ACTA PHYSIOLOGICA

The interstitium conducts extrarenal storage of sodium and represents a third compartment essential for extracellular volume and blood pressure homeostasis

Journal:	*Acta Physiologica*
Manuscript ID	APH-2017-06-0242.R2
Manuscript Type:	Review Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Wiig, Helge; University of Bergen, Department of Biomedicine Luft, Friedrich; Max-Delbrück Center for Molecular Medicine, Experimental and Clinical Research Center Titze, Jens; Vanderbilt University, Department of Medicine
Key Words:	blood pressure, extracellular fluid, extracellular matrix, hypertension, sodium, salt

The interstitium conducts extrarenal storage of sodium and represents a third compartment essential for extracellular volume and blood pressure homeostasis

Helge Wiig[1], Friedrich C Luft[2,3] and Jens Titze[3]

[1]Department of Biomedicine, University of Bergen, Bergen Norway

[2]Experimental and Clinical Research Center, Max-Delbrück Center for Molecular Medicine, Charité Medical Faculty, Berlin, Germany

[3]Division of Clinical Pharmacology, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

Running title: Interstitium and arterial hypertension

Corresponding author: Helge Wiig

Department of Biomedicine, University of Bergen

Jonas Lies vei 91, N 5009 Bergen, Norway

Tel: +47 55 58 63 87

e-mail: helge.wiig@uib.no

Abstract

The role of salt in the pathogenesis of arterial hypertension is not well understood. According to the current understanding, the central mechanism for blood pressure regulation relies on classical studies linking blood pressure and Na^+ balance, placing the kidney at the very centre of long-term blood pressure regulation. To maintain blood pressure homeostasis, the effective circulating fluid volume and thereby body Na^+ content has to be maintained within very narrow limits. From recent work in humans and rats, the notion has emerged that Na^+ could be stored somewhere in the body without commensurate water retention to buffer free extracellular Na^+, and that previously unidentified extrarenal, tissue-specific regulatory mechanisms are operative regulating the release and storage of Na^+ from a kidney-independent reservoir. Moreover, immune cells from the mononuclear phagocyte system not only function as local on-site sensors of interstitial electrolyte concentration, but also, together with lymphatics, act as systemic regulators of body fluid volume and blood pressure. These studies have established new and unexpected targets in studies of blood pressure control and thus the pathophysiology of hypertension; the interstitium-/extracellular matrix of the skin, its inherent interstitial fluid and the lymphatic vasculature forming a vessel network in the interstitium. Aspects of the interstitium in relation to Na^+ balance and hypertension are the focus of this review. Taken together, observations of salt storage in the skin to buffer free extracellular Na^+ and macrophage modulation of the extracellular matrix and lymphatics suggest that electrolyte homeostasis in the body cannot be achieved by renal excretion alone, but also relies on extrarenal regulatory mechanisms.

Keywords: Blood pressure, extracellular fluid, extracellular matrix, hypertension, sodium, salt.

Introduction

Cardiovascular diseases are the major cause of death worldwide and represent a dramatic socio-economic challenge. In a recent Global Burden of Disease Survey, high blood pressure or hypertension was, on a global basis, identified as the most important cause of premature death and disease[1]. Hypertension accounts for 18% of cardiovascular disease deaths in the Western countries, and is a major risk factor for stroke, coronary heart disease and heart failure[2] with an estimated cost of €169 billion in the European Union[3]. With an aging population, the prevalence of cardiovascular disease is expected to rise. The disease burden and related costs of hypertension is thus substantial and call for continuous effort to control this condition. Short and long-term blood pressure (BP) regulation is very complex and involve the actions of many cardiovascular, renal, neural and local tissue control systems as summarized in recent comprehensive reviews[4, 5]. Our focus here will be local tissue systems and in particular the role of salt storage in the interstitium. This area has been largely neglected by investigators even though Guyton et al, in their prescient systems model of blood pressure regulation included a major component in their model labelled tissue fluids, pressures, and gel[6]. The findings we describe are pertinent to not only this state-of-affairs, but also to long-forgotten earlier studies.

Salt intake has been implicated in blood pressure regulation and hypertension for over 5000 years. The role of salt in the pathogenesis of arterial hypertension is not well understood and is much debated[7, 8]. According to the current understanding, the central mechanism for blood pressure regulation relies on classical studies linking blood pressure

and Na^+ balance[9], placing the kidney at the very centre of long-term blood pressure regulation. Today, the kidney still has an "inextricable role" in hypertension[10] even though a role for other tissues, notably the skin, has emerged during the last years. As for the kidney, any imbalance between dietary salt intake and renal salt excretion leads to a progressive alteration in the filling of the vascular system and thus changes in blood pressure. To maintain blood pressure homeostasis, the effective circulating volume and thereby body Na^+ content has to be maintained within very narrow limits. However, already Cannon[11] suggested a role for the loose connective tissue in storage of salt and water in situations of "intolerable excess" designated as inundation, and that the skin could serve as a reservoir for sodium chloride that could be stored in an osmotically inactive form. Cannon made reference to work of Wahlgren, who in 1909, observed that one-third of the body's chloride (he could not measure sodium) was stored in the skin[12]. Moreover, Guyton et al suggested[13] that strongly negatively charged mucopolysaccharides (later called glycosaminoglycans, see below) could attract, and thus create, a higher density of cations such as Na^+, and that "tissue fluids, pressures and gel" could, as proposed in his extensive model, influence overall regulation of circulation[6]. Inspired by these earlier investigations and later experiments that might challenge the generally accepted concept of sodium homeostasis[14], Titze et al introduced a new paradigm with regard to handling of salt in the body. Based on several long-term studies in humans they found that considerable amounts of Na^+ are retained or removed from the subjects' bodies without commensurate water retention or loss[15]. From their work in humans and rats, it has emerged that Na^+ can be stored buffered in reservoirs in the body without commensurate water retention, and that previously unidentified extrarenal, tissue-specific regulatory mechanisms are operative regulating the release and storage of Na^+ from such a kidney-independent reservoir[16-22]. These studies suggested that

the interstium with its extracellular matrix and gel phase might be involved in Na^+ homeostasis, although, of course, not questioning the pivotal role of the kidney with regard to control of total body Na^+ and thereby extracellular volume[23]. Another unexpected aspect emerging from these studies on salt storage in skin is that immune cells from the mononuclear phagocyte system (MPS) including macrophages and dendritic cells, not only function as local on-site sensors of interstitial electrolyte concentration, but also, together with lymphatics, act as systemic regulators of body fluid and blood pressure homeostasis[24-26]. Together, the observations of Na^+ storage in the skin and macrophage modulation of tissue extracellular matrix composition and lymphatic structure suggest that effective clearance of electrolytes from the body cannot be achieved by renal excretion alone, but also relies on extrarenal regulatory mechanisms. Notwithstanding the fact that the role of the kidney is undisputed in fluid and blood pressure homeostasis, these studies have established new and unexpected targets in studies of blood pressure control and thus the pathophysiology of hypertension: The interstitium-/extracellular matrix of the skin, its inherent interstitial fluid and the lymphatic vasculature forming a vessel network in the interstitium of most tissues that has an important homeostatic role in the body, as recently emphasized by Bhave and Neilson[27] in light of new knowledge in the field. In this excellent review, they also explored how the interstitial storage and flow of Na^+ back into the circulation connects with renal Na^+ handling as part of an evolving model of body fluid dynamics. In the present review, aspects of the interstitium in relation to Na^+ balance and buffering in the skin and hypertension are the focus.

The interstitium

The interstitium is the space located between the capillary wall and cells[28]. It consists of a fiber framework where collagen is the major component, a gel phase of glycosaminoglycans (GAGs), and in tissues with continuous capillaries like skin and muscle in focus here, a salt solution containing plasma proteins like albumin and IgG and electrolytes. The major fluid compartments of the body are the extracellular fluid (ECF) and intracellular fluid (ICF), where the ECF is subdivided into plasma volume and interstitial fluid volume (ISF) that may be further subdivided depending on the site of the body and tissue type[27]. In the interstitium, the relative composition of the various components as well as the amount of ISF varies considerably between tissues. We will focus on the skin and muscle, where ISF is ≈50 % and ≈10% of wet tissue weight, respectively[29]. The composition, structure and function of the normal interstitium have been covered in several extensive reviews, and are thus only briefly described here[30-36]. GAGs, of particular interest here because of their negative charge[37], and the major fibrous structural elements (collagen) are important contributors to regulation of interstitial fluid volume and hydraulic conductivity[38], and have recently been addressed in this context by one of us[29]. Other components of the ECM include elastin, the cell adhesion proteins fibronectin, vitronectin, thrombospondins, and laminins[39-41].

1. Collagen. The collagens are a family of proteins in the ECM that have multiple functions, including a dominant scaffolding role in various tissues. All consist of three polypeptide α chains with triple-helical collagenous as well as non-collagenous domains. Their structures and functions have been comprehensively reviewed[41-45]. Collagen is the major component of

ECM in skin and muscle, amounting to ≈20%[29] and 1-10%[31] of wet tissue weight, respectively.

Whether or not the collagen fibril is charged at physiological pH is of interest in relation to Na^+ storage. Collagen is a polyampholyte and thus a protein having positively as well as negatively charged groups that self-compensates at pH 7.4. In general, collagen I fibrils carry a small positive charge (pI ≈8.0) at physiological pH[46, 47], estimated to +14 mol/mol in reconstituted collagen[48]. Collagen II has a zero net charge density in cartilage[49], resulting in a slight net positive charge on the molecule[46, 50]. Collagen has thereby almost no net charge, and behaves as an element without high local charge and with no Debye length, i.e. no persisting electrostatic effect[51].

2. Glycosaminoglycans (GAGs)

GAGs are unbranched polyanionic polysaccharide chains of variable length made up of repeating disaccharide units [37, 52]that have been proposed to be implicated in Na+ storage in skin[16] as discussed below. The disaccharide groups are fully charged at physiological pH, and are thus an important contributor to the negative charge of the extracellular matrix in vivo. The four major classes of GAGs that can divided into sulphated (heparin/heparan sulphate, chondroitin/dermatan sulphate, keratan sulphate) and non-sulphated (hyaluronan)[32, 52]. With the exception of hyaluronan, GAGs are all covalently bound to a protein backbone, and the combined macromolecule is called a proteoglycan[52]. Proteoglycans are structural molecules with multiple roles[35] that are immobilized in the interstitium, except for hyaluronan that is cleared to lymph[53] thus entering the systemic circulation.

The GAGs adopt highly extended conformations that are essential for hydrogel formation[54]. They attract counterions because of their high net negative charge density and

establish a Donnan distribution of diffusible solutes that results in an osmotic pressure thereby contributing to the hydration of the interstitium[55]. Hyaluronan, with molecular weight of several thousand kD, is a major GAG , particularly important for skin hydration[28]. Its quarternary random coil structure indicates that hyaluronan molecules will occupy a domain with radius 100-1000 times larger than that of the organic material[37]. Skin is the biggest pool of hyaluronan in our bodies (>50% of total in rats)[56], whereas <10% is found in skeletal muscle[57, 58]. Examples of the amounts of hyaluronan, GAGs and collagen in skin, muscle and lung have been summarized elsewhere[29]

3. Charge of the interstitium. GAGs carry 1-3 negatively charged side groups on each disaccharide unit at physiological pH[37] and will strongly affect the charge density of the tissue, that can also influence the composition of the ISF and fluid balance[27, 29]. For skin, this topic has not attracted much attention until it was shown that the skin GAG content and distribution and thereby charge density influences the storage capacity of Na^{+}[16]. Before turning to a discussion on ECM charge and skin Na^{+} storage, we will briefly recapitulate central elements from a more comprehensive discussion of whether the charge density affects the composition of ISF[29]. Interestingly, in cartilage, a tissue where such charge phenomena have been studied extensively, e.g.[49], polyanionic GAGs will attract monovalent cations into the interstitial gel, resulting in Na^{+} concentrations that may surpass the corresponding concentration in plasma by 100 mM[59].

In one of the few previous studies where the question of potential influence charged components in skin affect the composition of the ISF has been addressed, Haljamae et al[60] detected a higher K^{+} and Na^{+} and a lower Cl^{-} concentration in tissue and capsular fluid

sampled with micropipettes than in plasma. They attributed this discrepancy to an increased amount of GAGs in the two extravascular fluids. Differences in concentrations were ascribed to variation in types of GAGs and thus electrical charges in the two compartments of origin. It should be noted, though, that the tissue elements, including GAG-dense regions, will have to be electroneutral[61] even when binding increased amounts of cations in situations of high Na^+ intake. In contrast to Haljamäe et al[60], Gilanyi and Kovach[62], found no differences in ionic concentration between ISF and plasma, that led them to conclude that the ions distributed according to the Gibbs-Donnan equilibrium, namely that negatively charged plasma proteins (or GAGs in the interstitium) attract cations to increase Na^+ and K^+, and decrease Cl^- concentrations in plasma compared with the interstitium. Notwithstanding this fact, based on calculations they also concluded that this effect is negligible in the interstitium because of the high ionic strength and low protein concentration of the interstitial fluid[62].

Both of the methods used for ISF isolation have their potential caveats; Haljamäe et al aspirated carefully to isolate up to 50 nl fluid. For instance, such a small volume may represent challenges with respect to e.g. evaporation. Gilyanyi and Kovach collected 20-60 µl after a trauma inducing exposure of subcutaneous tissue by electrocoagulation. Apparently, based on these earlier studies it cannot be established whether the negatively charged ECM in skin results in an ISF deviating from plasma with respect to electrolyte concentration during steady state control conditions. Our own recent experiments, however, have shown that there are no Na^+ or osmolal gradients between plasma, interstitium and lymph[63]. Because we found no indications of total ion as well as cation gradients, our observations suggest that there are no anionic gradients, and thereby that the effect of Donnan forces on ISF and lymph ion distribution is negligible, in agreement with predictions discussed above[62]. Clearly, our knowledge on the interaction between ions and

the extracellular matrix elements, and how ions are stored in GAGs in normal as well as high salt situation is very limited and should be addressed in future studies.

4. Interstitial fluid – space and volume regulation.

The transcapillary exchange of water and solutes and formation of interstitial fluid is determined by properties of the capillary wall, hydrostatic pressure, and protein concentrations in the blood and interstitium that will exert a colloid osmotic or oncotic pressure[29]. The basic principles for such transport described by Starling more than a century ago[64] are still valid in spite of recent important modifications[65]. There is a transcapillary colloid osmotic pressure difference because the vessel wall is relatively impermeable to macromolecules and proteins, and a hydrostatic pressure gradient between the capillary pressure driving filtration and the pressure in the ISF. Commonly these hydrostatic and colloid osmotic pressures across the capillary are referred to as Starling forces. We will not address this topic further here, but rather refer to detailed, comprehensive reviews on transcapillary fluid exchange and interstitial fluid volume regulation[28, 29, 66-69].

A common feature of all tissues studied is that an enhanced net filtration will enhance ISF hydrostatic pressure and thus exert hydrostatic buffering and at the same time reduce ISF colloid osmotic pressure resulting in colloid osmotic buffering. Moreover, an augmented filtration will lead to increased lymph drainage thereby preventing the formation of edema, thus constituting "safety factors against edema"[13] contributing to maintenance of interstitial fluid volume. Actually, normal control of ISF volume is achieved through changes in ISF hydrostatic and colloid osmotic pressures that will counteract alterations in capillary fluid filtration and restore normal filtration, termed "autoregulation" of interstitial fluid volume[28, 55].

Lymph vessels and fluid composition

ISF is formed continuously by filtration, and lymph vessels are required for carrying fluid, interstitial proteins and peptides, and cells, notably immune cells, to the lymph nodes and back to the blood circulation[29]. Lymph vessels are introduced here because of their newly recognized role in blood pressure control as will be addressed in more detail below. For a more extensive discussion of lymphatic function we refer to several recent comprehensive reviews[70-72]. Moreover, the architecture of the initial part of the lymphatic system has been reviewed in detail[73, 74].

With some exceptions, lymph vessels are present in almost, although not in avascular tissues such as epidermis, cartilage, the eye lens, and cornea as well as some vascularized organs like retina and bone marrow[73]. Until recently, the brain was considered to be missing lymphatics, but recently vessels were discovered in dura mater but not in the brain proper[75, 76]. Originating in the interstitium, initial lymphatic vessels are non-contractile, considerably larger (10 – 60 μm[77]) than surrounding blood capillaries. These vessels are connected to the elastic fibers in the surrounding ECM via so-called anchoring filaments [78, 79]. Formed lymph drains to larger collecting lymphatics having smooth muscle cells, continuous endothelium, basement membrane and regular valves. Segments between valves in collecting lymphatics are called *lymphangions*, all propelling lymph centrally. From collecting vessels lymph passes through lymph nodes, thus making the lymph pre- or postnodal (alternatively afferent or efferent) with respect to the node, influencing lymph composition[73, 80] as well as passing immune cells[81].

As will be seen below, when discussing the role of the interstitium in salt homeostasis, it important to know whether the composition of sampled lymph is

representing ISF. Direct comparisons between prenodal lymph and ISF isolated with different techniques indicate that the protein concentration and composition is similar in steady state conditions[82-84], indicating that there is no detectable change in protein concentration and thereby no net water transport along prenodal collecting lymphatics. In the mesentery, however, prenodal lymphatics were found to be permeable to albumin[85], and capable of distributing lymph components to surrounding fat[86]. Moreover, lymphatic endothelial cells in dermis were recently found to be capable of albumin uptake[87]. Whether such transport also includes water and solutes, and whether collecting lymphatics are permeable to proteins in other vascular beds will have to be determined. Until such data are available, there seems to be no reason to abandon the generally accepted view that prenodal lymph is not modified during its passage to the lymph node and may be considered as representative for interstitial fluid[28, 55, 66, 88].

Lymph vessels and immune cell interaction.

In addition to their role in fluid homeostasis, the interaction between immune cells residing in the interstitium and lymphatics is crucial in immune homeostasis and also, as we shall see below, of relevance for blood pressure regulation. Whereas of limited interest until a few years ago, this interaction is now an area with substantial interest as reflected by many recent reviews e.g.[72, 89-92], briefly summarized here. Interestingly, lymphatic endothelial cells (LECs) themselves appear to play active roles in controlling their own transport function and communicating with immune cells to modulate downstream function[91]. Recent data suggest that LECs shape innate as well as adaptive immunity through expression of multiple cytokines, scavenge and process antigens for presentation to T-cells and also regulate fluid and solute transport[91]. For immune cells expressing the receptor

CCR7, notably dendritic cells (DCs), a chemoattractant gradient consisting of CCL21 produced by LECs is an important mediator of entrance into the initial lymphatics[72]. In the skin, each layer is inhabited by different immune cells populations having the same precursor[93]. Langerhans cells reside in epidermis, whereas dermis contains tissue resident dermal macrophages and dermal dendritic cells. Hypodermis contains macrophages and T-cells. Monocytes, macrophages and DCs are elements in mononuclear phagocyte system (MPS), a body-wide, specialized system of phagocytic cells. This system functions in the innate immune response, in support of the adaptive immune response and in the maintenance of tissue homeostasis[94], recently shown to be involved in salt homeostasis as discussed below.

Sodium storage in the interstitium

The extracellular fluid volume (ECV) in skin and muscle together constitute ≈60% of body's ECV[28] and are therefore likely reservoirs for storage of the dominantly extracellular ion Na^+ in situations of excess. The traditional textbook view is that upon excess intake of salt, some of it is retained and water conservation mechanisms activated such that the intravascular as well as interstitial part of the extracellular volume and thereby body weight is increased, and a new steady state established in 3-4 days when the salt excretion matches the intake[23, 95]. Although plasma volume increased concomitantly with NaCl intake, in two carefully controlled series of experiments in humans exposed to increased salt ingestion, Heer et al[96] found that total extracellular volume and body weight were not increased, thereby challenging "established" knowledge. The authors concluded that Na^+ did not result in a corresponding body water accumulation, but led to a shift of fluid from the interstitial to the intravascular fluid phase. Interestingly, blood pressure was unaffected by the salt ingestion in spite of significant dose dependent increases in plasma volume.

Although not applying to the study by Heer et al above[96], a common problem in Na^+ balance studies in humans is short duration and precise control of Na^+ intake and output. These problems have been avoided in a series of experiments with careful monitoring of Na^+ balance performed by the Titze group on persons in simulated space flights. In their first study in this series lasting 135 days involving variable Na^+ intake[21], they found sodium and water accumulation in the initial phase, but a decoupling of sodium and water towards the end of the study, suggesting that some of the sodium accumulated in an osmotically inactive form. The BP increased with Na^+ accumulation in two of the three subjects studied. This work was followed up by more extensive long term Na^+ balance studies simulating a space flight to Mars involving 105 and 205 days of intervention where the subjects ate of 12, 9 and 6 g salt every day in periods of 35 days[15]. During abrupt increases in salt intake they found the proposed by textbooks short-term increases in total-body Na^+ and extracellular water, rapid suppression of aldosterone urinary excretion, and expected adjustment in sodium urinary excretion to the next salt intake level. They also found a modest, variable salt effect on blood pressure, possibly due to Na^+ buffering in the interstitium. This effect was, however, delayed and required several weeks to achieve a plateau. Analysis of the data on a longer times-scale, however, showed that at constant salt intake, endogenous about-weekly rhythms in aldosterone and/or cortisol induced large and spontaneous variability in sodium excretion that was independent of salt intake, and importantly, that there was no relationship between changes in total body Na^+ and BP. In this context, it is interesting to note that in mice, Na^+ urinary excretion and aldosterone level in plasma is regulated by the circadian clock protein Per1[97, 98]. This gene may have been involved in the rhythmic variations described in the long-term experiments[15] and calls corresponding experiments in humans.

By additional analyses of data from the same ultra-long term balance studies the authors have found that notwithstanding the fact that elevated salt intake resulted in an expected increased sodium excretion, the water intake (ad libidum) was decreased[99]. This decrease, however, could be explained by water produced by body internal metabolic production induced by increased cortisone secretion as reflected by urinary excretion. This led them to the conclusion that humans react to high salt intake by urea-driven renal water conservation, and by glucocorticoid –associated endogenous water production, and thus that dietary salt has a catabolic effect. This way, the endogenously generated water facilitates the diuresis required to excrete excess sodium. Such salt-induced subclinical hyper-glucocortocoid production, that was confirmed in mechanistic experiments in mice[100], could link high-salt intake with diabetes mellitus, cardiovascular and several other major disease groups[99]. That high salt intake results in an increase in glucocorticoids have been shown in humans [101] as well as mice [102], and even though glucocorticoids promote salt-sensitive hypertension it is not known whether moderate excess contributes to increased BP [103].

In the studies discussed above performed in young healthy male volunteers, the association between total body sodium accumulation and BP was variable and thereby unclear. The development of ^{23}Na-MRI[104] enabled non-invasive and longitudinal quantification of Na^+ by imaging, and established that Na^+ accumulation in skin and muscle is associated with essential hypertension in patients. In the initial method description study[104], hyperaldosteronism gave significant Na^+ accumulation in muscle and increased BP that both were reversed after treatment with the aldosterone antagonist spironolactone. In a more extensive follow-up study, the same group used this method to assess tissue sodium in normal control subjects and 57 men and women with essential hypertension[105]. The group

found age-dependent increases in Na^+ content in muscle in men that was not associated with increase in water content, indicating water-free Na^+ storage. In skin, there was an increase in Na^+ content with age in men as well as women. Interestingly, when controlled for age, there was increased tissue Na^+ content in patients with refractory hypertension when compared with controls, linking sodium storage in skin and muscle to essential hypertension (Figure 1). Recently, Schneider et al[106] demonstrated that although skin sodium content correlated with systolic BP, skin sodium was an even stronger predictor of left ventricular mass in patients with chronic kidney disease. In these patients, it may be that the high skin Na^+ is reflecting the myocardium and thereby is a surrogate marker for elevated Na^+ in the heart and of BP-independent processes, e.g. mediated by macrophages attracted by high salt[107], in the myocardium. These studies call for direct measurements of cardiac sodium by MRI.

Above we have discussed salt accumulation in skin and muscle as a risk factor for development of essential hypertension, but recently high salt conditions, induced by 4% NaCl in the chow combined with 1% NaCl in the drinking water (named HSD), have been found to exacerbate autoimmune encephalitis in mice[108-110] and cancer in gerbils[111]. On the other hand, salt accumulation in the skin can actually be protective against infections by strengthening the antimicrobial barrier in humans and mice by boosting the classical macrophage activation and thereby host defence[112]. The role of Na^+ as a regulator of immunity has been reviewed recently[113] and will be discussed in more detail below in relation to the role of macrophages as driver of lymphangiogenesis.

Adaptations to and the pathophysiological importance of interstitial salt storage

Up till now, we have discussed studies showing that the accumulation of sodium in skin and muscle is associated with essential hypertension in humans, but these were preceded by a series of investigations in experimental animals all showing sodium without commensurate water accumulation[17-21, 114]. The data from the animal studies suggested that an increased amount of Na^+ could be made osmotically inactive by binding to tissue glycosaminoglycans that was shifted towards GAGs with increased Na^+ binding capacity in situations of high Na^+ load. Later this work led to mechanistic studies showing the role of mononuclear phagocyte system (MPS) cells and lymphatics in skin in blood pressure control, indicating that Na^+ homeostasis in the interstitium cannot be maintained by renal function only but will require additional extra-renal buffer mechanisms involving MPS-cells and skin lymphatics (Figure 2).

The observations of an incommensurate Na^+ and water accumulation led to the question of how salt could be stored in the interstitium without being osmotically active. Two mechanisms have been proposed; one is by increasing the content and charge density of the negatively charged GAGs that are an integral part of the extracellular matrix[22] and the other that Na^+ may be stored intracellularly[27]. Although it is well established that GAGs are important in maintaining hydration of the interstitial fluid phase, more recent studies have suggested that skin GAGs also play a role of in total body Na^+ and thereby regulation of fluid volume. In a series of papers from the Titze group it was first shown that upon high salt feeding there is an augmented GAG content in skin and cartilage[22]. Later the group determined osmotically active and inactive Na^+, defined as Na^+ accumulation with or without corresponding accumulation of water. In growing rats on a low compared with a high salt diet, they found that whereas total body Na^+ content relative to dry weight was reduced

during growth, there was an additional diet induced relative reduction of osmotically inactive Na^+ originating mainly from the skin[16]. A low salt diet resulted in a change skin GAG composition with increased hyaluronan and reduced sulphated proteoglycans (PG), thus lowering the charge density and water-free Na^+ binding to the ECM, whereas a high salt diet gave an increase in sulphated GAGs and an increased capacity for Na^+ binding. A shift in the composition of GAGs may represent an actively regulated mechanism for interstitial cation exchange that is involved in the control of extracellular fluid volume and blood pressure[16]. These suggestions based on experimental animal work were recently supported by data from humans showing that the skin may serve to buffer dietary salt[115] and moreover that glycosaminoglycans are involved in sodium storage[116].

In contrast, based on the observation that Na^+ associated with negatively charged purified GAGs in cartilage exhibits an osmotic coefficient similar to normal saline[117], Bhave and Neilson[27] concluded that Na^+ associated with GAGs in skin is osmotically active. According to their calculations this would mean that for skin, ≈20 -40 mmol/L gradients of Na^+ stored in skin GAGs (estimated from ashing experiments, e.g.[25]) would set up a colloid osmotic pressure from GAGs of 100 mm Hg, that would have to be balanced by a hydrostatic pressure set up by collagen to eliminate water movement. Normal transcapillary Starling pressures are normally way lower than this (e.g. reviewed in[28]), and one might expect that pressures in this range would result in substantial changes in skin fluid volumes that have not been observed in salt loading experiments. As an addition or alternative to storage in GAGs, they suggest intracellular Na^+ accumulation in exchange for K^+ or other intracellular osmolytes in situations of Na^+ surplus[27], that will work well in the cell rich muscle also shown to accumulate salt[17] but less well in the more cell-poor dermis. Significant amounts of Na^+ may, however, be stored in in the cell rich epidermis, shown to concentrate Na^+ by such

mechanisms[118, 119]. How Na^+ is stored in skin is therefore not established, but should be addressed in more detail for insight into the homeostasis of this important cation.

One part of this salt storage question is whether salt accumulation is reflected in interstitial fluid bathing all cells in the interstitium, that can be of importance for osmolar sensing by e.g. macrophages and clearance of electrolytes when ISF is converted to lymph after entering a lymphatic. Moreover, changes in GAGs induced by high salt diet (e.g.[16]) made it relevant to determine the concentration of Na^+ in proper ISF. The question is whether GAGs in skin establish a gradient in Na^+ different from what can be predicted by the Gibbs-Donnan effect for the capsule and ISF[120] (vide supra) and whether there is a gradient between the plasma and ISF that might attract fluid to the interstitium. We have addressed this issue in two different series of experiments reaching diverging conclusions. In the first series, we found that HSD (in rats 8% salt in the chow) gave salt accumulation, and resulted in increased tissue osmolality of ≈20 mosmol/kg as determined with a vapor pressure osmometer[26], showing that the salt storage affected the total skin osmolality. In a recent series of experiments, we addressed the question of whether the tissue hyperosmolality was reflected in the skin interstitial fluid phase by isolating ISF from implanted wicks or lymph draining skin from the tail[63]. Although we were able to verify that salt accumulation results in an increase in total skin osmolality, skin ISF and lymph osmolality was identical in low and highs salt diet and deoxycorticosterone (DOCA)-salt rats. Because lymph may be considered as the “gold standard” representative for interstitial fluid in steady state conditions[28, 29, 66], we concluded based on our lymph as well as wick data that interstitial fluid from skin is isosmotic with plasma even if the tissue is hyperosmotic because of salt accumulation[63]. An osmolal gradient between the skin tissue and its constituent ISF might seem counterintuitive, and should be sought explained by additional studies.

Because the previously observed significantly higher Na^+ concentration in lymphatics measured with the x-ray electron microprobe analysis than in plasma[26] could reflect intracutaneous electrolyte gradients, we eluted skin samples separated in epidermis-upper dermis (upper 0.5 mm) and lower dermis (subsequent 0.5 mm). We found that there was an osmolyte gradient from epidermis to dermis in skin that increased during salt accumulation, and that urea was an important contributor to this gradient (Figure 3). Our observations of a gradient increasing with salt accumulation support the notion that there is a counter-current exchange of osmolytes in the skin[118]. These findings, however, calls for a more detailed assessment of the gradient in higher resolution to be better able to understand the functional implications of this observation [63]. The role of urea in this context will be of particular interest to explore in light of the new observations that the body generates urea to conserve water and excrete salt[99]. The observed urea-gradient in the skin may contribute in this process, assisted by keratinocytes that control generation of NO in epidermis and upper dermis and thereby contribute in BP regulation[121]. Active transport by keratinocytes may moreover contribute to the generation of the observed skin osmolar gradient because of their ability to transport sodium being provided with epithelial sodium channels (ENaCs)[122]. This potential dual role of keratinocytes calls for a more detailed analysis of skin gradients and the role of keratinocytes in BP regulation.

Role of lymphatic vessels and innate immune cells in salt handling

Experiments in rats and mice have indicated that the response to high salt is initiated by activation of transcription factor tonicity-responsive enhancer binding protein (TonEBP, gene name Nfat5) in infiltrating MPS-cells from the innate immune system[24, 25]. When these cells, notably macrophages, are exposed to a hypertonic microenvironment they express

TonEBP, leading to release of vascular endothelial growth factor C (VEGF-C). VEGF-C is the primary lymphatic vessel growth factor, and its release results in hyperplasia of the lymph capillary network that facilitates sodium and chloride clearance from the tissue. Oppositely, failure of such macrophage-driven clearance results in skin electrolyte overload and hypertension [24, 25]. Recently, we presented more detailed knowledge on the role of MPS-cells in electrolyte and blood pressure homeostasis. By genetic deletion of TonEBP in MPS-cells, lymphangiogenesis was prevented, also resulting in skin electrolyte accumulation and salt-sensitive hypertension. Pharmacological blockade of VEGFR3 (receptor for VEGF-C) and skin specific trapping of VEGF-C in mice with selective overexpression of VEGFR3 in skin gave the same effect with respect to skin electrolyte accumulation and blood pressure. These experiments led us to conclude that MPS-cells via TonEBP-VEGF-C signaling exert homeostatic immune function in the skin by regulating electrolyte clearance via cutaneous lymph capillaries, thus contributing to blood pressure control.

The functional role of lymphangiogenesis in this context was however addressed in a recent study in rats using the multitargeted VEGF inhibitor sunitinib[123]. The question asked was whether sunitinib augmented the BP rise induced by HSD because of a concomitant attenuation of skin lymphangiogenesis. It was found that the HSD induced a rise in BP that was aggravated by sunitinib, apparently supporting the initial hypothesis. Whereas HSD was associated with an accentuated MPS cell infiltration and lymphangiogenesis in the skin, the lymphangiogenesis was not significantly impaired by sunitinib. This led to the conclusion that impaired skin lymphangiogenesis was not a major contributor to the rise in BP observed with sunitinib under HSD, thus calling for additional mechanistic studies on sunitinib and salt sensitivity.

Interestingly, it was recently shown that high salt conditions reduced the activation of innate immune cells that attenuate the tissue inflammation. These cells are called alternatively activated (M2) macrophages as opposed to classically activated (M1), proinflammatory macrophages. Such reduced M2 macrophage activation may result in a more pronounced proinflammatory response and an overall imbalance in immune cell homeostasis[124]. In line with this observation, skin macrophages from high-salt–treated mice with either genetic or pharmacologic inhibition of the cyclooxygenase-2 pathway expressed decreased M2 markers and VEGF-C production, and exhibited aberrant lymphangiogenesis[125]. These studies by Zhang et al suggested that cyclooxygenase-2–derived prostaglandin-E2 in hematopoietic cells plays an important role in skin as well as kidney in maintaining homeostasis in response to chronically increased dietary salt.

Although assigned an important role in BP control, the functional capacity for fluid transport of the newly generated lymphatics has not been evaluated. Accordingly, we do not know if the skin lymph flow is actually enhanced in high salt conditions. Kwon et al [126] have, however, assessed lymphatic contractile function in rats and mice on high salt diet, and were able to show dilated vessels having an increased contractility in the high salt diet animals. These observations were supported by data from isolated vessels showing an increased contractility during high salt conditions[127], but were complicated by data from the same researchers showing that there was a differential effect of high salt on afferent (reduced) and efferent (enhanced) collecting vessel contractility[128], that make it hard to decide the net effect of high salt on lymph flow. Whether the lymphatics formed by lymphangiogenesis are capable of transporting increased electrolyte loads back to the general circulation and thereby to the kidney for final disposal that might be reflected as an increased lymph flow has yet to be determined (Figure 2).

Above we have discussed the role of high salt conditions on MPS cells from the innate immune system, but high salt also influence cells from the adaptive immune system. It has recently been shown that T-cells exposed to high-salt conditions polarize into highly pro-inflammatory autoimmune Th17 phenotype cells that produce interleukin-17 (IL-17) and worsen experimental autoimmune encephalitis[109, 110] and also attenuates the immune suppression by impairing the function of $FOXP3^+$ regulatory T-cells[129]. In addition, autoimmune processes like systemic sclerosis may attract salt that may per se modulate, and be a biomarker, of the disease process[130]. The fact that high salt result in Th17 polarization and thus IL-17 production is interesting considering the recently appointed role of IL-17 in the pathogenesis of hypertension[131] and may indicate that this cytokine is involved in generation of salt-sensitive hypertension. Apparently, the many studies showing that high salt in the tissue microenvironment drives immune cell activation suggest that this results in an interstitium that is in a chronic inflammatory state, again resulting in vascular target organ damage, increased vessel stiffness[132] and elevated blood pressure. This mechanism may be of particular relevance in aging shown to result in salt accumulation in skin and muscle [105], and also be an extrarenal contributor microvascular stiffening[133] and thus to the malignant hypertension that may develop in autoimmune scarring diseases of the skin like scleroderma[134]

To *summarize*, we here discuss a new and unexpected, but hardly unknown target in studies of blood pressure control and thus the pathophysiology of hypertension: the interstitium-/extracellular matrix of the skin (and muscle) and its inherent interstitial fluid. As a part of the interstitial matrix, the lymphatic vasculature forms a vessel network in the interstitium of most tissues that has a role in BP control. It has emerged that Na^+ can be stored and

thereby buffered in the body without commensurate water retention. Although not questioning the "inextricable role" of the kidney in salt homeostasis and hypertension[10], there appear to be previously unappreciated extrarenal, tissue-specific regulatory mechanisms that are operative that regulate the release and storage of Na^+ from a kidney-independent reservoir and thereby buffer a salt load. Such sodium reservoirs may have evolved to facilitate survival during long fasts and in salt poor environments. Regarding salt storage in skin, molecular mechanisms have been identified by which cells from the mononuclear phagocyte system (MPS) including macrophages and dendritic cells, not only function as local on-site sensors of interstitial electrolyte concentration, but also contribute to body fluid and blood pressure homeostasis. This "immune cell" response leads to salt-sensitive hypertension[24-26]. Subcutaneous tissue macrophages express the transcription factor tonicity enhancer binding protein (TonEBP) in response to osmotic shifts induced by Na^+ storage in the interstitium. These cells secrete the major lymphatic vessel growth factor VEGF-C, binding to VEGFR-3 on lymphatic capillaries and thereby inducing hyperplasia of the lymph-capillary network[25, 26]. Taken together, observations of salt storage in the skin to buffer free extracellular Na^+ and macrophage modulation of the extracellular matrix and lymphatics suggest that electrolyte homeostasis in the body cannot be achieved by renal excretion alone, but also relies on extrarenal regulatory mechanisms.

Perspectives

When trying to place our discussion on extracellular electrolyte homeostasis in extrarenal tissues in the perspective of blood pressure control, we enter into the controversial field of how high salt intake might cause hypertension; by vasodysfunction[8] or by renal dysfunction characterized by impaired pressure natriuresis[7]. Although shown to be

present in long term sodium balance studies[135] and important for blood pressure control[136], a careful review of available data led Bie and Evans[137] to the conclusion that the pressure natriuresis mechanism is neither the only nor the predominating mechanism of sodium homeostasis. Age- and sex-dependent differences in skin and muscle Na^+ content paralleled by corresponding increase in blood pressure in the same subjects[105] might indicate that tissue Na^+ storage is either associated with, or mechanistically related to, the development of essential hypertension in humans and thus buffering in the extrarenal interstitium as discussed above. Clearly, the kidney is critical for sodium excretion and thereby for the regulation of body fluid homeostasis and blood pressure, but how can it be influenced by the Na^+ in the extrarenal interstitium? As suggested by Ivy and Bailey[136], this can occur via immune cells secreting VEGF-C thus inducing lymphangiogenesis when exposed to high salt. This way lymphatics may control the amount of sodium that is returned to the blood from the tissue store and thereby amount the kidney "sees". Other ways for buffered salt in the interstitium to influence blood pressure is through regulation of skin resistance vessel reactivity that has been shown to be increased by HSD[132], or through the generation of local reactive oxygen species that leads to vasoconstriction and elevated BP[112]. Elucidation of the how salt in the interstitium is connected to the vasculature and kidney will pave the way for new treatment modalities for salt-sensitive hypertension.

Acknowledgements

Financial support from the Research Council of Norway (project number 222278 and 262079) to HW is gratefully acknowledged.

References

1. Murray, CJ, Ezzati, M, Flaxman, AD, Lim, S, Lozano, R, Michaud, C, Naghavi, M, Salomon, JA, Shibuya, K, Vos, T, Wikler, D, Lopez, AD: GBD 2010: design, definitions, and metrics. *Lancet,* 380**:** 2063-2066, 2012.
2. Ezzati, M, Vander Hoorn, S, Lopez, AD, Danaei, G, Rodgers, A, Mathers, CD, Murray, CJL: Comparative quantification of mortality and burden of disease attributable to selected risk factors. In: *Global burden of disease and risk factors.* edited by LOPEZ, A. D., MATHERS, C. D., AEZZATI, M., JAMISON, D. T., MURRAY, C. J. L., New York, Oxford University Press, 2006.
3. Leal, J, Luengo-Fernandez, R, Gray, A, Petersen, S, Rayner, M: Economic burden of cardiovascular diseases in the enlarged European Union. *European heart journal,* 27**:** 1610-1619, 2006.
4. Brands, MW: Chronic blood pressure control. *Compr Physiol,* 2**:** 2481-2494, 2012.
5. Hall, JE, Granger, JP, do Carmo, JM, da Silva, AA, Dubinion, J, George, E, Hamza, S, Speed, J, Hall, ME: Hypertension: physiology and pathophysiology. *Compr Physiol,* 2**:** 2393-2442, 2012.
6. Guyton, AC, Coleman, TG, Granger, HJ: Circulation: overall regulation. *Annu Rev Physiol,* 34**:** 13-46, 1972.
7. Hall, JE: Renal Dysfunction, Rather Than Nonrenal Vascular Dysfunction, Mediates Salt-Induced Hypertension. *Circulation,* 133**:** 894-906, 2016.
8. Morris, RC, Jr., Schmidlin, O, Sebastian, A, Tanaka, M, Kurtz, TW: Vasodysfunction That Involves Renal Vasodysfunction, Not Abnormally Increased Renal Retention of Sodium, Accounts for the Initiation of Salt-Induced Hypertension. *Circulation,* 133**:** 881-893, 2016.
9. Guyton, AC, Coleman, TG, Cowley, AW, Manning, RD, Norman, RA, Ferguson, JD: A systems analysis approach to understanding long-range arterial blood pressure control and hypertension. *Circ Res,* 35**:** 159 - 176, 1974.
10. Coffman, TM: The inextricable role of the kidney in hypertension. *J Clin Invest,* 124**:** 2341-2347, 2014.
11. Cannon, WB: Organization for physiological homeostasis. *Physiological Reviews,* 9**:** 399-431, 1929.
12. Wahlgren, V: Über die Bedeutung der Gewebe als Chlordepots. *Arch Experim Pathol Pharmakol,* 61**:** 97-112, 1909.
13. Guyton, AC, Granger, HJ, Taylor, AE: Interstitial fluid pressure. *Physiol Rev,* 51**:** 527-563, 1971.
14. Ivanova, LN, Archibasova, VK, Shterental, I: [Sodium-depositing function of the skin in white rats]. *Fiziol Zh SSSR Im I M Sechenova,* 64**:** 358-363, 1978.
15. Rakova, N, Juttner, K, Dahlmann, A, Schroder, A, Linz, P, Kopp, C, Rauh, M, Goller, U, Beck, L, Agureev, A, Vassilieva, G, Lenkova, L, Johannes, B, Wabel, P, Moissl, U, Vienken, J, Gerzer, R, Eckardt, KU, Muller, DN, Kirsch, K, Morukov, B, Luft, FC, Titze, J: Long-term space flight simulation reveals infradian rhythmicity in human Na(+) balance. *Cell metabolism,* 17**:** 125-131, 2013.

16. Schafflhuber, M, Volpi, N, Dahlmann, A, Hilgers, KF, Maccari, F, Dietsch, P, Wagner, H, Luft, FC, Eckardt, KU, Titze, J: Mobilization of osmotically inactive Na+ by growth and by dietary salt restriction in rats. *Am J Physiol Renal Physiol,* 292**:** F1490-1500, 2007.
17. Titze, J, Bauer, K, Schafflhuber, M, Dietsch, P, Lang, R, Schwind, KH, Luft, FC, Eckardt, KU, Hilgers, KF: Internal sodium balance in DOCA-salt rats: a body composition study. *Am J Physiol Renal Physiol,* 289**:** F793-802, 2005.
18. Titze, J, Krause, H, Hecht, H, Dietsch, P, Rittweger, J, Lang, R, Kirsch, KA, Hilgers, KF: Reduced osmotically inactive Na storage capacity and hypertension in the Dahl model. *Am J Physiol Renal Physiol,* 283**:** F134-141, 2002.
19. Titze, J, Lang, R, Ilies, C, Schwind, KH, Kirsch, KA, Dietsch, P, Luft, FC, Hilgers, KF: Osmotically inactive skin Na+ storage in rats. *Am J Physiol Renal Physiol,* 285**:** F1108-1117, 2003.
20. Titze, J, Luft, FC, Bauer, K, Dietsch, P, Lang, R, Veelken, R, Wagner, H, Eckardt, KU, Hilgers, KF: Extrarenal Na+ balance, volume, and blood pressure homeostasis in intact and ovariectomized deoxycorticosterone-acetate salt rats. *Hypertension,* 47**:** 1101-1107, 2006.
21. Titze, J, Maillet, A, Lang, R, Gunga, HC, Johannes, B, Gauquelin-Koch, G, Kihm, E, Larina, I, Gharib, C, Kirsch, KA: Long-term sodium balance in humans in a terrestrial space station simulation study. *Am J Kidney Dis,* 40**:** 508-516, 2002.
22. Titze, J, Shakibaei, M, Schafflhuber, M, Schulze-Tanzil, G, Porst, M, Schwind, KH, Dietsch, P, Hilgers, KF: Glycosaminoglycan polymerization may enable osmotically inactive Na+ storage in the skin. *Am J Physiol Heart Circ Physiol,* 287**:** H203-208, 2004.
23. Walser, M: Phenomenological analysis of electrolyte homeostasis. In: *The Kidney: Physiology and Pathophysiology.* edited by SELDIN, D. W., New York, Raven Press, Ltd., 1992, pp 31-44.
24. Machnik, A, Dahlmann, A, Kopp, C, Goss, J, Wagner, H, van Rooijen, N, Eckardt, KU, Muller, DN, Park, JK, Luft, FC, Kerjaschki, D, Titze, J: Mononuclear phagocyte system depletion blocks interstitial tonicity-responsive enhancer binding protein/vascular endothelial growth factor C expression and induces salt-sensitive hypertension in rats. *Hypertension,* 55**:** 755-761, 2010.
25. Machnik, A, Neuhofer, W, Jantsch, J, Dahlmann, A, Tammela, T, Machura, K, Park, JK, Beck, FX, Muller, DN, Derer, W, Goss, J, Ziomber, A, Dietsch, P, Wagner, H, van Rooijen, N, Kurtz, A, Hilgers, KF, Alitalo, K, Eckardt, KU, Luft, FC, Kerjaschki, D, Titze, J: Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanism. *Nat Med,* 15**:** 545-552, 2009.
26. Wiig, H, Schroder, A, Neuhofer, W, Jantsch, J, Kopp, C, Karlsen, TV, Boschmann, M, Goss, J, Bry, M, Rakova, N, Dahlmann, A, Brenner, S, Tenstad, O, Nurmi, H, Mervaala, E, Wagner, H, Beck, FX, Muller, DN, Kerjaschki, D, Luft, FC, Harrison, DG, Alitalo, K, Titze, J: Immune cells control skin lymphatic electrolyte homeostasis and blood pressure. *J Clin Invest,* 123**:** 2803-2815, 2013.
27. Bhave, G, Neilson, EG: Body fluid dynamics: back to the future. *J Am Soc Nephrol,* 22**:** 2166-2181, 2011.
28. Aukland, K, Reed, RK: Interstitial-lymphatic mechanisms in the control of extracellular fluid volume. *Physiol Rev,* 73**:** 1-78, 1993.
29. Wiig, H, Swartz, MA: Interstitial fluid and lymph formation and transport: physiological regulation and roles in inflammation and cancer. *Physiol Rev,* 92**:** 1005-1060, 2012.

30. Gelse, K, Poschl, E, Aigner, T: Collagens--structure, function, and biosynthesis. *Adv Drug Deliv Rev,* 55**:** 1531-1546, 2003.
31. Kjaer, M: Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol Rev,* 84**:** 649-698, 2004.
32. Raman, R, Sasisekharan, V, Sasisekharan, R: Structural insights into biological roles of protein-glycosaminoglycan interactions. *Chem Biol,* 12**:** 267-277, 2005.
33. Fan, D, Creemers, EE, Kassiri, Z: Matrix as an interstitial transport system. *Circ Res,* 114**:** 889-902, 2014.
34. Halper, J, Kjaer, M: Basic components of connective tissues and extracellular matrix: elastin, fibrillin, fibulins, fibrinogen, fibronectin, laminin, tenascins and thrombospondins. *Adv Exp Med Biol,* 802**:** 31-47, 2014.
35. Theocharis, AD, Skandalis, SS, Gialeli, C, Karamanos, NK: Extracellular matrix structure. *Adv Drug Deliv Rev,* 97**:** 4-27, 2016.
36. Jarvelainen, H, Sainio, A, Koulu, M, Wight, TN, Penttinen, R: Extracellular matrix molecules: potential targets in pharmacotherapy. *Pharmacol Rev,* 61**:** 198-223, 2009.
37. Comper, WD, Laurent, TC: Physiological function of connective tissue polysaccharides. *Physiol Rev,* 58**:** 255-315, 1978.
38. Wiig, H, Gyenge, C, Iversen, PO, Gullberg, D, Tenstad, O: The role of the extracellular matrix in tissue distribution of macromolecules in normal and pathological tissues: potential therapeutic consequences. *Microcirculation,* 15**:** 283-296, 2008.
39. Jackson, RL, Busch, SJ, Cardin, AD: Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiol Rev,* 71**:** 481-539, 1991.
40. Miner, JH, Yurchenco, PD: Laminin functions in tissue morphogenesis. *Annual review of cell and developmental biology,* 20**:** 255-284, 2004.
41. Aszodi, A, Legate, KR, Nakchbandi, I, Fassler, R: What mouse mutants teach us about extracellular matrix function. *Annual review of cell and developmental biology,* 22**:** 591-621, 2006.
42. Kadler, KE, Baldock, C, Bella, J, Boot-Handford, RP: Collagens at a glance. *J Cell Sci,* 120**:** 1955-1958, 2007.
43. Myllyharju, J, Kivirikko, KI: Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet,* 20**:** 33-43, 2004.
44. Shoulders, MD, Raines, RT: Collagen structure and stability. *Annu Rev Biochem,* 78**:** 929-958, 2009.
45. Ricard-Blum, S: The collagen family. *Cold Spring Harb Perspect Biol,* 3**:** a004978, 2011.
46. Li, ST, Katz, EP: An electrostatic model for collagen fibrils. The interaction of reconstituted collagen with Ca++, Na+, and Cl. *Biopolymers,* 15**:** 1439-1460, 1976.
47. Reigle, KL, Di Lullo, G, Turner, KR, Last, JA, Chervoneva, I, Birk, DE, Funderburgh, JL, Elrod, E, Germann, MW, Surber, C, Sanderson, RD, San Antonio, JD: Non-enzymatic glycation of type I collagen diminishes collagen-proteoglycan binding and weakens cell adhesion. *J Cell Biochem,* 104**:** 1684-1698, 2008.
48. Katz, EP, Wachtel, EJ, Maroudas, A: Extrafibrillar proteoglycans osmotically regulate the molecular packing of collagen in cartilage. *Biochim Biophys Acta,* 882**:** 136-139, 1986.
49. Wachtel, E, Maroudas, A: The effects of pH and ionic strength on intrafibrillar hydration in articular cartilage. *Biochim Biophys Acta,* 1381**:** 37-48, 1998.
50. Grodzinsky, AJ: Electromechanical and physicochemical properties of connective tissue. *Crit Rev Biomed Eng,* 9**:** 133-199, 1983.

51. Wiig, H, Kolmannskog, O, Tenstad, O, Bert, JL: Effect of charge on interstitial distribution of albumin in rat dermis in vitro. *J Physiol,* 550**:** 505-514, 2003.
52. Schaefer, L, Schaefer, RM: Proteoglycans: from structural compounds to signaling molecules. *Cell Tissue Res,* 339**:** 237-246, 2010.
53. Tengblad, A, Laurent, UB, Lilja, K, Cahill, RN, Engstrom-Laurent, A, Fraser, JR, Hansson, HE, Laurent, TC: Concentration and relative molecular mass of hyaluronate in lymph and blood. *Biochem J,* 236**:** 521-525, 1986.
54. Frantz, C, Stewart, KM, Weaver, VM: The extracellular matrix at a glance. *J Cell Sci,* 123**:** 4195-4200, 2010.
55. Aukland, K, Nicolaysen, G: Interstitial fluid volume: local regulatory mechanisms. *Physiol Rev,* 61**:** 556-643, 1981.
56. Reed, RK, Lilja, K, Laurent, TC: Hyaluronan in the rat with special reference to the skin. *Acta Physiol Scand,* 134**:** 405-411, 1988.
57. Reed, RK, Lepsoe, S, Wiig, H: Interstitial exclusion of albumin in rat dermis and subcutis in over- and dehydration. *Am J Physiol,* 257**:** H1819-H1827, 1989.
58. Gyenge, CC, Tenstad, O, Wiig, H: In vivo determination of steric and electrostatic exclusion of albumin in rat skin and skeletal muscle. *J Physiol,* 552**:** 907-916, 2003.
59. Mobasheri, A: Correlation between [Na+], [glycosaminoglycan] and Na+/K+ pump density in the extracellular matrix of bovine articular cartilage. *Physiol Res,* 47**:** 47-52, 1998.
60. Haljamae, H, Linde, A, Amundson, B: Comparative analyses of capsular fluid and interstitial fluid. *Am J Physiol,* 227**:** 1199-1205, 1974.
61. Oien, AH, Wiig, H: Electrostatic, elastic and hydration-dependent interactions in dermis influencing volume exclusion and macromolecular transport. *J Theor Biol,* 400**:** 80-91, 2016.
62. Gilanyi, M, Ikrenyi, C, Fekete, J, Ikrenyi, K, Kovach, AG: Ion concentrations in subcutaneous interstitial fluid: measured versus expected values. *Am J Physiol,* 255**:** F513-519, 1988.
63. Nikpey, E, Karlsen, TV, Rakova, N, Titze, JM, Tenstad, O, Wiig, H: High-Salt Diet Causes Osmotic Gradients and Hyperosmolality in Skin Without Affecting Interstitial Fluid and Lymph. *Hypertension,* 69**:** 660-668, 2017.
64. Starling, EH: On the Absorption of Fluids from the Connective Tissue Spaces. *J Physiol,* 19**:** 312-326, 1896.
65. Levick, JR, Michel, CC: Microvascular fluid exchange and the revised Starling principle. *Cardiovasc Res,* 87**:** 198-210, 2010.
66. Michel, CC, Curry, FE: Microvascular permeability. *Physiol Rev,* 79**:** 703-761, 1999.
67. Curry, FR: Microvascular solute and water transport. *Microcirculation,* 12**:** 17-31, 2005.
68. Mehta, D, Malik, AB: Signaling mechanisms regulating endothelial permeability. *Physiol Rev,* 86**:** 279-367, 2006.
69. Scallan, J, Huxley, VH, Korthuis, RJ: Capillary Fluid Exchange: Regulation, Functions, and Pathology. In: *Colloquium Lectures on Integrated Systems Physiology-From Molecules to Function.* 2011/04/01 ed. edited by GRANGER, D. N., GRANGER, J. P., Morgan & Claypool Life Sciences, 2010.
70. Alitalo, K: The lymphatic vasculature in disease. *Nat Med,* 17**:** 1371-1380, 2011.
71. Aspelund, A, Robciuc, MR, Karaman, S, Makinen, T, Alitalo, K: Lymphatic System in Cardiovascular Medicine. *Circ Res,* 118**:** 515-530, 2016.

72. Randolph, GJ, Ivanov, S, Zinselmeyer, BH, Scallan, JP: The Lymphatic System: Integral Roles in Immunity. *Annu Rev Immunol*, 2016.
73. Schmid-Schonbein, GW: Microlymphatics and lymph flow. *Physiol Rev,* 70**:** 987-1028, 1990.
74. Ohtani, O, Ohtani, Y: Structure and function of rat lymph nodes. *Arch Histol Cytol,* 71**:** 69-76, 2008.
75. Aspelund, A, Antila, S, Proulx, ST, Karlsen, TV, Karaman, S, Detmar, M, Wiig, H, Alitalo, K: A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. *J Exp Med,* 212**:** 991-999, 2015.
76. Louveau, A, Smirnov, I, Keyes, TJ, Eccles, JD, Rouhani, SJ, Peske, JD, Derecki, NC, Castle, D, Mandell, JW, Lee, KS, Harris, TH, Kipnis, J: Structural and functional features of central nervous system lymphatic vessels. *Nature,* 523**:** 337-341, 2015.
77. Swartz, MA: The physiology of the lymphatic system. *Adv Drug Deliv Rev,* 50**:** 3-20, 2001.
78. Leak, LV, Burke, JF: Ultrastructural studies on the lymphatic anchoring filaments. *J Cell Biol,* 36**:** 129-149, 1968.
79. Leak, LV: The structure of lymphatic capillaries in lymph formation. *Fed Proc,* 35**:** 1863-1871, 1976.
80. Adair, TH, Moffatt, DS, Paulsen, AW, Guyton, AC: Quantitation of changes in lymph protein concentration during lymph node transit. *Am J Physiol,* 243**:** H351-359, 1982.
81. Randolph, GJ, Angeli, V, Swartz, MA: Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat Rev Immunol,* 5**:** 617-628, 2005.
82. Vreim, CR, Snashall, PD, Demling, RH, Staub, NC: Lung lymph and free interstitial fluid protein composition in sheep with edema. *Am J Physiol,* 230**:** 1650-1653, 1976.
83. Taylor, AE, Gibson, WH, Granger, HJ, Guyton, AC: The interaction between intracapillary and tissue forces in the overall regulation of interstitial fluid volume. *Lymphology,* 6**:** 192-208, 1973.
84. Fadnes, HO: Colloid osmotic pressure in interstitial fluid and lymph from rabbit subcutaneous tissue. *Microvasc Res,* 21**:** 390-392, 1981.
85. Scallan, JP, Huxley, VH: In vivo determination of collecting lymphatic vessel permeability to albumin: a role for lymphatics in exchange. *J Physiol,* 588**:** 243-254, 2010.
86. Kuan, EL, Ivanov, S, Bridenbaugh, EA, Victora, G, Wang, W, Childs, EW, Platt, AM, Jakubzick, CV, Mason, RJ, Gashev, AA, Nussenzweig, M, Swartz, MA, Dustin, ML, Zawieja, DC, Randolph, GJ: Collecting lymphatic vessel permeability facilitates adipose tissue inflammation and distribution of antigen to lymph node-homing adipose tissue dendritic cells. *J Immunol,* 194**:** 5200-5210, 2015.
87. Triacca, V, Guc, E, Kilarski, WW, Pisano, M, Swartz, MA: Transcellular Pathways in Lymphatic Endothelial Cells Regulate Changes in Solute Transport by Fluid Stress. *Circ Res*, 2017.
88. Renkin, EM: Lymph as a measure of the compositon of interstitial fluid. In: *Pulonary Edema.* edited by FISHMAN, A. P., RENKIN, E. M., Bethesda, MD, American Physiological Society, 1979, pp 145-159.
89. Aebischer, D, Iolyeva, M, Halin, C: The inflammatory response of lymphatic endothelium. *Angiogenesis,* 17**:** 383-393, 2014.
90. Betterman, KL, Harvey, NL: The lymphatic vasculature: development and role in shaping immunity. *Immunol Rev,* 271**:** 276-292, 2016.
91. Card, CM, Yu, SS, Swartz, MA: Emerging roles of lymphatic endothelium in regulating adaptive immunity. *J Clin Invest,* 124**:** 943-952, 2014.

92. Lund, AW, Medler, TR, Leachman, SA, Coussens, LM: Lymphatic Vessels, Inflammation, and Immunity in Skin Cancer. *Cancer Discov,* 6**:** 22-35, 2016.
93. Nestle, FO, Di Meglio, P, Qin, JZ, Nickoloff, BJ: Skin immune sentinels in health and disease. *Nat Rev Immunol,* 9**:** 679-691, 2009.
94. Hettinger, J, Richards, DM, Hansson, J, Barra, MM, Joschko, AC, Krijgsveld, J, Feuerer, M: Origin of monocytes and macrophages in a committed progenitor. *Nat Immunol,* 14**:** 821-830, 2013.
95. Rose, BD: Regulation of the effective circulating volume. In: *Clinical physiology of acid-base and electrolyte disorders.* 4 ed. edited by ROSE, B. D., New York, McGraw-Hill, 1994, pp 235-260.
96. Heer, M, Baisch, F, Kropp, J, Gerzer, R, Drummer, C: High dietary sodium chloride consumption may not induce body fluid retention in humans. *Am J Physiol Renal Physiol,* 278**:** F585-595, 2000.
97. Gumz, ML, Stow, LR, Lynch, IJ, Greenlee, MM, Rudin, A, Cain, BD, Weaver, DR, Wingo, CS: The circadian clock protein Period 1 regulates expression of the renal epithelial sodium channel in mice. *J Clin Invest,* 119**:** 2423-2434, 2009.
98. Richards, J, Cheng, KY, Ali, S, Skopis, G, Jeffers, L, Lynch, IJ, Wingo, CS, Gumz, ML: A role for the circadian clock protein Per1 in the regulation of aldosterone levels and renal Na+ retention. *Am J Physiol Renal Physiol,* 305**:** F1697-1704, 2013.
99. Rakova, N, Kitada, K, Lerchl, K, Dahlmann, A, Birukov, A, Daub, S, Kopp, C, Pedchenko, T, Zhang, Y, Beck, L, Johannes, B, Marton, A, Muller, DN, Rauh, M, Luft, FC, Titze, J: Increased salt consumption induces body water conservation and decreases fluid intake. *J Clin Invest,* 127**:** 1932-1943, 2017.
100. Kitada, K, Daub, S, Zhang, Y, Klein, JD, Nakano, D, Pedchenko, T, Lantier, L, LaRocque, LM, Marton, A, Neubert, P, Schroder, A, Rakova, N, Jantsch, J, Dikalova, AE, Dikalov, SI, Harrison, DG, Muller, DN, Nishiyama, A, Rauh, M, Harris, RC, Luft, FC, Wassermann, DH, Sands, JM, Titze, J: High salt intake reprioritizes osmolyte and energy metabolism for body fluid conservation. *J Clin Invest,* 127**:** 1944-1959, 2017.
101. Weber, CS, Thayer, JF, Rudat, M, Sharma, AM, Perschel, FH, Buchholz, K, Deter, HC: Salt-sensitive men show reduced heart rate variability, lower norepinephrine and enhanced cortisol during mental stress. *J Hum Hypertens,* 22**:** 423-431, 2008.
102. Craigie, E, Evans, LC, Mullins, JJ, Bailey, MA: Failure to downregulate the epithelial sodium channel causes salt sensitivity in Hsd11b2 heterozygote mice. *Hypertension,* 60**:** 684-690, 2012.
103. Hunter, RW, Ivy, JR, Bailey, MA: Glucocorticoids and renal Na+ transport: implications for hypertension and salt sensitivity. *J Physiol,* 592**:** 1731-1744, 2014.
104. Kopp, C, Linz, P, Wachsmuth, L, Dahlmann, A, Horbach, T, Schofl, C, Renz, W, Santoro, D, Niendorf, T, Muller, DN, Neininger, M, Cavallaro, A, Eckardt, KU, Schmieder, RE, Luft, FC, Uder, M, Titze, J: (23)Na magnetic resonance imaging of tissue sodium. *Hypertension,* 59**:** 167-172, 2012.
105. Kopp, C, Linz, P, Dahlmann, A, Hammon, M, Jantsch, J, Muller, DN, Schmieder, RE, Cavallaro, A, Eckardt, KU, Uder, M, Luft, FC, Titze, J: 23Na magnetic resonance imaging-determined tissue sodium in healthy subjects and hypertensive patients. *Hypertension,* 61**:** 635-640, 2013.
106. Schneider, MP, Raff, U, Kopp, C, Scheppach, JB, Toncar, S, Wanner, C, Schlieper, G, Saritas, T, Floege, J, Schmid, M, Birukov, A, Dahlmann, A, Linz, P, Janka, R, Uder, M,

Schmieder, RE, Titze, JM, Eckardt, KU: Skin Sodium Concentration Correlates with Left Ventricular Hypertrophy in CKD. *J Am Soc Nephrol*, 2017.

107. Muller, S, Quast, T, Schroder, A, Hucke, S, Klotz, L, Jantsch, J, Gerzer, R, Hemmersbach, R, Kolanus, W: Salt-dependent chemotaxis of macrophages. *PLoS One,* 8**:** e73439, 2013.
108. Hucke, S, Eschborn, M, Liebmann, M, Herold, M, Freise, N, Engbers, A, Ehling, P, Meuth, SG, Roth, J, Kuhlmann, T, Wiendl, H, Klotz, L: Sodium chloride promotes pro-inflammatory macrophage polarization thereby aggravating CNS autoimmunity. *J Autoimmun,* 67**:** 90-101, 2016.
109. Wu, C, Yosef, N, Thalhamer, T, Zhu, C, Xiao, S, Kishi, Y, Regev, A, Kuchroo, VK: Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. *Nature,* 496**:** 513-517, 2013.
110. Kleinewietfeld, M, Manzel, A, Titze, J, Kvakan, H, Yosef, N, Linker, RA, Muller, DN, Hafler, DA: Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature,* 496**:** 518-522, 2013.
111. Gaddy, JA, Radin, JN, Loh, JT, Zhang, F, Washington, MK, Peek, RM, Jr., Algood, HM, Cover, TL: High dietary salt intake exacerbates Helicobacter pylori-induced gastric carcinogenesis. *Infect Immun,* 81**:** 2258-2267, 2013.
112. Jantsch, J, Schatz, V, Friedrich, D, Schroder, A, Kopp, C, Siegert, I, Maronna, A, Wendelborn, D, Linz, P, Binger, KJ, Gebhardt, M, Heinig, M, Neubert, P, Fischer, F, Teufel, S, David, JP, Neufert, C, Cavallaro, A, Rakova, N, Kuper, C, Beck, FX, Neuhofer, W, Muller, DN, Schuler, G, Uder, M, Bogdan, C, Luft, FC, Titze, J: Cutaneous Na+ storage strengthens the antimicrobial barrier function of the skin and boosts macrophage-driven host defense. *Cell metabolism,* 21**:** 493-501, 2015.
113. Schatz, V, Neubert, P, Schroder, A, Binger, K, Gebhard, M, Muller, DN, Luft, FC, Titze, J, Jantsch, J: Elementary immunology: Na+ as a regulator of immunity. *Pediatr Nephrol,* 32**:** 201-210, 2017.
114. Ziomber, A, Machnik, A, Dahlmann, A, Dietsch, P, Beck, FX, Wagner, H, Hilgers, KF, Luft, FC, Eckardt, KU, Titze, J: Sodium-, potassium-, chloride-, and bicarbonate-related effects on blood pressure and electrolyte homeostasis in deoxycorticosterone acetate-treated rats. *Am J Physiol Renal Physiol,* 295**:** F1752-1763, 2008.
115. Selvarajah, V, Maki-Petaja, KM, Pedro, L, Bruggraber, SFA, Burling, K, Goodhart, AK, Brown, MJ, McEniery, CM, Wilkinson, IB: Novel Mechanism for Buffering Dietary Salt in Humans: Effects of Salt Loading on Skin Sodium, Vascular Endothelial Growth Factor C, and Blood Pressure. *Hypertension*, 2017.
116. Fischereder, M, Michalke, B, Schmockel, E, Habicht, A, Kunisch, R, Pavelic, I, Szabados, B, Schonermarck, U, Nelson, PJ, Stangl, M: Sodium storage in human tissues is mediated by glycosaminoglycan expression. *Am J Physiol Renal Physiol,* 313**:** F319-F325, 2017.
117. Maroudas, A: Distribution and diffusion of solutes in articular cartilage. *Biophys J,* 10**:** 365-379, 1970.
118. Hofmeister, LH, Perisic, S, Titze, J: Tissue sodium storage: evidence for kidney-like extrarenal countercurrent systems? *Pflugers Arch,* 467**:** 551-558, 2015.
119. Linz, P, Santoro, D, Renz, W, Rieger, J, Ruehle, A, Ruff, J, Deimling, M, Rakova, N, Muller, DN, Luft, FC, Titze, J, Niendorf, T: Skin sodium measured with (2)(3)Na MRI at 7.0 T. *NMR Biomed,* 28**:** 54-62, 2015.

120. Gilanyi, M, Kovach, AG: Effect of local pH on interstitial fluid pressure. *Am J Physiol,* 261**:** H627-631, 1991.
121. Cowburn, AS, Takeda, N, Boutin, AT, Kim, JW, Sterling, JC, Nakasaki, M, Southwood, M, Goldrath, AW, Jamora, C, Nizet, V, Chilvers, ER, Johnson, RS: HIF isoforms in the skin differentially regulate systemic arterial pressure. *Proc Natl Acad Sci U S A,* 110**:** 17570-17575, 2013.
122. Xu, W, Hong, SJ, Zeitchek, M, Cooper, G, Jia, S, Xie, P, Qureshi, HA, Zhong, A, Porterfield, MD, Galiano, RD, Surmeier, DJ, Mustoe, TA: Hydration status regulates sodium flux and inflammatory pathways through epithelial sodium channel (ENaC) in the skin. *J Invest Dermatol,* 135**:** 796-806, 2015.
123. Lankhorst, S, Severs, D, Marko, L, Rakova, N, Titze, J, Muller, DN, Danser, AH, van den Meiracker, AH: Salt Sensitivity of Angiogenesis Inhibition-Induced Blood Pressure Rise: Role of Interstitial Sodium Accumulation? *Hypertension,* 69**:** 919-926, 2017.
124. Binger, KJ, Gebhardt, M, Heinig, M, Rintisch, C, Schroeder, A, Neuhofer, W, Hilgers, K, Manzel, A, Schwartz, C, Kleinewietfeld, M, Voelkl, J, Schatz, V, Linker, RA, Lang, F, Voehringer, D, Wright, MD, Hubner, N, Dechend, R, Jantsch, J, Titze, J, Muller, DN: High salt reduces the activation of IL-4- and IL-13-stimulated macrophages. *J Clin Invest,* 125**:** 4223-4238, 2015.
125. Zhang, MZ, Yao, B, Wang, Y, Yang, S, Wang, S, Fan, X, Harris, RC: Inhibition of cyclooxygenase-2 in hematopoietic cells results in salt-sensitive hypertension. *J Clin Invest,* 125**:** 4281-4294, 2015.
126. Kwon, S, Agollah, GD, Chan, W, Sevick-Muraca, EM: Altered lymphatic function and architecture in salt-induced hypertension assessed by near-infrared fluorescence imaging. *J Biomed Opt,* 17**:** 080504-080501, 2012.
127. Mizuno, R, Isshiki, M, Ono, N, Nishimoto, M, Fujita, T: A high salt diet alters pressure-induced mechanical activity of the rat lymphatics with enhancement of myogenic characteristics. *Lymphat Res Biol,* 13**:** 2-9, 2015.
128. Mizuno, R, Isshiki, M, Ono, N, Nishimoto, M, Fujita, T: A High-Salt Diet Differentially Modulates Mechanical Activity of Afferent and Efferent Collecting Lymphatics in Murine Iliac Lymph Nodes. *Lymphat Res Biol,* 13**:** 85-92, 2015.
129. Hernandez, AL, Kitz, A, Wu, C, Lowther, DE, Rodriguez, DM, Vudattu, N, Deng, S, Herold, KC, Kuchroo, VK, Kleinewietfeld, M, Hafler, DA: Sodium chloride inhibits the suppressive function of FOXP3+ regulatory T cells. *J Clin Invest,* 125**:** 4212-4222, 2015.
130. Kopp, C, Beyer, C, Linz, P, Dahlmann, A, Hammon, M, Jantsch, J, Neubert, P, Rosenhauer, D, Muller, DN, Cavallaro, A, Eckardt, KU, Schett, G, Luft, FC, Uder, M, Distler, JH, Titze, J: Na+ deposition in the fibrotic skin of systemic sclerosis patients detected by 23Na-magnetic resonance imaging. *Rheumatology (Oxford)*, 2016.
131. Madhur, MS, Lob, HE, McCann, LA, Iwakura, Y, Blinder, Y, Guzik, TJ, Harrison, DG: Interleukin 17 promotes angiotensin II-induced hypertension and vascular dysfunction. *Hypertension,* 55**:** 500-507, 2010.
132. Helle, F, Karlsen, TV, Tenstad, O, Titze, J, Wiig, H: High-salt diet increases hormonal sensitivity in skin pre-capillary resistance vessels. *Acta Physiol (Oxf),* 207**:** 577-581, 2013.
133. Bartoloni, E, Pucci, G, Cannarile, F, Battista, F, Alunno, A, Giuliani, M, Cafaro, G, Gerli, R, Schillaci, G: Central Hemodynamics and Arterial Stiffness in Systemic Sclerosis. *Hypertension,* 68**:** 1504-1511, 2016.

134. Mouthon, L, Bussone, G, Berezne, A, Noel, LH, Guillevin, L: Scleroderma renal crisis. *J Rheumatol,* 41**:** 1040-1048, 2014.
135. Titze, J, Luft, FC: Speculations on salt and the genesis of arterial hypertension. *Kidney Int,* 91**:** 1324-1335, 2017.
136. Ivy, JR, Bailey, MA: Pressure natriuresis and the renal control of arterial blood pressure. *J Physiol,* 592**:** 3955-3967, 2014.
137. Bie, P, Evans, RG: Normotension, hypertension and body fluid regulation: brain and kidney. *Acta Physiol (Oxf),* 219**:** 288-304, 2017.

Figure legends

Figure 1. ^{23}Na magnetic resonance imaging (^{23}Na-MRI) of tissue Na^+. **A**. Representative ^{23}Na-MR image of the lower leg of a young normotensive man vs an older man with hypertension. Tubes with solutions containing 10, 20, 30, and 40 mmol/L of NaCl are arranged below the extremity, thereby allowing us to calibrate tissue Na^+. Tissue Na^+ content is increased in the old compared with the young subject. **B**. Tissue water in the same young and old man detected with conventional ^{1}H-MRI. No difference in muscle water content is visible to the naked eye. Reproduced from Kopp et al, Hypertension 2013[105] with permission from The American Heart Association.

Figure 2. The traditional research approach for body electrolyte balance and blood pressure homeostasis is based on the concept of passive body fluid equilibrium in closed systems where known forces are balanced and electrolyte concentrations are not remarkably different between blood volume and interstitial volume. The extended research approach for body electrolyte balance and blood pressure homeostasis presented here is based on the finding that interstitial electrolyte concentrations are higher than in blood ("skin sodium storage"). Interstitial electrolyte balance is not achieved by renal blood purification alone, but instead relies on additional extrarenal regulatory mechanisms within the skin interstitium (gel of glycosaminoglycans). Macrophages act as local osmosensors that

regulate local interstitial electrolyte composition via a tonicity-enhancer–binding protein(TonEBP)/vascular endothelial growth factor C (VEGF-C)–dependent mechanism, enhancing electrolyte clearance via VEGF-C/vascular endothelial growth factor R3 (VEGFR3)–mediated modulation of the lymph capillary network in the skin. Cl, chloride; eNOS, endothelial nitric oxide synthase; Na^+, sodium; NO, nitric oxide; VEGFR, vascular endothelial growth factor receptor.

Figure 3. A. Individual values of osmolal content after elution in distilled H_2O of epidermis and associated upper dermis (Epidermis) and corresponding lower dermis (Dermis) isolated from rats on low salt diet (LSD) (n=11), high salt diet (HSD) (n=9) and DOCA salt (n=6). (*: $p<0.05$, †: $p<0.01$ and ****$p<0.0001$ when comparing Epidermis and Dermis with Student *t* tests in corresponding tissues or ANOVA followed by Dunnett test when comparing HSD and DOCA with LSD Epidermis). B: Individual values of urea content of epidermis and associated upper dermis (Epidermis) and corresponding lower dermis (Dermis) isolated from rats on low salt diet (LSD) (n=10), and high salt diet (HSD) (n=9) and DOCA salt (n=5). (*: $p<0.05$ and ***$p<0.001$, Student's t-test). Figure panels modified from Nikpey at al, Hypertension 2017[63] with permission from The American Heart Association.

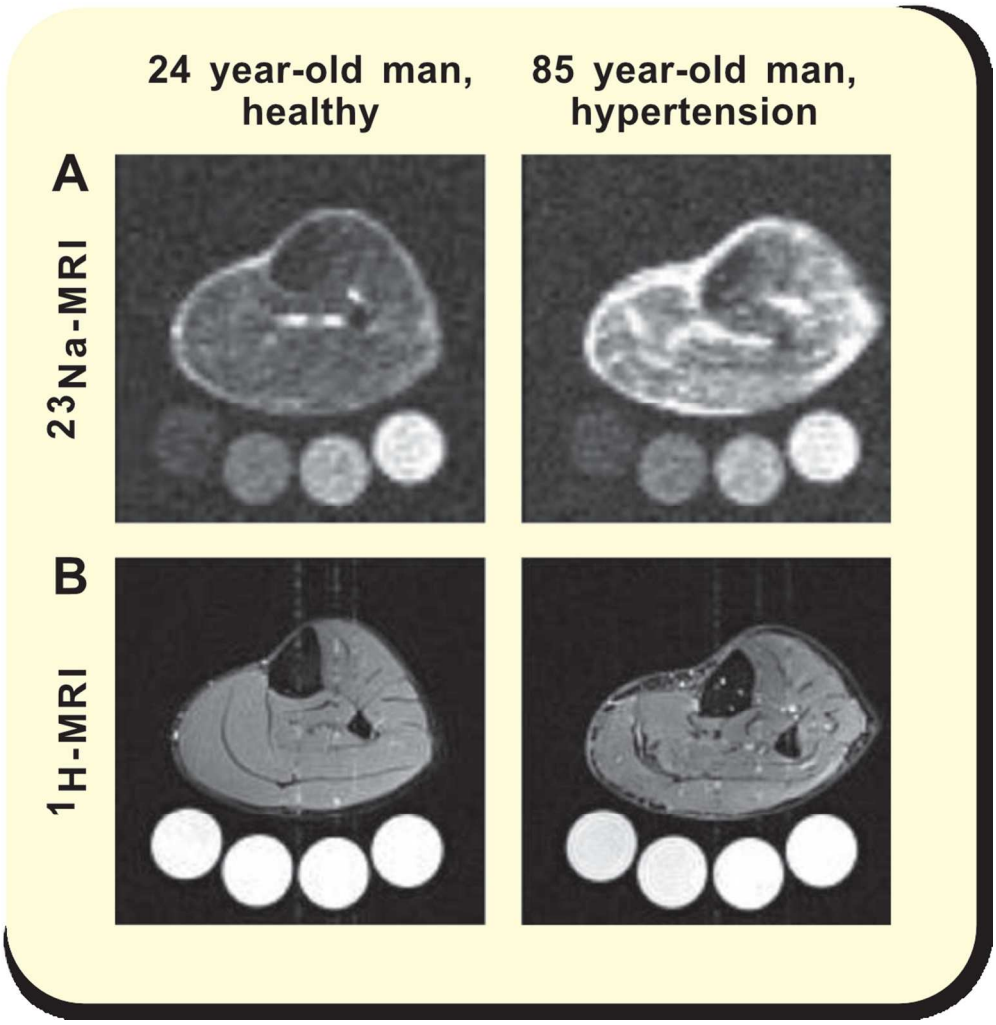
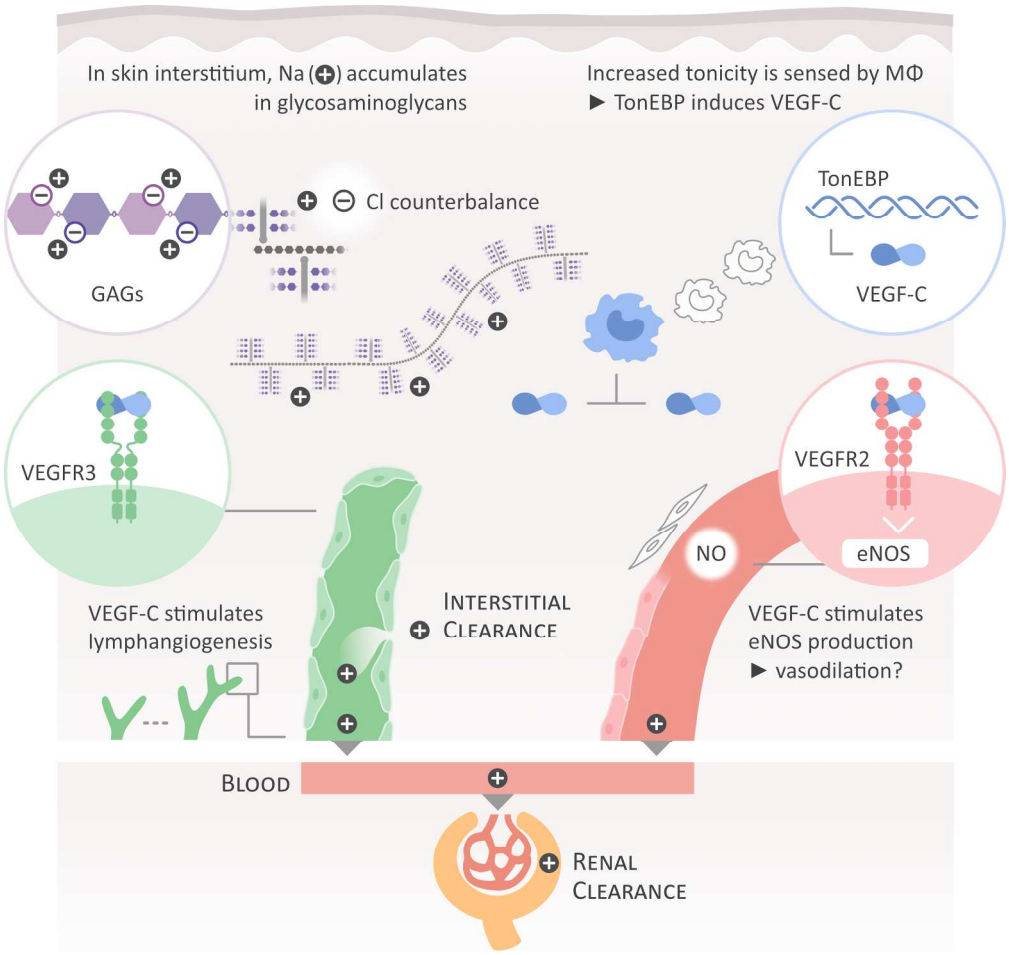
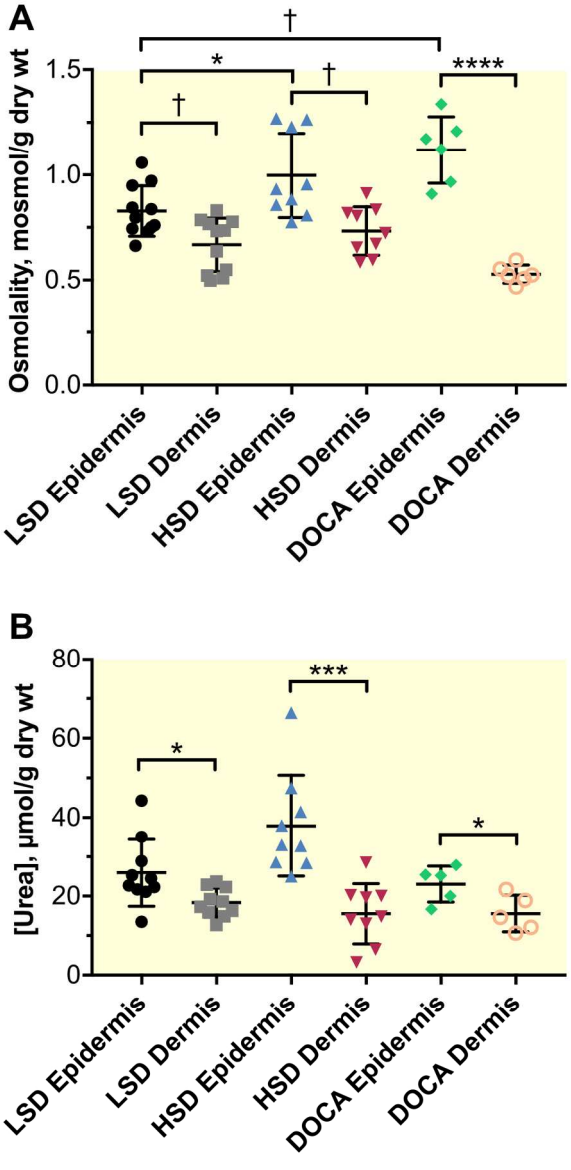


Figure 1. 23Na magnetic resonance imaging (23Na-MRI) of tissue Na+. A. Representative 23Na-MR image of the lower leg of a young normotensive man vs an older man with hypertension. Tubes with solutions containing 10, 20, 30, and 40 mmol/L of NaCl are arranged below the extremity, thereby allowing us to calibrate tissue Na+. Tissue Na+ content is increased in the old compared with the young subject. B. Tissue water in the same young and old man detected with conventional 1H-MRI. No difference in muscle water content is visible to the naked eye. Reproduced from Kopp et al, Hypertension 2013105 with permission from The American Heart Association.



The traditional research approach for body electrolyte balance and blood pressure homeostasis is based on the concept of passive body fluid equilibrium in closed systems where known forces are balanced and electrolyte concentrations are not remarkably different between blood volume and interstitial volume. The extended research approach for body electrolyte balance and blood pressure homeostasis presented here is based on the finding that interstitial electrolyte concentrations are higher than in blood ("skin sodium storage"). Interstitial electrolyte balance is not achieved by renal blood purification alone, but instead relies on additional extrarenal regulatory mechanisms within the skin interstitium (gel of glycosaminoglycans). Macrophages act as local osmosensors that regulate local interstitial electrolyte composition via a tonicity-enhancer–binding protein(TonEBP)/vascular endothelial growth factor C (VEGF-C)–dependent mechanism, enhancing electrolyte clearance via VEGF-C/vascular endothelial growth factor R3 (VEGFR3)–mediated modulation of the lymph capillary network in the skin. Cl, chloride; eNOS, endothelial nitric oxide synthase; Na+, sodium; NO, nitric oxide; VEGFR, vascular endothelial growth factor receptor.



Individual values of osmolal content after elution in distilled H2O of epidermis and associated upper dermis (Epidermis) and corresponding lower dermis (Dermis) isolated from rats on low salt diet (LSD) (n=11), high salt diet (HSD) (n=9) and DOCA salt (n=6). (*: $p<0.05$, †: $p<0.01$ and ****$p<0.0001$ when comparing Epidermis and Dermis with Student t tests in corresponding tissues or ANOVA followed by Dunnett test when comparing HSD and DOCA with LSD Epidermis). B: Individual values of urea content of epidermis and associated upper dermis (Epidermis) and corresponding lower dermis (Dermis) isolated from rats on low salt diet (LSD) (n=10), and high salt diet (HSD) (n=9) and DOCA salt (n=5). (*: $p<0.05$ and ***$p<0.001$, Student's t-test). Figure panels modified from Nikpey at al, Hypertension 201763 with permission from The American Heart Association.