) in comparison to low-sodium diet, after high-sodium diet and saline infusion. The decrease of total heparan sulfates disaccharides was mainly caused by a decline of the nonsulfated and monosulfated heparan sulfate disaccharides (fig. 3B1). Relative to total heparan sulfates, only the monosulfated fraction altered significantly (fig. 3B2).

Figure 1. Transcapillary escape rate (TERalb) and urinary radioactivity after the three sodium interventions.



(A) TERalb did not differ between low-sodium diet (7.0 [5.5 to 8.6]%) and high-sodium diet (7.0 [4.5 to 9.5]%) but increased significantly after saline infusion to 9.7 (7.8 to 11.6)%.
**P = 0.04 compared to low-sodium diet.*
(B) Urinary radioactivity was also significantly increased after saline infusion.
*#P = 0.006 compared to low-sodium diet. *P = 0.006 compared to high-sodium diet. The data are represented as means and 95% CI (n = 12). The P values were derived from Bonferroni post hoc tests.*
CPM = counts per minute; kreat = creatinine in urine.

Figure 2. Sublingual perfused boundary region corrected for erythrocyte (red blood cell [RBC]) increases after saline infusion.

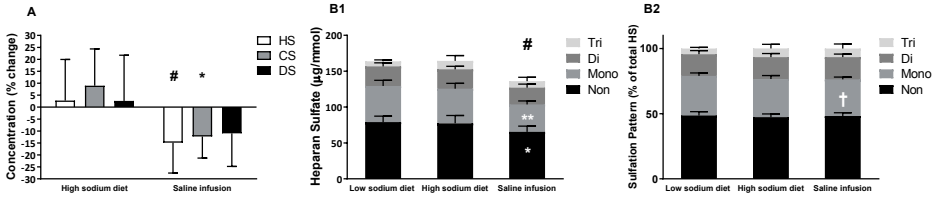


The correction of perfused boundary region (PBR) for erythrocyte showed no changes in erythrocyte-corrected perfused boundary region values after saline infusion (P = 0.14) compared to the low-sodium diet. The data are represented as the means and 95% CI (n = 12).

No changes in sublingual microvascular perfusion after sodium interventions

Sublingual microvascular density was similar after low-sodium diet (2,526 [2,341 to 2,712] μm/mm²), high-sodium diet (2,612 [2,244 to 2,980] μm/mm²) and after saline infusion (2,724 [2,513 to 2,935] μm/mm²). RBC filling showed no differences between diets (low-sodium diet: 74.4 [72.7 to 76.2]%; high-sodium diet: 75.1 [73.5 to 76.7]%) or after saline infusion (75.5 [73.8 to 77.3]%).

Figure 3. Urinary glycosaminoglycan disaccharide concentrations decrease after saline infusion.



*(A) Saline infusion induced a decrease of urinary heparan sulfate (HS) and chondroitin sulfate (CS) glycosaminoglycans. #P = 0.03 compared to low-sodium diet; *P = 0.01 compared to low-sodium diet. The urinary amount of dermatan sulfate (DS) disaccharides did not change after saline infusion. (B1) The decrease of total heparan sulfates disaccharides was mainly caused by a decline of the nonsulfated and monosulfated heparan sulfates.*

*(B2) Only the monosulfated fraction (% of total heparan sulfates) altered significantly. #Total heparan sulfate concentration: versus low-sodium diet, P = 0.02; *Non: versus low-sodium diet, P = 0.01; **Mono: versus lowsodium diet, P = 0.04, and versus high-sodium diet, P = 0.03. †Mono: versus low-sodium diet P = 0.03. The data are represented as the means and 95% CI (n = 12). The P values derived from Bonferroni post hoc tests. Di = disulfated disaccharides (D0S6 and D2S00); Mono = monosulfated disaccharides (D0S0, D0A6, and D2A0); Non = nonsulfated disaccharides (D0A0); Tri = trisulfated disaccharides (D2S6).*

Change in plasma electrolytes and fractional sodium excretion

Both sodium loading experiments induced a significant increase of plasma sodium, chloride, and osmolality, whereas plasma potassium remained stable. Hematocrit decreased after saline infusion. The sodium/creatinine ratio and fractional excretion of sodium were both increased after the high-sodium diet and the saline infusion compared to the low-sodium diet, with the highest levels after high-sodium diet. Table 2 gives an overview of the plasma and urine characteristics.

Table 2. Plasma and urine characteristics.

Characteristics (N=12)	Low-sodium Diet Mean (95%CI)	High-sodium Diet Mean (95%CI)	Saline infusion Mean (95%CI)	P value
Plasma				
Hematocrit (L/L)	0.43 (0.41-0.44)	0.42 (0.41-0.43)	0.39 (0.38-0.40)	<0.001*
Sodium (mmol/L)	138 (137-139)	140 (139 -141)	141 (140-141)	<0.001†
Potassium (mmol/L)	3.9 (3.7-4.1)	3.9 (3.9-4.0)	4.1 (3.8-4.4)	0.11
Chloride (mmol/L)	100 (99-101)	103 (102-105)	105 (104-106)	<0.001*
Glucose (mmol/L)	4.9 (4.7-5.1)	5.0 (4.7-5.2)	4.9 (4.6-5.2)	0.73
Osmolality (mOsm/kg)	285 (283-287)	290 (287-292)	290 (288-292)	0.005‡
Creatinine (µmol/L)	84 (78-90)	77 (71–83)	-	<0.001
Estimated glomerular filtration rate mL/min per 1.73m^2)	112 (103-121)	120 (112-127)	-	0.001
NTproBNP, median (IQR), ng/L	8.5 (5.2-11.9)	16.4 (9.3 – 37.2)	9.1 (5.3-12.3)	0.11
Urine				
Sodium/Creatinine ratio	1.3 (0.9-1.7)	24.6 (20.2-29.0)	6.3 (3.3-9.4)	<0.001§
Fractional excretion of sodium (%)	0.08 (0.06-0.1)	1.4 (1.1-1.7)	0.4 (0.2-0.6)	<0.001‖

Bonferroni post hoc test results:
**Low-sodium diet versus saline infusion, P < 0.001; low-sodium diet versus high-sodium diet, P < 0.001.*
†Low-sodium diet versus saline infusion, P < 0.005; low-sodium diet versus high-sodium diet, P < 0.005.
‡Low-sodium diet versus saline infusion, P < 0.001; low-sodium diet versus high-sodium diet, P < 0.05.
§Low-sodium diet versus saline infusion, P < 0.01; low-sodium diet versus high-sodium diet, P < 0.001; high-sodium diet versus saline infusion, P < 0.001.
‖Low-sodium diet versus saline infusion, P < 0.01; low-sodium diet versus high-sodium diet, P < 0.001; high-sodium diet versus saline infusion, P < 0.001
IQR = interquartile range; NTproBNP = N-terminal pro b-type natriuretic peptide.

DISCUSSION

We demonstrate that acute intravenous sodium loading causes an increase of microvascular permeability, whereas chronic dietary sodium loading does not affect microvascular permeability, despite similar increases in plasma sodium, chloride, and osmolality. Both interventions have no significant impact on blood pressure, whereas chronic dietary sodium loading increases body weight substantially. The increase of microvascular permeability after saline infusion coincides with increased plasma volume, decreased hematocrit reflecting hemodilution, and decreased urinary glycosaminoglycan excretion, which were not observed after a high-sodium diet. Both sodium loading interventions had no significant impact on sublingual microcirculatory density and RBC filling. These results suggest that an acute intravenous sodium load has direct adverse effects on the endothelial surface layer, compared to chronic high sodium intake, independent of blood pressure.

To our knowledge, this is the first *in vivo* study in humans that demonstrates increased microvascular permeability after saline infusion. Our observation is in agreement with earlier studies that studied the effects of plasma volume expansion. Parving *et al.*[30] have demonstrated that infusion of both 25% human serum albumin and 6% dextran in healthy volunteers resulted in increased plasma volume and increased permeability measured with TERalb. Furthermore, volume loading with colloids (5% albumin or 6% hydroxyethyl starch 130/0.4) containing 0.9% NaCl is associated with a decrease in endothelial surface layer volume[31] and increased shedding of endothelial surface layer constituents into the circulation,[22] indicating the damaging effects of these infusions on the barrier function of the endothelial surface layer. However, the microvascular effects of hypertonic saline infusion are mainly studied in the context of shock and the subsequent phase of fluid resuscitation. A recent study in a rat model of hemorrhagic shock and fluid resuscitation demonstrated that changes in microvascular permeability were correlated with alterations in both glycocalyx thickness, as well as with changes in plasma endothelial surface layer components.[32]

Apart from the increase in microvascular permeability, we observed an increase in urinary radioactivity after hypertonic saline infusion. Because there are no reasons to assume that hypertonic saline results in a dissociation of the ^{125}I-labeled albumin complex (*i.e.*, leading to higher "free" fraction ^{125}I), the increased urinary radioactivity after saline infusion indicates either increased transglomerular passage or decreased tubular reabsorption of ^{125}I-labeled albumin. For now, we cannot make differentiation between these two possibilities. However, given the fractional sodium excretion of less than 1%, reflecting normal tubular function, increased glomerular permeability to albumin may represent the best explanation. Of note, because urinary albumin was in

all subjects below lower limit of quantification at all interventions, we were not able to quantify total albuminuria in this group of healthy young men.

We also demonstrated a decrease of the fractions of urinary heparan sulfate (low sulfated disaccharides in particular) and chondroitin sulfate, which are important constituents of the endothelial surface layer. A possible explanation might be that due to the increased vascular permeability, heparan sulfate and chondroitin sulfate have already leaked into the extravascular space. This seems to be corroborated by a study demonstrating that urinary excretion of heparan sulfate was decreased in diabetic patients, who are characterized with reduced endothelial surface layer, compared with nondiabetic controls, and that a more distinct decrease was present in patients with diabetic nephropathy (*i.e.*, subjects with more severe endothelial surface layer loss as compared to diabetic patients without nephropathy).[33] One might have expected increased excretion of urinary glycosaminoglycans, because increased shedding is thought to be a marker of endothelial surface layer damage. However, data regarding shedding of glycosaminoglycans after fluid challenges are limited. Chappell *et al.*[22] measured serum and urine heparan sulfate after infusion of 6% hydroxyethyl starch 130/0.4 and were not able to demonstrate differences before and after infusion in heparan sulfate concentrations, neither in urine nor in serum. These observations indicate that it remains difficult to interpret changes of endothelial surface layer components in the context of endothelial surface layer damage, especially after fluid resuscitation.

Although the dietary sodium load caused a comparable increase in plasma sodium and osmolality as the intravenous sodium load and induced weight gain indicating (extracellular) volume expansion, this intervention was not associated with microvascular or hemodynamic changes. This may indicate that our young and healthy cohort may be capable of compensating for endothelial surface layer and microvascular damage (*i.e.*, increased permeability of the microcirculation) in response to a dietary sodium load. These results correspond with our findings that in healthy male subjects sublingual detected changes of the endothelial surface layer and microcirculation are only manifest in subjects that demonstrate a sodium-sensitive blood pressure response (personal communication, Liffert Vogt, M.D., Ph.D., Division of Nephrology, Department of Internal Medicine, Academic Medical Center, Amsterdam, The Netherlands, 2016).

Mechanistically, the increase of microvascular permeability after saline infusion can be the result of endothelial surface layer damage due to either the sodium contents of the infusion or the increment of plasma volume. The endothelial surface layer consists of the endothelial glycocalyx and the adsorbed plasma proteins. It has been shown that hemodilution caused by infusion with artificial fluids can lead to reduced

blood flow resistance, indicating reduction of the endothelial surface layer, as demonstrated in a metaanalysis of experimental animal studies.[34] Saline-based infusions increased vascular permeability to a greater extent than infusates containing plasma products.[34,35] Infusions containing sodium might cause more washout of the adsorbed plasma proteins, whereas infusions containing proteins may be able to restore the endothelial surface layer and its vascular barrier properties. This observation can further be supported by the concurrent increase of plasma volume after the saline infusion, because this may also suggest compaction of the endothelial surface layer volume[36] indicative of reduced endothelial surface layer thickness.[31] Another possible contributor to the observed increased microvascular permeability might be atrial natriuretic peptide (ANP). In addition to the well known diuretic and natriuretic effects of ANP, this hormone is also associated with increased microvascular permeability and increased shedding of endothelial surface layer constituents[22,37,38] and rapid shifts of fluids from the intravascular to interstitial space.[39] Volume loading with 6% hydroxyethyl starch 130/0.4 before elective surgery resulted in increased plasma concentrations of ANP.[22] Furthermore infusion of 2 l of 0.9% NaCl (308 mmol Na^+) is known to cause an increase in plasma ANP levels.[40] Unfortunately we were not able to measure ANP. We did measure NTproBNP, another member of the natriuretic peptide family. We did not detect changes in NTproBNP. Our observations of NTproBNP are corresponding with data from two previous studies,[41,42] that show that b-type natriuretic peptide but not ANP remains unaffected after acute sodium loading. ANP could therefore explain the observed effects on the endothelial surface layer after saline infusion. However, this remains merely speculative.

A limitation of this study is that TERalb, urinary shedding products, and the perfused boundary region are indirect measurements of the endothelial surface layer. Therefore, we are not able to provide results about the structure, volume, and thickness of this layer. Moreover, it remains unclear whether urinary glycosaminoglycan shedding concentrations are a reflection of endothelial surface layer damage and are related to shedding of plasma glycosaminoglycans. We did attempt to measure plasma glycosaminoglycans with high performance liquid chromatography with mass spectrometry/mass spectrometry, but the plasma levels of glycosaminoglycan disaccharides were not quantifiable. Second, the other microcirculatory parameters were visualized sublingually, and it remains uncertain whether our findings also apply to other microvascular beds. Furthermore, we cannot differentiate whether the plasma volume expansion or the hypertonic sodium contents of the infusion, causing an acute increase in plasma sodium and osmolality, are responsible for the observed increase of TERalb. We also cannot distinguish whether the increase in TERalb is explained to some extent by an increase of paracellular hyperfiltration or diffusion *via* the transcellular pathway.

Future studies comparing different infusion fluids, *e.g.*, 0.9% NaCl, other crystalloids and colloid infusions, should be carried out to determine whether our results can be explained either by the increased plasma volume or by the sodium contents of the infusion.

CONCLUSION

In conclusion, our study shows that a dietary sodium load and an acute intravenous sodium load in healthy male subjects have different effects on the endothelial surface layer and microcirculation despite comparable osmolar changes. An acute intravenous sodium load induced increased microvascular permeability and plasma volume, which was accompanied by a decrease in urinary fractions of heparan sulfate and chondroitin sulfate, indicating damage to the endothelial surface layer. Although we found no hemodynamic changes, our study demonstrates deleterious microvascular effects of saline infusion in healthy subjects. This might indicate that fluid therapy with hypertonic saline, often used in hospital settings and in critically ill patients, can be harmful in these patient groups. At the same time, we were not able to demonstrate effects of a dietary sodium load on the endothelial surface layer, microcirculation, and blood pressure. This seems to indicate that healthy subjects are able to adjust to the impact of a dietary sodium load. Further research regarding potentially regenerative effects of the endothelial surface layer in the context of a chronic high sodium load is needed to provide more insight into underlying mechanisms of sodium intake and sodium-mediated increase of blood pressure.

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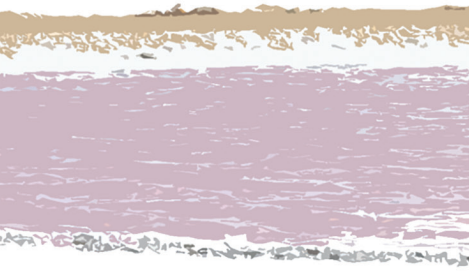

6 The Blood Pressure Lowering Potential of Sulodexide a Systematic Review and Meta-Analysis

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ABSTRACT

Aims Sulodexide is a highly purified mixture of glycosaminoglycans that has been studied for its anti-albuminuric potential. Considering the effects of glycosaminoglycans on endothelial function and sodium homeostasis, we hypothesized that sulodexide may lower blood pressure (BP). In this meta-analysis, we therefore investigated the antihypertensive effects of sulodexide treatment.

Methods We selected randomized controlled trials that investigated sulodexide treatment of at least 4 weeks and measured BP at baseline and after treatment. Two reviewers independently extracted data on study design, risk of bias, population characteristics and outcome measures. In addition, we contacted authors and pharmaceutical companies to provide missing data.

Results Eight studies, totalling 3019 subjects (mean follow-up 4.4 months) were included. Mean age was 61 years and mean baseline BP was 135/75 mmHg. Compared with control treatment, sulodexide resulted in a significant systolic (2.2 mmHg [95% CI 0.3 - 4.1], P=0.02) and diastolic BP reduction (1.7 mmHg [95% CI 0.6 - 2.9], P=0.004). Hypertensive patients displayed the largest systolic BP and diastolic BP reductions (10.2/5.4 mmHg, P<0.001). Higher baseline systolic and diastolic BP were significantly associated with larger systolic (R^2=0.83, P<0.001) and diastolic BP (R^2=0.41, P=0.02) reductions after sulodexide treatment. In addition, systolic (R^2=0.41, P=0.03) and diastolic BP reductions (R^2=0.60, P=0.005) were significantly associated with albuminuria reduction.

Conclusion Our data suggest that sulodexide treatment results in a significant BP reduction, especially in hypertensive subjects. This indicates that endothelial glycosaminoglycans might be an independent therapy target in cardiovascular disease. Future studies should further address the BP lowering potential of sulodexide.

INTRODUCTION

Hypertension is the most important risk factor for cardiovascular and all-cause mortality worldwide and its prevalence is still increasing[1]. However, half of all hypertensive patients have an uncontrolled blood pressure (BP) and even in patients who have their BP controlled the residual cardiovascular risk remains high[2-5]. New therapeutic interventions may therefore help to control the cardiovascular burden of hypertension.

Sulodexide is a highly purified mixture of glycosaminoglycans (GAGs) that is currently marketed in a number of countries in Europe, South America and Asia for various cardiovascular conditions. GAGs are large, negatively charged, linear polymers that are present on the surface of all endothelial cells and in the extracellular matrix. Here, GAGs interact with a wide range of processes that are involved in the development of cardiovascular disease, including shear mediated nitric oxide (NO) production and non-osmotic sodium storage[6]. Sulodexide has been shown to improve endothelial function and lipid profiles, exert anti-inflammatory, anti-thrombotic and fibrinolytic activity, inhibit leucocyte adhesion and diminish platelet aggregation[7]. Because of these vasoprotective effects, sulodexide has been studied in numerous clinical trials. For instance, sulodexide has been shown to decrease claudication symptoms in peripheral artery disease patients and to prevent atherothrombotic events after acute myocardial infarction[8,9]. In addition, a series of small studies demonstrated that sulodexide decreased albuminuria[10]. However, two recently performed large randomized controlled trials could not reproduce these findings[11,12]. Noticeably, no clinical trials have thus far investigated the antihypertensive potency of sulodexide.

In this meta-analysis, we have therefore investigated whether sulodexide treatment results in a significant BP reduction when compared with control treatment in adult patients.

METHODS

The primary objective of this systematic review and meta-analysis was to investigate the effect of sulodexide on BP in adult patients, after correction for control treatment.

Information sources and searches

In this meta-analysis, we adhered to PRISMA guidelines. MEDLINE, EMBASE and Cochrane library databases were searched (until October 2014) for clinical trials in which sulodexide was administered to adult subjects. The electronic search strategy was designed by two authors (ROE, NR) who were trained in systematic review searches (Supplementary Data). In addition, we used bibliographies of previously published

narrative reviews and editorials concerning sulodexide to search for eligible clinical trials. Articles were first evaluated based on title and abstract. Case reports, guidelines, editorials and reviews were excluded, as well as abstracts with a combination of title and abstract that indicated that the article could not meet the requirements of this review.

Study selection

For this review we considered randomized controlled trials in adult patients that investigated the effects of sulodexide on any medical condition. Studies were included when sulodexide treatment lasted at least 4 weeks and BP data were reported. We excluded studies with active treatment in the control arm. To ensure that the data set was as complete as possible we contacted corresponding authors and sulodexide manufacturers of studies that mentioned BP measurements, but not reported BP values. Two reviewers (ROE and NR) independently assessed the eligibility of each study. Disagreement was resolved through final discussion with a third reviewer (LV).

Data collection process and data items

We extracted data using a standardized data abstraction form. Data extraction was done by two independent reviewers (ROE and NR). We extracted data on BP changes in sulodexide and control groups. In addition, we collected data on key demographics such as age, gender, body mass index (BMI), baseline BP, plasma creatinine, diabetes prevalence, presence of albuminuria and use of renin-angiotensin system (RAS) inhibitors, and study characteristics such as study size, mean follow-up duration, publication year and inclusion criteria, and the incidence of adverse events. Adverse events were defined as serious adverse events or adverse events that led to study discontinuation of the patient.

Risk of bias in individual studies

In individual studies, two authors (ROE and NR) assessed the risk of bias according to the Cochrane Handbook Guidelines. The risk of bias was assessed for random sequence generation, allocation concealment, blinding of personnel and participants, blinding of outcome assessment, incomplete outcome data and selective reporting.

Summary measures and synthesis of results

Quantitative analyses of outcomes were based on intention-to-treat analysis whenever possible. We calculated mean BP changes and 95% confidence intervals (CI) between baseline and after sulodexide treatment for each study to combine outcomes across trials. To correct for placebo effects and regression to the mean, we adjusted the mean BP difference for the observed BP change in parallel control groups (i.e. control-

subtracted effects). To investigate the effects of sulodexide both in normotensive and hypertensive subjects, we performed a stratified analysis for studies with baseline BP ≥140/90 mmHg and <140/90 mmHg.

We calculated the (anti-)albuminuric and proteinuric effects of sulodexide in percentage change from baseline (mean and standard deviation), corrected for control groups. To combine incidences of adverse events among trials, we calculated risk ratios for each study.

Statistical heterogeneity was identified by calculating I^2 that describes the percentage of total variation across studies that is due to heterogeneity[13]. We examined funnel plot asymmetry to explore the potential presence of publication bias. Data were analyzed using a random effects model.

Sensitivity and meta-regression analyses

The robustness of our results was tested by sensitivity analyses excluding open label trials and trials that did not keep track of antihypertensive treatment during follow-up. We used meta-regression analyses to test whether BP changes induced by sulodexide were associated with albuminuria reduction, a surrogate endpoint for both cardiovascular and renal outcome[14, 15], or patient characteristics such as age, gender, sulodexide dose, use of renin-angiotensin system inhibition and baseline BP. In these analyses, studies were weighted according to the inverse variance of the BP changes. Risk ratios for adverse events were log-transformed for linear regression analyses. Data were analyzed using Cochrane Review Manager Software (Review Manager 5.2) and SPSS (Version 21.0, SPSS, Inc., Chicago, IL, USA).

RESULTS

Study selection

A total of 638 records were found after searching in MEDLINE, EMBASE and the Cochrane database and 93 full-text articles were reviewed (Figure 1). Eight studies containing thirteen comparisons, totalling 3019 participants, were included[10-12, 16-20].

Study characteristics

In seven studies, sulodexide treatment was compared with placebo while one study compared sulodexide with a control group that did not receive any treatment. Six out of eight studies investigated possible anti-albuminuric effects of sulodexide in diabetic patients. Three of these studies only included micro-albuminuric patients[11, 17, 20], one study only included macro-albuminuric patients[12] and two studies included both micro- and macro-albuminuric patients[10, 18]. In addition, one study investigated the effects of sulodexide on proteinuria from non-diabetic origin (IgA nephropathy)[16]. The remaining

study investigated the effects of sulodexide on ulcer healing in patients with chronic venous insufficiency[19]. BP measurements were reported after 3 to 6 months of sulodexide therapy with an average treatment period of 4.4 months.

Patient characteristics

The mean age of participants was 61 (11) years, 73% were male and mean systolic BP (SBP) (135 (15) mmHg) and diastolic BP (DBP) (75 (10) mmHg) were within normal range (Table1). The average BMI was 31.8 (11.5) kg m^{-2} and mean serum creatinine was 141 (62) μmol l^{-1}. In six studies sulodexide was given on top of RAS inhibition[10-12, 16, 18, 20]. The mean administered sulodexide dose was 185 mg day^{-1} and ranged from 50 to 400 mg among studies.

Figure 1. Selection process for studies included in the meta-analysis according to the PRISMA 2009 flow diagram.

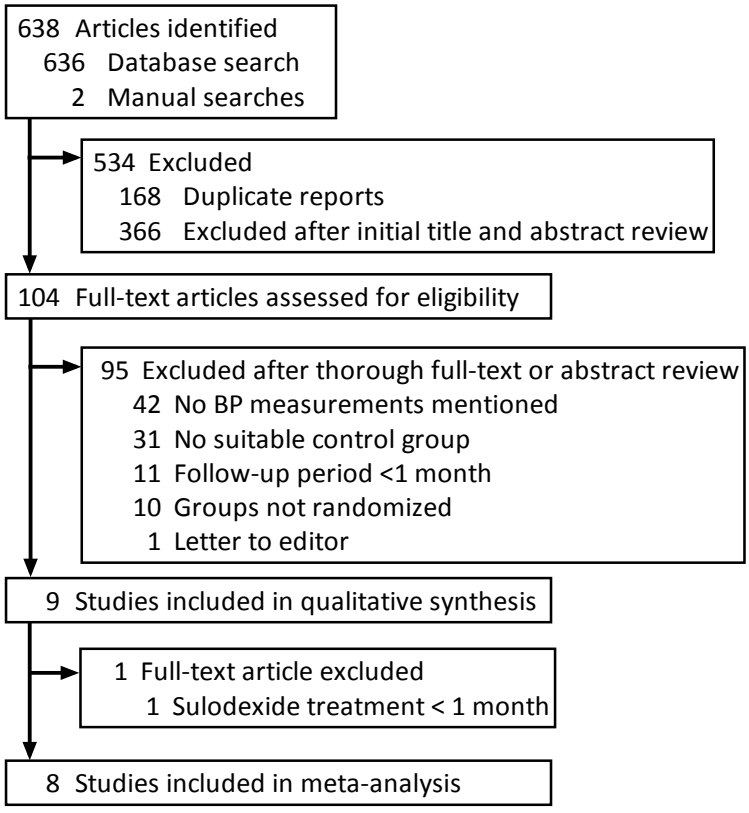


Table 1. Characteristics of included studies.

Study	Population	N	Treatment	FU *	Age (yrs)	Male (%)	DM1/ DM2 (%)	RASi (%)
Bang 2012[16]	Macroalbuminuric IgA nephropathy	28	SUL 150 mg	6	40	50	0/0	100
		25	SUL 75 mg		42	36	0/0	100
		24	Placebo		43	50	0/0	100
Coccheri 2002[19]	Chronic venous insufficiency	120	SUL 60 mg im 20d, 100 mg oral 70d	3	63	44	NA/NA	NA
		110	Placebo		64	48	NA/NA	NA
Gambaro 2002[10]	Micro- and macro-albuminuric	55	SUL 200 mg	4	47	NA	56/44	58
		56	SUL 100 mg		47	NA	59/41	48
		56	SUL 50 mg		49	NA	54/46	48
		56	Placebo		47	NA	54/46	54
Heerspink 2008[20]	Microalbuminuric	52	SUL 400 mg	6	61	73	0/100	100
		50	SUL 200 mg		64	72	0/100	100
		47	Placebo		60	70	0/100	100
Lewis 2011[11]	Microalbuminuric	524	SUL 200 mg	6	62	75	0/100	100
		532	Placebo		62	77	0/100	100
Packham 2012[12]	Macroalbuminuric	619	SUL 200 mg	3	62	62	0/100	100
		629	Placebo		64	60	0/100	100
Solini 1997[18]	Hypertensive micro- and macro-albuminuric	12	SUL 100 mg	4	52	NA	0/100	17
		12	Placebo				0/100	
Velussi 1996[17]	Hypertensive microalbuminuric	24	SUL 100 mg	6	67	67	0/100	NA
		24	No treatment				0/100	

** when last BP measurements were performed in the entire cohort during sulodexide treatment.*
DM, diabetes mellitus; FU, follow-up in months; im, intramuscular; RASi, renin-angiotensin system inhibition; SUL, sulodexide; NA, not available.

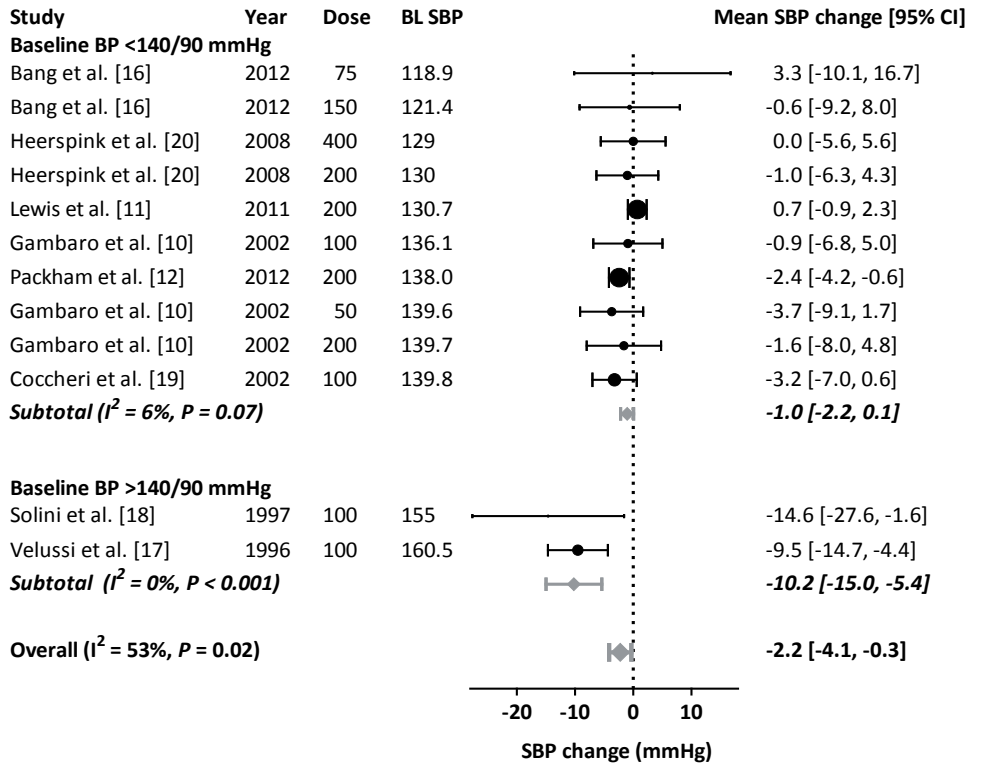
Risk of bias within and across studies

Seven out of eight studies were double-blinded. Three studies explicitly stated that no change in antihypertensive treatment was made during sulodexide or placebo treatment[10, 17, 18]. Four studies reported methods for BP measurements, all calculating mean values of three seated BP measurements after at least 5 min rest. Corresponding authors provided (additional) BP data for three studies. BP data for one study was retrieved after contact with the manufacturer (Alfa Wasserman, Bologna, Italy). Funnel plots were symmetrical by visual inspection suggesting that no publication bias was present.

Synthesis of results

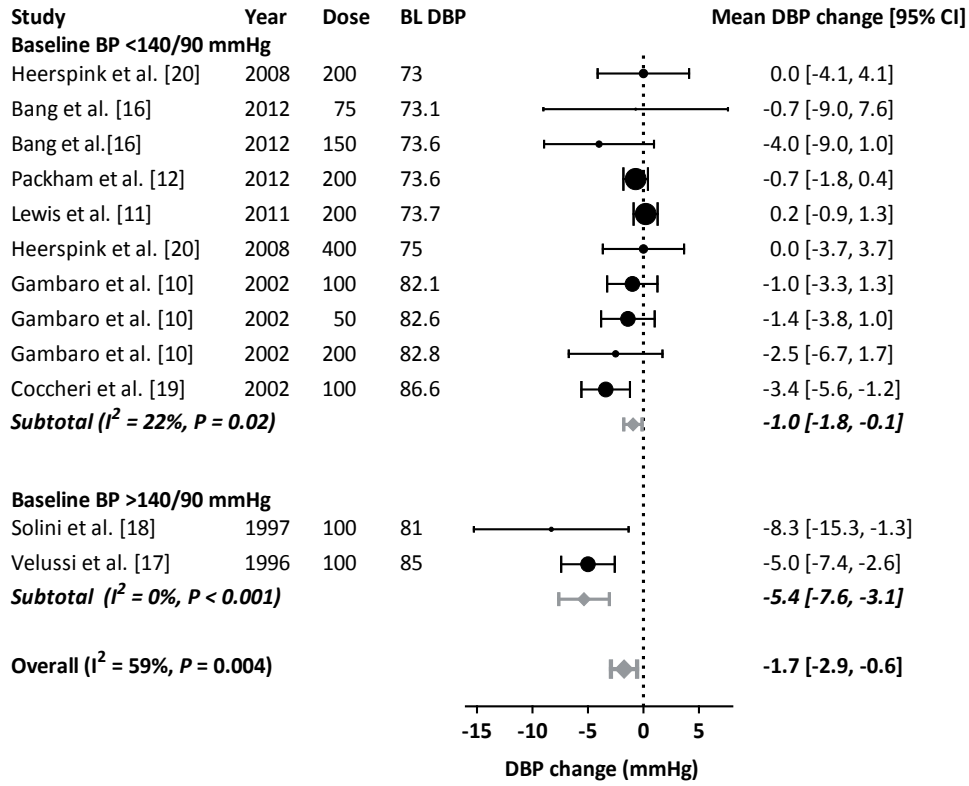
Sulodexide treatment led to a significant control-subtracted BP reduction (Figure 2). SBP decreased by 2.2 mmHg (P=0.022; I^2=53%) while DBP decreased by 1.7 mmHg (P=0.004; I^2=59%). In two studies that included patients with an average uncontrolled BP at baseline (i.e. >140/90 mmHg) we observed a large SBP (10.2 mmHg, P<0.001) and DBP reduction (5.4 mmHg, P<0.001), while studies that included patients with a controlled BP at baseline showed a lesser SBP (1.0 mmHg, P=0.07) and DBP reduction (1.0 mmHg, P=0.02) (Figure 2). In the subgroups of patients with an average controlled or uncontrolled BP we found no heterogeneity for the outcomes of SBP and DBP reduction (I^2 <50%). Sensitivity analyses did not lead to a significant change in treatment effect.

Figure 2A. Forest plot of SBP changes.



Studies have been separated according to mean baseline BP as hypertensive (>140/90 mmHg) or non-hypertensive (<140/90 mmHg). Studies were weighted by the inverse of variance assuming random effects. The diameter of the point estimate (circle), representing mean BP changes, is proportional to the weight of the study.
BL, baseline; DBP, diastolic blood pressure; SBP, systolic blood pressure.

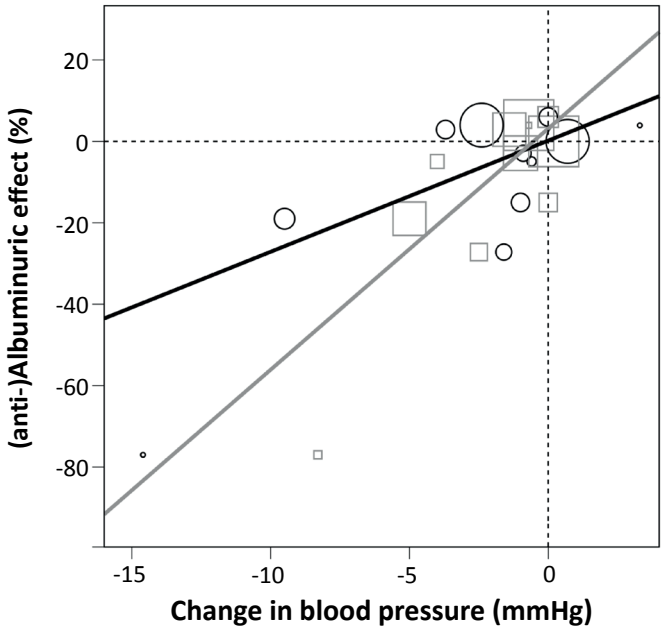
Figure 2B. Forest plot of DBP changes.



Studies have been separated according to mean baseline BP as hypertensive (>140/90 mmHg) or non-hypertensive (<140/90 mmHg). Studies were weighted by the inverse of variance assuming random effects. The diameter of the point estimate (circle), representing mean BP changes, is proportional to the weight of the study.
BL, baseline; DBP, diastolic blood pressure; SBP, systolic blood pressure.

Six comparisons demonstrated a reduction in albuminuria or proteinuria after sulodexide treatment while five comparisons, including two large recent trials, did not. The mean effect of sulodexide on albuminuria or proteinuria was a non-significant decrease of 6% (95% CI −35% - +23%, *P*=0.70). The change in albuminuria and proteinuria after sulodexide treatment was significantly associated with the degree of SBP (R^2=0.41, *P*=0.034) and DBP reduction (R^2=0.60, *P*=0.005) (Figure 3). Seven out of eight trials reported the incidence of adverse events during sulodexide and placebo treatment. Comparable incidences of adverse events were found for sulodexide and placebo (risk ratio 1.07 [95% CI 0.93 - 1.22], *P*=0.33). Most adverse events that were reported were not believed to be related to the study medication.

Figure 3. Association between BP and anti-albuminuric effects.



Linear regression analysis of the association between SBP (black) and DBP (grey) reduction and anti-albuminuric effects after sulodexide treatment. Changes in albuminuria were significantly associated with SBP (R^2=0.41, P=0.034) and DBP changes (R^2=0.60, P=0.005) induced by sulodexide.

Meta-regression analyses

We observed a significant positive association between baseline SBP and the observed drop in SBP (R^2=0.83, *P*<0.001) as well as baseline DBP and the DBP reduction (R^2=0.41, *P*=0.024) after sulodexide treatment. SBP reduction showed a significant positive association with total cholesterol concentrations (R^2=0.65, *P*=0.029). In addition, higher total cholesterol concentrations and lower BMI were significantly associated with larger DBP reductions. These associations, however, did not remain significant after correction for baseline BP. Sulodexide dose, mean age, gender, length of follow-up, study size and serum creatinine were not associated with the effects of sulodexide on BP. The risk of adverse events was not associated with baseline BP, observed BP changes during treatment or sulodexide dose.

DISCUSSION

The findings of this meta-analysis demonstrate that sulodexide has antihypertensive potency. Because included studies were randomized controlled trials of good methodological quality and we corrected for BP changes in parallel control groups, the observed BP lowering effects are neither caused by a placebo effect nor by regression to the mean. The significant SBP and DBP decrease in patients with uncontrolled hypertension equals BP reductions achieved after monotherapy with other classes of antihypertensive drugs[21]. In patients with controlled BP, sulodexide resulted in a minor, significant reduction in DBP, while SBP was not significantly reduced. These effects were observed in subjects with high cardiovascular risk of which the majority was already being treated with antihypertensive drugs.

We could not observe a dose-dependent association between sulodexide dose and the degree of BP reduction. Because baseline BP was a strong covariate that had a large influence on the degree of BP reduction, this analysis cannot exclude possible dose-dependent effects of sulodexide. In three studies that investigated multiple sulodexide doses within one study, in patients with similar baseline BP, we could not observe a dose-dependent BP effect. Because patients in these studies had controlled BP, it cannot be excluded that the BP reduction in patients with uncontrolled BP may be larger. A higher incidence of adverse events may be anticipated for higher doses of sulodexide[22]. However, the rate of adverse events during sulodexide treatment was similar to placebo and higher doses were not associated with an increase in adverse events.

The BP lowering effects of sulodexide may relate to both increased NO production and non-osmotic sodium storage. Sulodexide has been demonstrated to increase NO availability in a rat model of chronic kidney disease[23]. This may be because of a reduction in inflammation or oxidative stress, both of which have been observed after sulodexide treatment and are known to decrease NO bioavailability[24-26]. An increase in endothelial surface layer (ESL) volume may be another mechanism by which sulodexide could increase NO production. The ESL is a dynamic layer on the luminal side of the endothelial cell that is home to a large amount of GAGs, especially heparan sulphate. Sulodexide is distributed to the ESL where it has been shown to restore reduced ESL dimensions present in diabetic patients[27-29]. As the ESL is an important mediator of shear-induced NO production, an increase in ESL volume following sulodexide treatment may lead to an increase in NO availability[30-32]. BP reductions by sulodexide therefore seem a logical result of endothelial function improvement that appears to be the common pathway of many actions exerted by sulodexide[33-35]. Non-osmotic sodium storage may also contribute to the antihypertensive potency of

sulodexide[6]. Sulodexide consists of negatively charged GAGs, which have been shown to be able to bind and osmotically inactivate sodium ions in the skin interstitium[36-38]. In addition, GAGs in the ESL have been shown to be able to bind sodium under flow conditions[39]. Considering the large systemic volume of the ESL, non-osmotic sodium storage in the ESL may have significant implications for BP and extracellular volume regulation[40]. Sulodexide may therefore increase the capacity for non-osmotic sodium storage and prevent sodium from deteriorating endothelial cell function or expanding extracellular volume and causing BP to rise[29].

By increasing NO availability and the non-osmotic ESL buffer capacity for sodium, sulodexide may be particularly beneficial in salt-sensitive hypertension and result in an additional BP reduction on top of other antihypertensive treatments. As salt-sensitivity is a major problem in resistant hypertension, sulodexide may contribute to the treatment of resistant hypertension[41]. This is supported by the results of our meta-analysis, in which most patients received sulodexide on top of antihypertensive treatment and showed an additional BP reduction. In addition, sulodexide has favourable characteristics that may reduce cardiovascular risk beyond BP. Sulodexide has been shown to diminish platelet aggregation and to exert anti-inflammatory, lipid lowering, anti-thrombotic and fibrinolytic actions[7]. It is therefore conceivable that sulodexide may be able to affect beneficially the residual risk of hypertensive patients that remains high despite maximum antihypertensive treatment[3]. Furthermore, as recently hypothesized by us and others, an increase in non-osmotic sodium storage capacity may help to control fluid overload in patients with heart failure and chronic kidney disease[6, 42]. A cardiovascular outcome trial in 3986 myocardial infarction patients demonstrated that sulodexide was able to reduce mortality and reinfarction rate compared with standard therapy, excluding antiplatelet and anticoagulant therapy[9]. Because a highly significant risk reduction of death from heart failure in the first months was not accompanied by a risk reduction of reinfarction rate, other mechanisms than the hypothesized anti-coagulant activity may have contributed to the cardiovascular benefit including BP lowering effects and an increase in non-osmotic sodium binding capacity.

In this meta-analysis, most included studies have investigated the ability of sulodexide to reduce albuminuria or proteinuria. Various underlying mechanisms have been suggested for the proposed anti-albuminuric/proteinuric effects of sulodexide, all of them assuming that sulodexide specifically targets the kidney. Our data show that greater reductions in albuminuria by sulodexide are associated with larger BP reductions. This is consistent with previous studies that have demonstrated that lower BP is associated with less albuminuria, also in the lower BP ranges of the studies that were included in this meta-analysis[43]. This suggests that systemic effects of sulodexide

should not be overlooked and may explain the contrasting finding of previous studies on albuminuria endpoints to a certain extent. Future studies investigating the kidney-specific effect of sulodexide should therefore correct for systemic BP reductions.

We acknowledge some limitations in the interpretation of the data from this meta-analysis. First of all, methods of BP measurements were only provided in three trials and could be retrieved in one more after correspondence. Second, three studies did not keep track of antihypertensive medication use. This is most likely because these studies included patients with controlled BP. Although these limitations may induce bias, their influence is probably minor since seven out of eight studies were double-blinded and BP was not regarded as a primary outcome in any of the studies.

CONCLUSION

This meta-analysis provides evidence that sulodexide treatment results in a significant BP reduction, especially in hypertensive patients. Considering the anti-inflammatory and anti-thrombotic actions, it is conceivable that sulodexide may render additional cardioprotective benefits as compared with regular classes of antihypertensive agents. Future studies are needed to confirm the antihypertensive potency of sulodexide and investigate the mechanisms underlying the BP reducing effects. Finally, optimal dosing and combination strategies with current antihypertensive treatment for BP control deserve exploration.

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Supplemental Data 1 Characteristics and the risk of bias table of included studies.

Bang 2012

Methods	Randomized, double-blind, placebo-controlled, multi-center trial in Korea between March 2007 and April 2009.
Participants	77 macroalbuminuric, non-diabetic, IgA nephropathy patients with a mean age of 42, 46% male, baseline SBP/DBP of 120/73 and mean eGRF 62 ml/min. All patients were treated with RAS inhibition.
Interventions	Sulodexide 150 mg/day Sulodexide 75 mg/day Placebo All patients were treated for 6 months
Outcomes	Achievement of 50% reduction in urinary protein/creatinine ratio
Notes	No baseline differences of dose and frequency of anti-hypertensive medication No significant differences in adverse events

Risk of bias table

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	**Low risk**	'Patients who met the inclusion criteria proceeded to randomization and were randomly assigned, based on a computer-generated randomization schedule, to treatment with placebo, sulodexide 75 mg or sulodexide 150 mg daily at a 1:1:1 ratio.'
Allocation concealment (selection bias)	**Unclear risk**	Computer-generated randomization schedule
Blinding of participants and personnel (performance bias)	**Low risk**	Double blind 'A pharmaceutist from the Clinical Trials Center'without any of the patients' information distributed the drugs. And 'Patients were instructed to take their medication orally with water 30 minutes prior to morning and evening meals'.
Blinding of outcome assessment (detection bias)	**Low risk**	Blood pressure was regularly measured at study visits and was no primary or secondary outcome.
Incomplete outcome data (attrition bias)	**High risk**	'Of the 104 randomized patients, 77 completed the study. Twenty-seven patients discontinued due to adverse events, were lost to follow-up, or spontaneously withdrew from the study.' No significant differences in adverse events.
Selective reporting (reporting bias)	**Low risk**	Data provided of all patients that completed the study

Coccheri 2002

Methods	Randomized, double-blind, placebo-controlled multi-center trial in 31 Italian centres between July 1998 and August 2000.
Participants	235 patients with leg ulcers due to chronic venous insufficiency greater than 2 cm with a mean age of 62, 46% male, baseline SBP/DBP of 140/82.
Interventions	Sulodexide intramuscular injection 60 mg for the first 20 days, and then 100 mg in two 50 mg capsules daily by the oral route for 70 days Matching placebo. All patients were treated for 90 days
Outcomes	Complete ulcer healing at two months
Notes	Blood pressure data retrieved via Alfa Wasserman No significant differences in adverse events No data on antihypertensive medication

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	**Low risk**	Patients were blindly allocated to sulodexide or matching placebo
Allocation concealment (selection bias)	**Unclear risk**	No data on allocation concealment
Blinding of participants and personnel (performance bias)	**Low risk**	Double blind. Both intramuscular injections and oral sulodexide was replaced by matching placebo.
Blinding of outcome assessment (detection bias)	**Low risk**	Blood pressure was no primary or secondary outcome.
Incomplete outcome data (attrition bias)	**Low risk**	Intention to treat analysis
Selective reporting (reporting bias)	**Low risk**	Data provided of all patients that completed the study

Gambaro 2002

Methods	A randomized double-blind, placebo-controlled multicenter trial in Czech Republic, Poland, Slovak Republic and Italy between October 1996 and December 1998.
Participants	223 micro- and macroalbuminuric diabetic patients with a mean age of 48, baseline SBP/DBP of 139/82 and mean serum creatinine of 92 mmol/L. 52% of patients was treated with RAS blockade.
Interventions	Sulodexide 200 mg/day, Sulodexide 100 mg/day, Sulodexide 50 mg/day, Placebo
	All patients were treated for 4 months
Outcomes	Albumin excretion rate
Notes	No significant differences in adverse events 'During the whole study period, patients did not alter their usual diet of antihypertensive treatment'

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	**Low risk**	'After screening and baseline evaluation, a computer-generated block randomization list (8 per block) prepared by the Sponsor's Medical Department was used to assign all eligible patients to treatment with 25 mg, 50 mg , or 100 mg oral sulodexide twice daily or placebo.'
Allocation concealment (selection bias)	**Low risk**	Clinical trial drug supply was managed by Unival, UK. The study medication and placebo were packaged indisstinguishably and labelled with a patient number
Blinding of participants and personnel (performance bias)	**Low risk**	Double blind
Blinding of outcome assessment (detection bias)	**Low risk**	Blood pressure was regularly measured at study visits and was no primary or secondary outcome.
Incomplete outcome data (attrition bias)	**Low risk**	'Treatment and follow-up were completed by 209 of 223 patients. Fourteen patients dropped out due to adverse effect, were lost at follow-up, or spontaneously withdrew. The quality -control evaluation committee recognized protocol violations or incorrect diagnosis in another 14 patients.' There was no substantial difference between groups for the number of unevaluable patients or prevalence and type of adverse events.' Analyses were performed according to the intent-to-treat model on all randomized patients in their assigned groups, regardless of adherence to treatment regimen.'
Selective reporting (reporting bias)	**Low risk**	Data provided of all patients that were randomized

Heerspink 2008

Methods	A randomized, double-masked placebo-controlled multi-center study.
Participants	149 microalbuminuric, diabetic patients with a mean age of 62, 36% male, baseline SBP/DBP of 129/74 and mean serum creatinine of 1.14 mg/dL. All patients were treated with maximally dosed RAS inhibition
Interventions	Sulodexide 400 mg/day Sulodexide 200 mg/day Placebo All patients were treated for 24 weeks
Outcomes	Conversion to normoalbuminuria and a 25% reduction in albumin-creatinine ratio from the baseline level, or a 50% reduction in albumin-creatinine ratio from baseline.
Notes	Office blood pressure was taken using a sphygmomanometer or an automated blood pressure device in the sitting position after at least 10 min rest. Three seated blood pressures are taken 1 min apart and the average was used for calculation. Exact data retrieved after contact with corresponding author. The incidence of possible related adverse events was similar in the three treatment groups

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	**Low risk**	Those patients who met the inclusion criteria proceeded to randomization and were randomly assigned, based on a computer-generated randomization schedule, to treatment with placebo, sulodexide 200 mg or sulodexide 400 mg at a 1:1:1 ratio.
Allocation concealment (selection bias)	**Unclear risk**	Computer-generated randomization schedule
Blinding of participants and personnel (performance bias)	**Low risk**	Double blind
Blinding of outcome assessment (detection bias)	**Low risk**	Blood pressure was regularly measured at study visits and was no primary or secondary outcome.
Incomplete outcome data (attrition bias)	**Low risk**	Intention to treat analysis
Selective reporting (reporting bias)	**Low risk**	Data provided of all patients that were randomized

Lewis 2011

Methods	A randomized, double-blinded, placebo-controlled multicenter study at 99 sites in Europe/Israel, 75 sites in North America and 26 sites in the Asia/Pacific region between October 2005 and June 2008
Participants	1056 microalbuminuric, diabetic patients with a mean age of 62, 76% male, baseline SBP/DBP of 131/74 and a mean eGFR of 78 ml/min.
Interventions	Sulodexide 200 mg/day Placebo All patients were treated for 6 months
Outcomes	Conversion to normoalbuminuria and a 25% reduction in albumin-creatinine ratio from the baseline level, or a 50% reduction in albumin-creatinine ratio from baseline.
Notes	Three seated blood pressures were obtained 1 minute apart and averaged for the blood pressure of record. No significant differences in adverse events. Follow-up data retrieved after contact with corresponding author.

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	**Unclear risk**	'Patients who met inclusion criteria at the end of the run-in period proceeded to randomization. Patients were randomly assigned to treatment with placebo or sulodexide, 100 mg, twice daily at a 1:1 ratio' Randomization method unknown
Allocation concealment (selection bias)	**Unclear risk**	No data on allocation concealment
Blinding of participants and personnel (performance bias)	**Low risk**	Double blind
Blinding of outcome assessment (detection bias)	**Low risk**	Blood pressure was regularly measured at study visits and was no primary or secondary outcome.
Incomplete outcome data (attrition bias)	**Low risk**	'All analyses in this report were performed using a modified intention to-treat basis.'
Selective reporting (reporting bias)	**Low risk**	Data provided of all patients that were randomized

Packham 2012

Methods	A randomized, double-blinded, placebo-controlled multicenter study at 114 sites in Europe/Israel, 77 sites in North America and 27 sites in the Asia/Pacific region between August 2005 and February 2008
Participants	1248 macroalbuminuric diabetic patients with a mean age of 63, 61% male, baseline SBP/DBP 138/73 and a mean eGFR of 31 ml/min.
Interventions	Sulodexide 200 mg/day Placebo Mean follow-up of patients was 11 months
Outcomes	Time until the first occurrence of a confirmed doubling of baseline serum creatinine or ESRD
Notes	After 1029 person-years of follow-up, no significant differences were detected between sulodexide and placebo. The study was therefore terminated early. Data on specific antihypertensive medications used during follow-up were not available. Blood pressure data is reported after 3 months of follow-up

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	**Low risk**	Randomization between sulodexide and placebo
Allocation concealment (selection bias)	**Low risk**	Central computer-based allocation service
Blinding of participants and personnel (performance bias)	**Low risk**	Double blind
Blinding of outcome assessment (detection bias)	**Low risk**	Blood pressure was regularly measured at study visits and was no primary or secondary outcome.
Incomplete outcome data (attrition bias)	**High risk**	'Twenty-seven patients withdrew consent and five others were lost to follow-up.' 'No differences in side effect profiles of sulodexide and placebo and, importantly, no serious adverse events were ascribed to the trial drug.' No data is provided whether these cases were particularly in the sulodexide or placebo group
Selective reporting (reporting bias)	**Low risk**	Early termination of a trial may introduce bias and overestimate effects. Since blood pressure data after 3 months is used in this analysis, the influence of early termination is only minor. In addition, blood pressure is not an endpoint on which termination has been decided.

Solini 1997

Methods	A randomized, double-blind, placebo-controlled, single center cross-over study
Participants	12 hypertensive micro- and macroalbuminuric diabetic patients with a mean age of 52, baseline SBP/DBP of 155/81 and a mean eGFR of 97 ml/min.
Interventions	Sulodexide 100 mg/day Placebo All patients were treated for 4 months
Outcomes	Albumin excretion rate
Notes	All patients were under antihypertensive pharmacological treatment from at least one year and none had registered any change in therapeutic scheme, either during the 6 months preceding enrolment in our trial or during the study period. Blood pressure measurements were performed were made by the same person, who had a Hawskley random-zero sphygmomanometer with two different cuff sizes.

Risk of bias table

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	**Unclear risk**	Patients were randomized between starting with sulodexide or placebo. Method of randomization unknown
Allocation concealment (selection bias)	**Unclear risk**	No data on allocation concealment
Blinding of participants and personnel (performance bias)	**Low risk**	Double blind
Blinding of outcome assessment (detection bias)	**Low risk**	Blood pressure was regularly measured at study visits and was no primary or secondary outcome.
Incomplete outcome data (attrition bias)	**Low risk**	All patients completed the study
Selective reporting (reporting bias)	**Low risk**	All patients completed the study

Velussi 1996

Methods	Randomized, placebo-controlled, single center, open-label cross-over study
Participants	24 microalbuminuric diabetic patients with a mean age of 67, 67% male, baseline SBP/DBP of 161/85 and baseline serum creatinine of 0.95 mg/dL.
Interventions	Sulodexide
Outcomes	Albuminuria, plasma fibrinogen and pain-free walking distance
Notes	Patients were not under anti-hypertensive treatment at enrolment nor was such treatment started ruing the study. Blood pressure measurements were performed by the same person during the study. After five minutes rest, three blood pressure measurements were performed of which the mean was calculated.

Risk of bias table

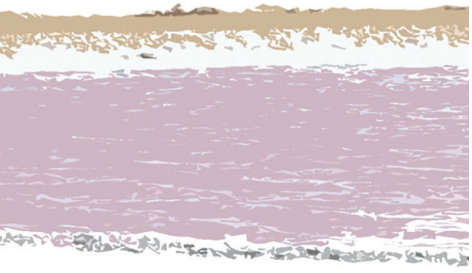
Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	**Unclear risk**	'The 24 patients were randomly divided in two groups which followed an inverse cross-over therapy schedule.' No data on the sequence generation
Allocation concealment (selection bias)	**Unclear risk**	No data on allocation concealment.
Blinding of participants and personnel (performance bias)	**High risk**	No blinding of participants or personnel
Blinding of outcome assessment (detection bias)	**Low risk**	Blood pressure was regularly measured at study visits and was no primary or secondary outcome.
Incomplete outcome data (attrition bias)	**Low risk**	'No dropouts were observed during the whole study period.'
Selective reporting (reporting bias)	**Low risk**	'No dropouts were observed during the whole study period.'

Supplemental Data 2

Search strategy:

"sulodexide" [All Fields] OR "Iduronylglycosaminoglycan sulfate" [All Fields] OR "glucuronyl glucosamine glycan sulfate"[Supplementary Concept] OR "glucuronyl glucosamine glycan sulfate"[All Fields]

Because we did not want to limit our search to certain diseases states or populations, we did not add any condition or population characteristic to the search strategy.



7 Summary and Perspectives

SUMMARY

This thesis titled **'Sodium-induced changes of the endothelial surface layer and microcirculation'** is aimed to study the influence of sodium loading (either by diet or infusion) on the microcirculation and endothelial surface layer. Our aim is to improve our understanding of sodium induced microcirculatory changes in regard to blood pressure changes.

CHAPTER 1 introduced the main subjects of this thesis. Historically, sodium has influenced our society in many ways and continues to do so in the field of health and disease. Epidemiological studies have associated high sodium intake with high blood pressure and increased risk of cardiovascular events. Conversely, reduction of sodium intake has resulted in reduced blood pressure and subsequently decreased risk of cardiovascular disease. These observations have led to lifestyle recommendations to limit dietary sodium intake to a maximum of 2 g (≈ 5 g salt) per day. Reduction of sodium intake has been recognized as a target for improvement of global health and lowering of medical costs. However, this target has not been met in any country and the optimum of maximum daily sodium intake remains controversial. Furthermore, it is also not fully understood *how* sodium loading leads to increased blood pressure and increased risk of cardiovascular events, and *why* the blood pressure response following sodium loading is so heterogeneous among individuals. Some people respond to high salt intake with increased blood pressure, also known as salt-sensitivity, while others do not (called salt-resistant blood pressure). We propose that sodium-associated microcirculatory changes might explain the -not yet clarified - link between sodium and high blood pressure and discuss two new mechanisms regarding sodium homeostasis. First, nonosmotic sodium storage is introduced, a mechanism that has challenged the classical two compartment view of sodium and volume homeostasis. It has been shown that glycosaminoglycans are able to bind sodium without water retention (thus store it in osmotically inactive form). Second, we describe a new view on sodium-sensitive hypertension, that centralizes vasodysfunction (i.e. inability to adjust vascular tone) as the explaining mechanism of sodium-sensitive hypertension. This contrasts with the classical view that focused on the role of the kidney and assigned expansion of extracellular volume and increased cardiac output as causes of elevated blood pressure. Both new concepts of nonosmotic sodium storage and vasodysfunction are suggested to be connected.

CHAPTER 2 reviewed the concept of nonosmotic sodium storage and its influence on sodium homeostasis. We focused on the role of the endothelial surface layer, a dynamic layer consisting of membrane bound proteoglycans, glycosaminoglycans and plasma proteins located at the luminal side of the microcirculation. Glycosaminoglycans have demonstrated to possess sodium binding characteristics in skin interstitium. We suggested that the endothelial surface layer, also containing these glycosaminoglycans, can also function as an intravascular buffer for nonosmotic sodium storage. Therefore the endothelial surface layer may serve as a target for therapy in treatment of high blood pressure and in conditions with expanded extracellular volume.

In **CHAPTER 3** we examined the contribution of nonosmotic sodium storage in twelve healthy volunteers following intravenous sodium loading on a low-sodium diet. We compared observed changes in plasma sodium and urinary cation excretion during four hours following infusion with the expected values as calculated with two commonly used formulas used to guide treatment of dysnatriemia (Adrogue-Madias and Nguyen-Kurtz formulas). Directly after infusion the average observed increase in plasma sodium (+3.5 mmol/L) was similar to the predicted changes (+3.3 mmol/L Adrogue-Madias; +3.1 mmol/L Nguyen-Kurtz). Four hours after infusion the observed and predicted plasma sodium changes were totally different (observed –1.8 mmol/L, Adrogue-Madias +0.4 mmol/L, Nguyen-Kurtz –0.9 mmol/L). Furthermore observed urinary cation excretion after four hours was only 47% (Adrogue-Madias) and 55% (Nguyen-Kurtz) of expected urinary cation excretion, indicating that healthy volunteers are able to osmotically inactivate a significant amount of sodium following intravenous saline infusion.

In **CHAPTER 4** we explored whether high salt intake influences sublingual microvascular density using SDF videomicroscopy, and assessed whether changes in body weight impact these microvascular changes in eighteen normotensive volunteers. All subjects followed a high sodium diet (>200 mmol/day) and low sodium diet (<50 mmol/day) for fourteen days each in randomized order. Sublingual nitroglycerin was administered to induce vasodilation and maximize residual microvascular capacity. We observed no changes in blood pressure or microvascular density between diets. We demonstrated that higher salt intake is correlated with decreased sublingual microvascular density following administration of nitroglycerin. Larger changes in body weight following high salt intake are accompanied by larger reductions of microvascular density. Changes in microvascular density occurred without blood pressure effects, indicating that a high dietary salt load independently contributes to microvascular changes.

In **CHAPTER 5** we studied the effects of acute intravenous sodium loading and chronic dietary sodium loading on microvascular permeability and the endothelial surface layer, and assessed whether these interventions differently affect these endpoints. Twelve healthy males were randomized to both a low sodium diet (<50 mmol/day) and high sodium diet (>200 mmol/day) for eight days, separated by a crossover period of at least 1 week. Following low sodium diet subjects received intravenous hypertonic saline infusion over the course of 30 minutes. Both sodium interventions did not alter blood pressure. Acute intravenous sodium loading and chronic dietary sodium loading resulted in similar increases of plasma sodium, chloride and osmolality, but had a different effect on microvascular permeability, measured with transcapillary escape rate of ^{125}I-labeled albumin. Acute intravenous sodium loading increased microvascular permeability, but chronic dietary sodium loading did not. Increased microvascular permeability following saline infusion coincided with decreased urinary glycosaminoglycan excretion, indicating damage to the endothelial surface layer, occurring independently of blood pressure. We demonstrate that hypertonic saline infusion has direct deleterious microvascular effects in healthy subjects. This might imply that fluid therapy with saline, often used in hospital settings, can be harmful for patients.

In **CHAPTER 6** we analyzed with a systematic review and meta-analysis whether restoration of the endothelial surface layer with sulodexide, a highly purified mixture of glycosaminoglycans, leads to decreased blood pressure. Sulodexide is marketed for various cardiovascular indications (e.g. reduction of claudication symptoms in peripheral vascular disease) but there are no clinical trials investigating the antihypertensive capability of this drug. We searched MEDLINE, EMBASE and Cochrane library databases for clinical trials that included administration of sulodexide to adult subjects, and included the studies that reported blood pressure and treatment of at least 4 weeks. Eight studies with totally 3019 subjects were included, with seven studies comparing sulodexide to placebo, and one study comparing to a control group that did not receive any treatment. In six studies sulodexide was administered on top of renin-angiotensin system inhibiting medication. Sulodexide treatment resulted in significant reduction of systolic blood pressure (-2.2 mmHg, P=0.02) and diastolic blood pressure (-1.7 mmHg, P=0.004). Blood pressure reduction was larger in patients with uncontrolled blood pressure at baseline (systolic blood pressure -10.2 mmHg, P<0.001; diastolic blood pressure -5.4 mmHg, P<0.001).

FINAL CONSIDERATIONS AND PERSPECTIVES

This thesis illustrates that our knowledge regarding sodium in (cardiovascular) health and disease continues to be challenged. We have demonstrated that healthy volunteers are able to osmotically inactivate sodium following saline infusion. Furthermore, we have shown that both a dietary sodium load and an acute intravenous sodium load have damaging effects on the endothelial surface layer and microcirculation, independent of blood pressure. We propose that sulodexide, a mixture of constituents of the endothelial surface layer, might be a new interesting drug to lower blood pressure. Our studies provide new insights into the relation between sodium, endothelial surface layer and microcirculation, but also generate new questions.

First, we have challenged the classical two compartment view on sodium and volume homeostasis[1,2]. However, we were not able to localize the nonosmotically stored sodium. Further research, with currently available techniques such as ^{23}Na-MRI[3], should provide more insight where sodium is stored and what the clinical impact is of nonosmotic sodium storage. Until now, it has been demonstrated that tissue sodium measured with ^{23}Na-MRI increases with age in men[4] and increased tissue sodium content is found in patients with hypertension, hyperaldosteronism, heart failure, end-stage renal disease or infection[3-7]. Interventions with diuretics, sodium glucose cotransporter 2 (SGLT-2) inhibition, dialysis, desmopressin and hypotonic fluid treatment in hypernatriemia have demonstrated to reduce tissue sodium content[5,7-9].

Furthermore, we demonstrated effects of acute intravenous and chronic dietary sodium loading on the endothelial surface layer in healthy volunteers without blood pressure effects[10,11]. It will be interesting to study these interventions also in patients that already have high blood pressure, or in those who are prone for developing high blood pressure or sodium-sensitivity, such as diabetic patients[12], to examine whether these microvascular manifestations are also present in these patient groups, and if blood pressure will be affected. Moreover, studies comparing different infusion fluids, for example 0.9% NaCl, other crystalloids and colloids, should be carried out to determine whether our results regarding intravenous sodium loading can be explained either by increased plasma volume or by sodium contents of the infusion.

Additionally, we have pointed out the antihypertensive potential of sulodexide[13]. Sulodexide may achieve these blood pressure lowering effects due to increased nitric oxide production and increased nonosmotic sodium storage. Because of these possible mechanisms of action that differ from currently available medications, sulodexide might be an interesting addition to the group of antihypertensive drugs. Future research regarding optimal dosage, the underlying mechanism of blood pressure lowering, and comparisons to other antihypertensive drugs are needed to confirm the results of our meta-analysis.

In general, more research is needed to fully understand the relationship between sodium, endothelial surface layer, nonosmotic sodium storage, sodium-sensitive hypertension and microcirculation in order to improve treatment of hypertension, dysnatriemia and other conditions involving disturbed sodium and water homeostasis.

The salt shaker of sodium related research questions is still far from empty …

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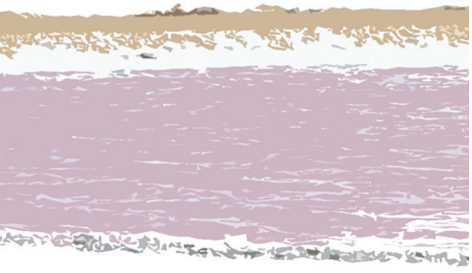

8 Nederlandse Samenvatting

SAMENVATTING

Dit proefschrift, getiteld **'Sodium-induced changes of the endothelial surface layer and microcirculation'** oftewel **'Natrium geïnduceerde veranderingen van de endotheliale oppervlaktelaag en de microcirculatie**' is er op gericht om de invloed van natriumbelasting (door middel van een dieet of infusie) op de microcirculatie en de endotheliale oppervlaktelaag te onderzoeken, zodat we ons begrip van de door natrium veroorzaakte veranderingen van de microcirculatie in relatie tot veranderingen in bloeddruk kunnen verbeteren.

HOOFDSTUK 1 geeft een introductie ten aanzien van de belangrijkste onderwerpen in dit proefschrift. In het verleden heeft natrium onze maatschappij op vele manieren beïnvloed en deze invloed is nog steeds aanwezig op het gebied van gezondheid en ziekte. Epidemiologische studies hebben verbanden tussen hoge natrium inname en zowel hoge bloeddruk als verhoogd risico op hart- en vaatziekten aangetoond. En een afname van de natriuminname zorgt voor een daling in bloeddruk en daaropvolgend ook een verminderd risico op hart- en vaatziekten. Dit heeft er toe geleid dat er leefstijladviezen worden gegeven om de dagelijkse inname van natrium te beperken tot 2 gram (ongeveer 5 gram zout) per dag. Vermindering van natriuminname wordt gezien als een belangrijk doel om wereldwijd gezondheid te verbeteren en medische kosten te verlagen. Dit doel wordt echter nog niet behaald en de optimale maximale hoeveelheid van natrium in het dieet blijft een punt van discussie. Verder begrijpen we nog niet goed *hoe* natriumbelasting leidt tot verhoogde bloeddruk en een verhoogd risico op hart- en vaatziekten, en *waarom* de bloeddruk reactie op een grote hoeveelheid natrium zo verschillend is tussen individuen. Sommige mensen reageren op een hoge zoutinname met een verhoogde bloeddruk, ook wel bekend als zoutgevoeligheid, terwijl andere geen verhoogde bloeddruk krijgen (zoutresistentie genoemd). We stellen voor dat veranderingen in de microcirculatie ten gevolge van natrium het verband, dat we tot op heden niet ontrafeld hebben, tussen natrium en hoge bloeddruk zouden kunnen verklaren. We bespreken twee nieuwe mechanismen die van belang zijn voor de natriumbalans. Ten eerste wordt het begrip van niet-osmotische natriumopslag geïntroduceerd, een mechanisme dat het klassieke twee compartimenten model van de natrium- en volumebalans uitdaagt. Studies hebben laten zien dat glycosaminoglycanen, negatief geladen suikerketens die onder andere aanwezig zijn in het interstitium van de huid, aan natrium kunnen binden zonder water vast te houden (en het daarmee kunnen opslaan in een osmotisch inactieve vorm). Ten tweede beschrijven we een nieuwe visie op zoutgevoelige hoge bloeddruk, dat vaatdysfunctie (d.w.z. de vaatspanning niet kunnen aanpassen) een centrale rol geeft om het

mechanisme hierachter uit te leggen. Dit staat in tegenstelling tot de klassieke opvatting dat de nier verantwoordelijk is voor verhoogde bloeddruk door het vasthouden van vocht, leidend tot toename van het extracellulair volume en de daaropvolgende verhoogde cardiale output. Beide nieuwe concepten van niet-osmotische natriumopslag en vaatdysfunctie lijken een connectie te hebben.

HOOFDSTUK 2 beschrijft het concept van niet-osmotische natrium opslag en de invloed hiervan op de natriumbalans. We gaan hier met name in op de rol van de endotheliale oppervlaktelaag, een dynamische laag die uit membraan gebonden proteoglycanen, glycosaminoglycanen en plasma eiwitten bestaat, en gelokaliseerd is aan de luminale zijde van de microcirculatie. Er is aangetoond dat glycosaminoglycanen in het interstitium van de huid natriumbindende eigenschappen hebben. Wij geven in dit hoofdstuk de suggestie dat de endotheliale oppervlaktelaag, die ook deze glycosaminoglycanen bevat, kan dienen als een intravasculaire buffer voor niet-osmotische natriumbinding. Deze endotheliale oppervlaktelaag zou daardoor kunnen fungeren als doelwit voor behandeling van hoge bloeddruk en andere condities waarbij het extracellulair volume toegenomen is.

In **HOOFDSTUK 3** hebben we de bijdrage van de niet-osmotische natriumopslag in twaalf gezonde vrijwilligers onderzocht nadat zij een natriumbelasting via het infuus kregen tijdens het volgen van een laag natrium dieet. We hebben de waargenomen verandering van het plasma en urine natrium gedurende vier uur na infusie vergeleken met de verwachte veranderingen, die we berekenden met behulp van twee vaak gebruikte formules voor de behandeling van dysnatriëmie (Adrogue-Madias and Nguyen-Kurtz formules). Direct na infusie kwamen de waargenomen gemiddelde waarde van het plasma natrium (+3.5 mmol/L) en de verwachte stijgingen overeen (+3.3 mmol/L Adrogue-Madias; +3.1 mmol/L Nguyen-Kurtz). Vier uur na infusie waren de waargenomen en verwachte waarden echter totaal verschillend (waargenomen –1.8 mmol/L, Adrogue-Madias +0.4 mmol/L, Nguyen-Kurtz –0.9 mmol/L). Bovendien was de geobserveerde urine kation uitscheiding na 4 uur slechts 47% (Adrogue-Madias) en 55% (Nguyen-Kurtz) van de verwachte urine kation uitscheiding. Dit laat zien dat gezonde vrijwilligers in staat zijn om een significante hoeveelheid natrium osmotisch te inactiveren na een intraveneuze natriumbelasting.

In **HOOFDSTUK 4** hebben we met behulp van SDF videomicroscopie gekeken of hoge zoutinname van invloed is op de microvasculaire dichtheid van de vaatjes onder de tong bij achttien vrijwilligers met een normale bloeddruk. Ook hebben we onderzocht of veranderingen in het lichaamsgewicht de microvasculaire veranderingen

kunnen beïnvloeden. Alle proefpersonen volgden een hoog natriumdieet (>200 mmol/dag) en een laag natrium dieet (<50 mmol/dag) gedurende 14 dagen in gerandomiseerde volgorde. Nitroglycerine werd onder de tong toegediend om vaatverwijding te bewerkstellingen en de resterende microvasculaire capaciteit te maximaliseren. We hebben geen veranderingen aangetoond in de bloeddruk of microvasculaire dichtheid tussen de twee diëten. We laten zien dat een hogere zoutinname samenhangt met een daling van de microvasculaire dichtheid onder de tong na toediening van nitroglycerine en dat grotere gewichtsveranderingen na hoge zoutinname samengaan met grotere daling van de microvasculaire dichtheid. De veranderingen in microvasculaire dichtheid traden op zonder effecten op de bloeddruk. Dit geeft een indicatie dat de hoge zoutbelasting op zichzelf bijdraagt aan microvasculaire veranderingen.

In **HOOFDSTUK 5** hebben we de effecten van acute intraveneuze natriumbelasting en chronische natriumbelasting via het dieet op de microvasculaire doorlaatbaarheid en endotheliale oppervlaktelaag bestudeerd, en bekeken of deze interventies een verschillend effect hebben op beiden. Twaalf gezonde mannen werden gerandomiseerd en volgden zowel een laag natrium (<50 mmol/dag) als een hoog natrium dieet (>200 mmol/dag) gedurende acht dagen, gescheiden door een cross-over periode van tenminste 1 week. Na het laag natrium dieet kregen de proefpersonen een intraveneuze hypertone zoutinfusie gedurende 30 minuten. Beide natrium interventies zorgden niet voor bloeddruk veranderingen. Acute intraveneuze natriumbelasting en chronische diëtaire natriumbelasting gaven dezelfde stijging in plasma natrium, chloride en osmolaliteit, maar hadden een verschillend effect op de microvasculaire doorlaatbaarheid, zoals gemeten met de transcapillaire ontsnappingssnelheid van ^{125}I-gelabeld albumine. Acute intraveneuze natriumbelasting verhoogde de microvasculaire doorlaatbaarheid maar dat deed de chronische diëtaire natriumbelasting niet. De verhoogde microvasculaire doorlaatbaarheid ging samen met verminderde glycosaminoglycanen uitscheiding via de urine, dat kan wijzen op schade van de endotheliale oppervlaktelaag. Dit alles trad op onafhankelijk van de bloeddruk. We laten daarmee zien dat infusie met hypertoon zout direct nadelige effecten heeft op de microcirculatie van gezonde proefpersonen. Dit zou kunnen betekenen dat infusie therapie met zout, dat vaak gebruikt wordt in het ziekenhuis, ook schadelijk kan zijn voor patiënten.

In **HOOFDSTUK 6** hebben we met behulp van een systematische review en meta-analyse onderzocht of herstel van de endotheliale oppervlaktelaag met sulodexide, een medicijn dat bestaat uit een mengsel van glycosaminoglycanen, leidt tot verlaging van

de bloeddruk. Sulodexide is voor verschillende hart- en vaatziekten op de markt beschikbaar, zoals het reduceren van claudicatie symptomen bij perifeer vaatlijden maar er zijn geen klinische onderzoeken die de mogelijkheden voor bloeddrukverlaging hebben onderzocht. We hebben in de MEDLINE, EMBASE en Cochrane bibliotheek databases gezocht naar klinische studies die sulodexide gaven aan volwassen patiënten. De studies die bloeddruk rapporteerden en ten minste 4 weken behandeling gaven, hebben we meegenomen in onze meta-analyse. In totaal hebben we acht studies met 3019 patiënten geïncludeerd, waarvan zeven studies sulodexide vergeleken met placebo, en één studie de vergelijking maakte met een controle groep die helemaal geen behandeling kreeg. In zes studies werd sulodexide voorgeschreven bovenop andere bloeddrukmedicatie, namelijk renine-angiotensine systeem blokkerende medicatie. Sulodexide zorgde voor een significante afname van de systolische bloeddruk (-2.2 mmHg, P=0.02) en diastolische bloeddruk (-1.7 mmHg, P=0.004). De bloeddruk verlaging was groter in de groep patiënten die een te hoge bloeddruk hadden bij aanvang (systolische bloeddruk -10.2 mmHg, P<0.001; diastolische bloeddruk -5.4 mmHg, P<0.001.

SLOT OPMERKINGEN EN TOEKOMSTPERSPECTIEF

Dit proefschrift beschrijft dat onze kennis aangaande de rol van natrium in (cardiovasculaire) gezondheid en ziekte nog steeds uitgedaagd wordt. We hebben aangetoond dat gezonde vrijwilligers in staat zijn om natrium osmotisch te inactiveren na een zoutinfusie. Bovendien hebben we laten zien dat zowel een diëtaire natriumbelasting als een acute intraveneuze natriumbelasting schadelijke effecten heeft op de endotheliale oppervlaktelaag en de microcirculatie, onafhankelijk van de bloeddruk. We stellen voor dat sulodexide, een mengsel van bouwstoffen van de endotheliale oppervlaktelaag, een nieuw interessant medicijn is om de bloeddruk te verlagen. Onze studies geven nieuwe inzichten in de relatie tussen natrium, de endotheliale oppervlaktelaag en de microcirculatie, maar werpen ook nieuwe vragen op.

Ten eerste hebben we het klassieke twee compartimenten model van de natrium- en volumebalans uitgedaagd[1,2]. We zijn echter niet in staat geweest om het niet-osmotisch opgeslagen natrium te lokaliseren. Er is meer onderzoek nodig, met technieken zoals ^{23}Na-MRI[3], om inzicht te krijgen waar het natrium wordt opgeslagen en wat de klinische betekenis van niet-osmotische natrium opslag is. Tot nu toe is er aangetoond dat weefsel natrium leeftijdsafhankelijk toeneemt bij mannen en dat verhoogd weefsel natrium aanwezig is in patiënten met hypertensie, hyperaldosteronisme, hartfalen, eindstadium nierfalen of infecties[3-7]. Interventies met diuretica, sodium glucose cotransporter 2

(SGLT-2) remmers, dialyse, desmopressine en hypotone vloeistoffen in het geval van hypernatriëmie hebben laten zien dat ze de hoeveelheid weefsel natrium kunnen laten dalen[5,7-9].

Bovendien hebben we effecten van acute intraveneuze en chronische diëtaire natriumbelasting laten zien in gezonde vrijwilligers zonder bloeddrukeffecten[10,11]. Het is interessant om deze interventies ook te doen in patiënten die al hoge bloeddruk hebben, of in diegene die meer gevoelig zijn voor het ontwikkelen van hoge bloeddruk of zoutgevoeligheid, zoals diabetici[12], om te onderzoeken of de microvasculaire effecten ook aanwezig zijn in deze patiëntengroepen en of de bloeddruk beïnvloed zal worden.

Vervolgens zouden er studies uitgevoerd kunnen worden die verschillende infusie vloeistoffen (zoals 0.9% NaCL en andere kristalloïden en colloïden) vergelijken om te bepalen of onze resultaten ten aanzien van intraveneuze natriumbelasting het gevolg zijn van het verhoogde plasma volume, of komen door de natrium eigenschappen van de vloeistof. Ook hebben we gewezen op de bloeddrukverlagende capaciteiten van sulodexide[13]. Het is mogelijk dat sulodexide deze bloeddrukdalingen veroorzaakt door de stikstofoxide productie en niet-osmotische natriumopslag te verhogen. Doordat het werkingsmechanisme verschillend is dan dat van andere beschikbare medicamenten, kan sulodexide een interessante toevoeging zijn aan de groep van bloeddruk verlagende middelen. Toekomstig onderzoek zal meer informatie op kunnen leveren ten aanzien van de optimale dosis, het onderliggende mechanisme van de bloeddruk verlaging en in vergelijking tot andere bloeddrukverlagende medicatie om de resultaten van onze meta-analyse te bevestigen.

In het algemeen kunnen we stellen dat er meer onderzoek nodig is om de relatie tussen zout, de endotheliale oppervlaktelaag, niet-osmotische natriumopslag, zoutgevoelige hypertensie en microcirculatie compleet te begrijpen, zodat we de behandeling van hoge bloeddruk, dysnatriëmie en andere condities die een verstoorde water- en zoutbalans veroorzaken, kunnen verbeteren.

Het zoutvaatje met zoutgerelateerde onderzoeksvragen is nog lang niet leeg …

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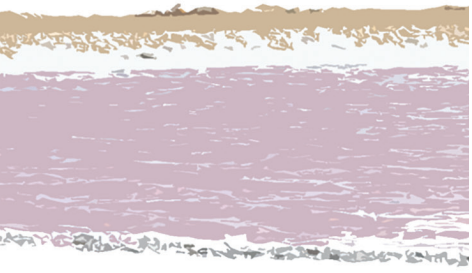

9 Appendices

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List of Publications
PhD portfolio
Curriculum Vitae
Dankwoord

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   Biol Blood Marrow Transplant. 2014 20:564-570.

PhD PORTFOLIO

Name Nienke Marja Geeske Rorije
PhD period January 2014 - May 2016
PhD supervisors Prof. dr. J.J. Homan van der Heide, dr. L. Vogt, dr. B.J.H. van den Born

PhD training

General Courses	Year	ECTS
Medline/Embase	2015	0.1
Oral Presentation	2015	0.8
Citation analysis and impact factor	2015	0.1
Project Management	2015	0.6
Scientific Writing	2015	1.5
Searching for a systematic review	2014	0.1
Clinical Epidemiology: Systematic Reviews	2014	0.7
Practical Biostatistics	2014	1.1
BROK: Legislation and Organization for Clinical Researchers	2014	1.0
Specific courses		
TIAS NIMBAS and Medical Business Education Summer Academy	2016	2.0
Kidney Week Early Program: 'Diagnosis and Management of Disorders of Acid-Base, Fluid, and Electrolyte Balance.	2014	1.0
Winterschool Dutch Kidney Foundation	2014	1.1
Seminars, workshops and masterclasses		
Weekly department seminars, Division of Nephrology	2014-2016	2.5
Weekly department seminars, Hypertension Research group	2014-2016	2.5
Journal Club, Division of Vascular Medicine	2014-2016	0.5
Discipline Overstijgend Onderwijs Medisch Leiderschap	2016	0.25
Wetenschap, Inspiratie, Talent festival	2016	0.1
Spinoza Masterclass, Steven Goodman 'How do you deal with biomedical journals?'	2016	0.1
Medical Business Masterclass	2016	0.6
Stichting Medical Business en Deloitte kennisavond	2015	0.1
Consensus meeting Microcirculation, AMC, Amsterdam	2015	0.1
Regional meeting Nephropathology, AMC, Amsterdam	2014	0.1
Parameters of Esteem: Awards and Prizes		
Accommodation Grant, 25th European Meeting on Hypertension and Cardiovascular Protection, Milan, Italy	2015	
AMC Graduate School PhD Poster Award, outstanding poster	2015	
Travel Grant, ASN Kidney Week, Philadelphia, USA	2014	

Presentations	Year	ECTS
Oral		
Dialysis Initiatives Meeting, Doorn, the Netherlands	2017	0.5
25th European Meeting on Hypertension and Cardiovascular Protection, Milan, Italy	2015	0.5
52nd ERA-EDTA Congress, London, United Kingdom	2015	0.5
Dutch-Belgian-Swiss Hypertension meeting, Antwerp, Belgium	2015	0.5
Fall Symposium, Dutch Federation of Nephrology, Utrecht, the Netherlands	2014	0.5
Poster		
AMC PhD Poster Awards	2015	0.5
American Society of Nephrology, Kidney Week, Philadelphia	2014	0.5
Fall Symposium, Dutch Federation of Nephrology, Utrecht, the Netherlands	2014	0.5
(Inter)national Conferences		
4th New Kids on the Block symposium, Amsterdam, the Netherlands	2016	0.25
Jong AMC/Jong VUMC Symposium, Amsterdam, the Netherlands	2015	0.1
3rd New Kids on the Block symposium, Amsterdam, the Netherlands	2015	0.25
25th European Meeting on Hypertension and Cardiovascular Protection, Milan, Italy	2015	1.0
52nd ERA-EDTA Congress, London, United Kingdom	2015	1.0
Dutch-Belgian-Swiss Hypertension meeting, Antwerp, Belgium	2015	0.5
ASN Kidney Week, Philadelphia, United States of America	2014	1.0
PLAN Day, Leiden, the Netherlands	2014	0.25
Fall Symposium, Dutch Federation of Nephrology, Utrecht, NL	2014	0.25
2nd New Kids on the Block symposium, Amsterdam, the Netherlands	2014	0.25
APROVE Science Night, Amsterdam, the Netherlands	2014	0.1
4th Cardiovascular Conference, Ermelo, the Netherlands	2014	0.5
National Hypertension Congress, Zeist, Netherlands	2014	0.1
Other		
Organizing committee yearly field hockey tournament AMC	2014	0.5
Organizing committee Dam to Dam cycle tour, fund raiser for Dutch Kidney Foundation	2014	0.5
Teaching - Supervising students		
Noori Guman, bachelor Medicine	2015-2016	0.7
Berta Esteve Soler, research internship Medical Informatics	2015	0.7
Robin Bokelaar, master thesis Medicine	2014	1.0

CURRICULUM VITAE

Nienke Marja Geeske Rorije, daughter of Eduard Willem Rorije en Marja Geeske Rorije – de Boer, was born on March 27, 1988 in Leiderdorp, the Netherlands. She was raised in Oegstgeest and graduated from high school at the Stedelijk Gymnasium Leiden in 2005. In the same year she started her medical training at the University of Groningen. After clinical rotations at the University Medical Center Groningen and the Medisch Centrum Leeuwarden she went abroad in 2012 for two internships. At the Diakonessenhuis in Paramaribo, Suriname, she completed her tropical medicine rotation. Her research clerkship at the Brigham and Women's hospital and Dana Farber Cancer Institute in Boston, United States of America resulted in her master thesis "BK Virus following allogeneic stem cell transplantation: a cohort analysis" and a publication in a peer reviewed journal.

Nienke completed her medical training with senior clinical clerkships in internal medicine at the Kennemer Gasthuis in Haarlem and intensive care medicine at the Onze Lieve Vrouwe Gasthuis in Amsterdam. In 2013 she received her medical degree and started working as a resident at the departments of Internal Medicine and Cardiology of the Kennemer Gasthuis hospital in Haarlem.

In January 2014 she commenced her PhD trajectory at the section of Nephrology of the Academic Medical Center in Amsterdam under the supervision of dr. L. Vogt, dr. B.J.H. van den Born and prof. dr. J.J. Homan van der Heide. The results of this project are presented in this thesis.

In September 2016 she started her internal medicine residency training. She currently works at the Onze Lieve Vrouwe Gasthuis in Amsterdam.

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Amsterdam
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