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Sodium, Interstitium, Lymphatics, and Hypertension:

a Tale of Hydraulics

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ABSTRACT (250)

Blood pressure is regulated by vascular resistance and intravascular volume. However, exchanges of electrolytes and water between intra and extracellular spaces and filtration of fluid and solutes in the capillary beds blur the separation between intravascular and interstitial compartments. Contemporary paradigms of microvascular exchange posit filtration of fluids and solutes along the whole capillary bed and a prominent role of lymphatic vessels, rather than its venous end, for their reabsorption. In the last decade, these concepts have stimulated greater interest in and better understanding of the lymphatic system as one of the master regulators of interstitial volume homeostasis.

Here we describe the anatomy and function of the lymphatic system and focus on its plasticity, in relation to the accumulation of interstitial sodium in hypertension. The pathophysiological relevance of the lymphatic system is exemplified in the kidneys, which are crucially involved in the control of blood pressure, but also in hypertension-mediated organ damage at large. Preclinical modulation of lymphatic reserve for tissue drainage has shown promise but also generated conflicting results thus far. Better understanding the hydraulic element of hypertension and the role of lymphatics in maintaining fluid balance can open new approaches to prevent and treat hypertension and its consequences, such as heart failure.

In the multifactorial, mosaic [1,2] conceptualization of arterial hypertension (HTN), sodium has long been known to play a prominent role in its pathophysiology and associated organ damage. In order to reduce the burden of deaths and lost quality-adjusted life years caused by HTN, WHO committed to reduce the global population's intake of sodium 30% by 2025 [3]. Although the optimal thresholds for intake reduction remain debated [4,5], the validity of a large-scale approach was recently confirmed by a cluster-randomized trial of salt substitutes with reduced sodium levels, resulting in lower blood pressure and lower rates of cardio-cerebrovascular events and deaths in rural China [6]. On a more individualized perspective, however, the effects of sodium intake on blood pressure are highly heterogeneous. 'Salt-sensitivity', variably defined in the past but generally described as an exaggerated change in blood pressure (BP) in response to a sodium load and/or depletion [7,8], is a phenotype that demonstrates how it's not just a matter of intake but, more substantially, of handling and, as we came to learn, distribution [9] (+ companion review).

Most of our body sodium is normally confined to the extracellular volume (ECV) and the master regulator of its balance is the kidney [10]. Well-established, superbly sophisticated neuro-hormonal mechanisms continuously operate and adapt to minuscule changes in sodium, classically coupled to water by the laws of canonical physiology, in order to maintain a steady state. Any modification in the effective arterial blood volume, a correlate and hemodynamic surrogate of body sodium, induces renal adjustments of sodium excretion to preserve homeostasis. Impairments in these mechanisms would mediate salt-sensitive HTN (SSHTN) via pressure natriuresis [11–13]. The intravascular site of sodium distribution and its role in BP regulation are embodied into the reductionist Volume-Resistance approach to HTN [14], with sodium representing the "Volume" end of its spectrum. Over the last few years, however, our recognition of this volume component largely expanded to include body volumes other than the vascular space [15–17] (+ companion review). Sodium was shown to accumulate in tissues in HTN, as well as in many other cardiovascular conditions and risk factors, and its interstitial storage was suggested to induce local responses that contribute to BP

control [17] (+ companion review). This shift of attention toward the interstitium in the understanding of HTN pathophysiology led to a resurged attention to lymphatic vessels, the main controllers of interstitial homeostasis. After more than 20 years from the original proposition of a lymphatic etiology for HTN by Mekarski [18], his then 'working hypothesis' has recently found more solid ground.

In this review we will provide an overview of the anatomy and physiology of the lymphatic system in general and in specific organs relevant to HTN. We will explore its regulation by mechanical factors, salt and fluid *per se* and its proposed implication in the pathogenesis of HTN and associated organ damage, but also highlight recent data that conversely point towards a potentially less important role of the lymphatic system and prompt further research on the connection with tissue sodium connection in HTN.

The lymphatic system: a hydraulic perspective

One key reason for the traditional neglect of lymphatic vessels in the context of cardiovascular disease resides in their anatomical and functional elusiveness since the original discovery [19–21]. Despite remarkable recent progress [22,23], imaging the lymphatic system remains challenging, particularly in the clinical setting and for its most peripheral vessels which are actively implicated in the fundamental lymphatic functions: the hydraulic/chemical homeostasis of the interstitial fluid and tissue immunosurveillance [24,25].

These peripheral or "initial" lymphatic vessels, also called lymphatic capillaries, are a network of thin-walled, blind-ended, highly permeable structures present in nearly all vascularized tissues. They are lined with lymphatic endothelial cells (LECs), a cell lineage with a unique molecular signature (Table 1) distinct from blood vessel ECs [26,27], that lack a continuous basement membrane and form button-like cell-cell junctions [28], thus offering the ideal site of entry for interstitial fluid

and macromolecules, pathogens, and immune cells. These capillaries coalesce into specialized lymphatic muscle cell-lined lymphatic collectors, which connect to regional lymph nodes and eventually, via progressively larger post-nodal vessels, drain into the thoracic duct and the right lymphatic duct, returning lymph to the veins of the neck and to the blood circulation (Figure 1) [29].

We have already reviewed the active role of lymphatic vessels and LECs in the trafficking of immune cells, the phenomenon of inflammation-associated lymphangiogenesis and their particular relevance in the kidney for the regulation of BP [30,31], in relation to the established role of inflammation in HTN and hypertensive organ damage [32–35] and of any contribution that salt intake or its tissue accumulation can have on it [36–39]. In this review we would rather focus on the importance of the lymphatic system in hydraulic interstitial homeostasis. This contribution has long been considered ancillary or even trivial, but contemporary and direct experimental reappraisal of the Starling-Landis forces, traditionally held to favor fluid filtration and reabsorption at the arterial and venular end of the capillary bed, respectively, indicates filtration along the entire bed in most conditions. Lymphatics, by returning liters of fluid per day to the central circulation, thus offer the major – not ancillary - route for drainage of the interstitium [29,40–42]. Quantitative relevance of this route in humans was confirmed by early studies of cervical thoracic duct surgical cannulation, revealing average lymph flows of 1400 ml/day in healthy subjects, increasing to values as high as 480 ml/h in severely congested heart failure patients [43]. These values are comparable to daily urinary outputs. In fact, also considering their role in removing waste products from the cell surroundings, one may regard lymphatics as peripheral kidneys, that “centralize” fluids and solutes for their ultimate excretion. If the parallel stands, including the concept of pressure-natriuresis, higher arteriolar pressure observed in HTN would likely result in increased fluid extravasation via capillary filtration and increased functional demand on the lymphatic system *per se*, particularly when accompanied by angiotensin II (Ang II) or salt-induced damage to the endothelial glycocalyx

[44,45]. The concept finds full confirmation in old experiments with one-kidney, one-clip Goldblatt dog models of HTN [46].

Mechanics and plasticity of lymphatic function

The above concept of "demand" inherently ties with those of functional reserve and exhaustion: contrarily to diffuse beliefs, lymphatic vessels are not passive routes for transport, but conduits with a remarkably sophisticated complexity that, on an evolutionary basis, stands for a non-dispensable role in both physiology and in disease.

Lymphatic capillaries are anchored to the extracellular matrix by thin fibrillar structures [47] that permit the expansion of these vessels and the opening of "flap valves" between discontinuous endothelial junctions, when pulled apart. As such interstitial pressure modulates the movement of fluids into the system since its very terminal branches. Of note, at euvolemia (typical of most hypertensive conditions) the interstitium has low compliance, *i.e.*, steep changes in interstitial pressure occur with even minimal (subclinical) changes in interstitial volume or capillary filtration rate [48,49] (Figure1): this simultaneously facilitates lymphatic drainage and prevents further filtration, and overt edema accordingly [50].

Once lymph has entered the vessels, propulsion for its transport is provided by multiple driving forces, which vary in their relative contribution in different parts of the body [51]. These physical factors include:

1) extrinsic factors, including intestinal peristalsis [52], intrathoracic pressure and respiration [53,54], and the "tissue pump" activated by active or passive motion of the skeletal muscle, *i.e.*, that same physical activity that is well known to reduce salt sensitivity and BP [55–57]. In the brain, an organ considered devoid of lymphatic vessels until recently, the flow and exchange of

cerebrospinal/interstitial fluid through perivascular spaces that terminally constitute the cellular waste-draining 'glymphatic system' [58,59] is largely driven by arterial pulsations and is reduced in hypertensive elderly subjects [60] and rodent models [61,62], in which arteries are stiffened and their pulsatility reduced [61].

2) the "vis a tergo", resulting from capillary filtration (see above) and the outflow pressure opposing lymph flow [63]. The latter component appears more relevant in clinical settings other than HTN, characterized by an overt increase in central venous pressure and edema, *i.e.*, heart failure [64–66].

3) the unique intrinsic pumping activity of the collecting lymphatic vessels [67], organized in a series of coordinated functional units, each called a "lymphangion", separated by intraluminal one-way valves and capable of spontaneous contractions generated by a specialized lymphatic muscle cell layer that shares biochemical and functional aspects with both blood vascular and cardiac muscle [68]. This physiology has similarities with heart function, including coordinated pace-making [68–70], diastolic and systolic phases and modulation by pre- and after-load volume [71,72], which confers remarkable plasticity in response to increased requirements and reflects tissue-level mechanobiological control mechanisms, including calcium and nitric oxide (NO) dynamics [73].

In striking analogy, BP itself is an expression of hydraulics and mechanobiology. Pulsatile stretch and shear stress components on vessels [74], modulation by long-established and newly identified [75] baroreceptors, and its tie with fluid volume homeostasis across vascular and non-vascular compartments [17] all affect BP. In relation to the intrinsic functional sophistication of lymphatic vessels and their role in controlling fluid electrolytes and volume balance, would changes in lymphatic contractility affect systemic BP? Studies in spontaneously hypertensive rats (SHR) revealed an impaired collecting lymphatic vasomotion profile [76] and decreased endothelium-dependent and endothelium-independent relaxation of the thoracic duct [77] in comparison to

normotensive controls. The defective vasodilatation in the hypertensive animals was partially rescued by oxidative stress scavenging or p38 MAPK inhibition. Of note, lymphatic dysfunction was not observed in young rats and its development was prevented by antihypertensive treatment [77]. This observation makes the precise cause-and-effect relationship unclear and warrants further investigation, but lymphatic dysfunction could contribute to a vicious HTN-perpetuating circle and to hypertensive organ damage, as discussed below.

Vascular endothelial growth factors and salt-associated-lymphangiogenesis

Plasticity of the lymphatic system to meet increased drainage demand is broadly considered to involve not only a modulation of the contractile function, but also expansion of the vascular network. Our anatomical and molecular understanding of lymphangiogenesis via sprouting from existing vessels [78,79], de novo lymphovasculogenesis from local progenitors [80], and maintenance of lymphatic vascular integrity has dramatically evolved in the last decade, as reviewed elsewhere [81,82]. In this context, the most relevant regulator of embryonic and postnatal lymphatic vessel growth is signaling via vascular endothelial growth factor receptor 3 (VEGFR-3, encoded by FLT4), predominantly activated by VEGF-C and, although dispensable in development, VEGF-D. VEGF-C, and to some degree VEGF-D, also binds to the pan-endothelial VEGFR-2 that drives blood vessel determination, proliferation and survival (classically associated with VEGF-A binding) [83].

The seminal observations linking VEGFR-3 signaling with HTN, and particularly SSHTN, came from studies in rodents addressing the phenomenon of tissue salt accumulation. Machnik *et al.* first identified a local control system in the skin, whereby the tissue mononuclear phagocyte system (MPS) sensed the salt-overloaded interstitium via activation of tonicity responsive enhancer binding protein (TonEBP, also known as nuclear factor of activated T-cells 5, NFAT5) that triggered VEGF-C expression [84], VEGFR-3-mediated expansion of the lymphatic capillary network and

VEGFR-2-mediated increased endothelial NO synthase (eNOS) expression in blood vessels. Disruption of the signaling axis by genetic deletion of TonEBP in the MPS cells or their depletion, VEGF-C trapping and, eventually, selective blockade of VEGFR-3 leaving VEGFR-2 intact prevented the lymphangiogenic response, resulting in excess skin sodium accumulation and SSHTN [84–86]. This phenomenon, herein conceptualized as "salt-associated lymphangiogenesis" (SAL) in analogy to the "inflammation-associated lymphangiogenesis" (IAL) that we previously reviewed [30,87], suggested that the plasticity of the peripheral lymphatic network plays an important role in the pathogenesis of SSHTN. Very recent data, however, suggest more cautions conclusions: in contrast to previous studies, high salt diet could not induce an increase in BP or sodium storage in mice with either hypoplastic or absent dermal lymphatic vessels; DOCA-salt did, but to the same extent in mice with dysfunctional lymphatic vessels and WT controls [88]. In light of a lymph flow - assessed by tracer washout technique - that was unaffected by any diet or genetically determined breadth of the lymphatic network, the authors suggested that fluid/electrolyte transport is not necessarily synonymous with lymphangiogenesis and what the latter can functionally achieve is not yet settled. Also of note, circulating levels of VEGF-C in patients may not necessarily reflect activation of VEGFR-3 signaling in tissues, since data are discordant across different human studies. The original report of high concentrations of VEGF-C in refractory HTN [84] conflicts with an independent study showing lower concentrations, along with unaffected density of skin lymphatic vessels, compared to normotensive controls [89]. VEGF-C was also found to decrease with aging (a condition typically associated with salt-sensitivity [8]), to parallel an age-dependent increase in skin sodium and to be even further reduced in elderly patients with end stage kidney disease [90,91]. These findings could be jeopardized by technical heterogeneity in the matrix used for VEGF-C measurement, patient selection, ongoing antihypertensive treatment, and lack of control of salt intake, which was shown to directly increase plasma VEGF-C levels [89].

Overall, data supporting a role for SAL in facilitating tissue sodium clearance and for a defective lymphatic response, or reduced reserve, in contributing to SSHTN still require conclusive confirmation. Notably, low arterial serum VEGF-C values were shown to independently predict all cause and cardiovascular mortality in patients with HTN, but not normotensive patients at high cardiovascular risk [92].

Use of VEGF inhibitors (VEGFI) [93] as anti-angiogenic treatments in a wide range of malignancies is known to be associated with an increase in BP. Of note, many VEGFI have action beyond the specificity for VEGF-A/VEGFR-2 action and instead target the receptor tyrosine kinase (RTK) activity of all VEGFRs (or beyond). Whether their effect on BP is in part exerted via lymphatic-related mechanisms remains to be proven, but we have preliminary evidence that VEGFI-induced HTN is salt-sensitive [94,95]. For example, treatment of Wistar–Kyoto rats with sunitinib, a drug inhibiting all VEGFR family member RTK activity, exacerbated the accumulation of sodium and water in the skin and the BP increase upon high salt diet [96], while showing a tendency to decrease the observed SAL. Recent pilot data in normotensive cancer patients confirmed the HTN effect, an increase in skin sodium content, and a decrease in plasma VEGF-C levels [97].

Mechano-induction of lymphatic plasticity

Whether or not salt *per se* can induce SAL, still debated based on the aforementioned conflicting findings [84,86,88], a high salt diet in salt-sensitive rodents was reported to increase the contractile function and remodeling of collectors, as assessed by in vivo dynamic near-infrared fluorescence imaging [98] and *ex vivo* video microscopy of isolated vessels mounted on pressure myographs [99]. Importantly, the responses appear to be species, anatomical location, and duration-of-treatment specific [100]. This modulation of the lymphatic pump could in part depend on VEGFR-3 signaling [101], as for SAL, but is likely to depend also on many other locally and systemically acting, agonistic

and antagonistic mechanisms [102]. The net result of a functional "expansion", with or without an anatomical component in its definition, would eventually be an increased total lymph flow, shown in high salt fed rats by optical imaging in both skin and muscle [103]. The trigger for the stimulatory effect of tissue sodium on lymphatic network and function was initially identified in TonEBP, in the context of a proposed water-independent (*i.e.*, hypertonic) pattern of interstitial sodium accumulation [104]. Recent evidence led to reconsider this phenomenon, highly prevalent in HTN and cardio-nephro-vascular disease [105], as systemic and paralleled at least in part by water, thus reflecting subclinical ECV expansion in many cases [106,107 (+ companion review)]. Despite the isotonicity, sodium accumulation could still activate TonEBP, as biomechanical stretch and compressive strain were recently reported to do in vascular smooth muscle cells [108] and macrophages [109], respectively, regardless of osmolarity. As discussed, even minimal volume changes induced by water-paralleled sodium accumulation induce steep pressure changes in the interstitium, thus substantially affecting the biophysical milieu of lymphatic cells (Figure 1). It is worth mentioning that others previously noted how "*the cell signaling pathways activated by dietary salt are reminiscent of mechanoreceptor signaling*" [110].

In fact, an experimental increase in the volume of the interstitial fluid was found to induce an expansion of the lymphatic network via β1 integrin-dependent mechano-induction of VEGFR-3 phosphorylation [111]. Since this report over a decade ago, our understanding of the mechano-transduction pathways involved in lymphatic growth and biology has grown substantially, as reviewed [112]. The responsible mechanical signals span from biophysical characteristics of the extracellular matrix to lymph flow and shear stress, and centripetally impact lymphatic structures from the most peripheral capillary anchoring filaments to more central collectors and their one-way valves [113–124]. While mechano-dependent morphogenesis and maintenance of valves is well elucidated, the exact mechanisms by which shear stress and intraluminal fluid pressure are sensed by lymphatic muscle cells and modulate their contractility [125,126] remain largely elusive. However,

electro-mechanical coupling through connexins [70] and the chloride channel Ano1 [69] are known to play an indispensable role.

Deletions or mutations in force transducers (*i.e.*, integrins or elastin microfibril interfacer-1 (Emilin-1), the main constituent of the anchoring filaments) or other key mechano-signaling axes are associated with defects in lymphatic drainage in experimental animal models and/or with clinical lymphedema in humans (Table 2). Of note, for many of the genes involved there is circumstantial evidence of association with BP values or hypertensive organ damage from GWAS studies, although mechanistic confirmation and proof of lymphatic tissue-specificity are lacking in most cases. For example, smaller genetic studies linked specific haplotypes of Emilin1 with HTN [127,128] and its deletion is known to feature increased BP [129] along with defects in the integrity and function of lymphatic vessels [130]. However, there are no lymphatic-specific phenotype studies, similar to others performed on vascular smooth muscle cells [131], to confirm or disprove any hypothesis of causality in salt-related forms of HTN.

Prototypic interstitial mechanophysiology relevant to hypertension: the renal lymphatics

The organ that most obviously provides translational relevance to the above, while simultaneously serving as a key regulator of the entire body fluid homeostasis and BP, accordingly, is the kidney. Kidneys are encapsulated organs: as such, their function is exquisitely sensitive to increases in intracapsular pressure [132,133]. Since early functional studies, these peculiar aspects prompted a much larger interest in the renal lymphatic system physiology than in any other organ. In particular, renal lymphatics have traditionally been regarded as a "safety system" against high intrarenal pressures [134,135], providing a route for fluid drainage alternative to ureteral and venous, as discussed below.

The renal lymphatic system anatomy (Figure 2) and development have been superbly reviewed elsewhere [82,136,137], including consideration for species-specific differences. In brief, and in sole

relation to humans or relevant mammals for the purpose of this review, the centripetally-arranged, hierarchical microscopic anatomy of capillaries and collectors discussed in the previous sections is similar between kidneys and other organs [138,139], with the exception that lymphatics are relatively abundant in the cortex and very rare, if at all present, in the medulla. Cortical lymph drains into arcuate, interlobar, and eventually hilar lymphatics [140], progressively larger and segmented by unidirectional valves, or toward a capsular lymphatic plexus [141]. The hilar and capsular systems are connected by communicating vessels [142]. In contrast, the early experimental evidence that interstitial fluid and proteins in the medulla are not drained by lymphatics, but the ascending vasa recta (AVR) [143,144], found recent support in the appreciation of a hybrid nature of these vessels [145]. Not only do AVR have a highly fenestrated, large diameter structure, a discontinuous basement membrane similar to lymphatic capillaries, and are characterized by high-volume but low-flow of fluid transport to prevent washout of the medullary osmotic gradient [137], but they also express some markers specific to lymphatic lineage, like VEGFR-3 and PROX-1. Prevention of AVR development at the time of late gestation resulted into rapid accumulation of fluid and cysts in the medullary interstitium, and decreased urine concentrating ability [145].

In the fluid economy of the kidney and its three routes for fluid exit, the relative orders of magnitude are such that every day approximately 180 L of fluid is filtered by human glomeruli but only 2 L of urine is produced. Therefore, most of the filtered fluid is drained back into the systemic circulation. Early indirect estimates suggested that the renal lymph flow, particularly high compared to subcutaneous tissues and muscle because of a uniquely positive – rather than sub atmospheric – interstitial pressure [146], is comparable to urine flow [147]. By reason of hydraulics, the ureteral, venous, and lymphatic systems "bear relationships to one another and operate reciprocally" [148], with intrarenal hydrostatic pressure – and particularly renal interstitial hydrostatic pressure (RIHP) – sitting at their crossroad. Importantly, RIHP and its changes are closely coupled with systemic arterial pressure [149] and natriuresis [133,150], as shown in experiments of "direct renal interstitial

volume expansion" [151,152], and its development/sensing differs between salt-sensitive and salt-resistant animals [153].

Models of venous HTN or ligation, primarily representative of conditions of renal venous congestion like heart failure [154,155], but also of the highly SSHTN induced by obesity [156], consistently revealed an increase in renal lymphatic pressure and flow, preceded by a rise in interstitial pressure and limited to a critical point of intrarenal lymphatics collapse [157]. Urine flow and sodium excretion simultaneously decreased, likely due to a decreased renal blood flow that promotes sodium reabsorption [148,158,159]. Experiments of ureteral obstruction overall led to similar results: lymphatic vessels are recruited and lymph flow increases [134,148,160], with capsular diversion [142], possibly due to intrarenal venous obstruction [136]. Of note, secondary lymphangiogenesis in rats paralleled fibrosis deposition in the tubulo-interstitial area and was dependent on transforming growth factor-ß – VEGF-C signaling [161]. Finally, acute extracellular fluid expansion *per se* increased lymph pressure and flow [162].

More directly relevant to our contention, *i.e.*, a role for lymphatic defect in HTN, are the "opposite" studies of impeded renal lymphatic drainage. Ligation of hilar and capsular lymphatics consistently led to: 1) an increase in venous flow, consistent with a "reciprocal three-way-valve system" and with hydrodynamic findings in limbs [163] likely explained by reduced capillary fluid extravasation, which could also suggest relative tissue ischemia and/or impaired metabolic substrate delivery; and 2) swollen and tense kidneys, with interstitial edema and a marked increase in ipsilateral urine flow and solute excretion, *i.e.* acute isosthenuria secondary to both interstitial HTN and un-dissipated osmotic pressure [164–170]. This would intuitively argue against any direct salt-retaining HTN effect of renal lymphatic disruption. However, careful dissection of the effects on instrumented and non-instrumented sides and of an additional saline challenge revealed that lymphatic ligation indeed increased the excretion of sodium, without major changes in its filtration or other renal hemodynamics under basal conditions [169–171], but not after salt loading [170]. In the contralateral, non-

ligated kidney natriuresis was rather reduced compared to sham-treated animals, resulting in an overall reduced total sodium excretion (Figure 3). We speculate that activation of systemic neurohormonal sodium-retentive axes is involved. Unfortunately, the rats undergoing sympathetic activation or blockade were not simultaneously salt-loaded [170] and the renin-angiotensin aldosterone system (RAAS) was not assessed in the original study. However, RAAS activation is known to occur in similar conditions of increased intrarenal pressure, even despite saline infusion [172,173], and HTN develops accordingly [174,175]. Of note, the parallel potassium- and particularly water-wasting phenotype of salt-loaded ligated rats (Figure 3) have recently been shown to induce HTN *per se,* via metabolic resetting toward aestivation-like motifs and peripheral vasoconstriction [176]. While the experimental setting used by Wilcox and colleagues in rats [170] was unsuitable to track BP changes, ligation of all hilar and capsular lymphatics in 5 dogs acutely induced a transient 20 mmHg increase in BP that paralleled all the changes discussed above, which gradually but incompletely returned toward baseline over the following 15 weeks [177]. With the available data, some degree of persisting systolic HTN at that time cannot be excluded. Similarly, it's unknown whether these animals developed any salt-sensitivity or if unresolved edema could slowly progress to interstitial fibrosis [178] and reduced creatinine clearance, as suggested by others in rats [179].

In summary, renal lymphatics play a key role in kidney homeostasis and function, particularly natriuresis, via modulation of RIHP. Acute insults on lymphatics lead to HTN via multiple intrarenal hydraulic-driven changes, which appear to be compensated in baseline conditions but also to favor salt retention under conditions of salt-load. Conversely, conditions of excess hydraulic load require renal lymphatic expansion. Goodwin and Kaufman [134], almost 60 years before the conceptualization of any chronic edema as "lymphatic edema" [42], argued that renal interstitial edema (*i.e.*, high RIHP) is a result of renal lymphatics reaching their capacity to carry away interstitial fluid. If this capacity were reduced in HTN subjects, as shown for patients with more advanced cardiovascular syndromes [64], SSHTN would be a lymphatic disease.

Renal lymphatics and hypertension: molecular approaches

Compared to early physiological studies, the recent years provided us with more sophisticated tools to investigate the association between renal lymphatics and HTN. We first detected a renal lymphatic network expansion, paralleled by increased VEGF-C and VEGFR-3 expression and by inflammatory cell infiltration and cytokine signatures, in SHR prone to renal injury compared to controls [180]. Therefore, we tested the hypothesis that by further improving the efficiency of lymphatic vessels, and the exfiltration of inflammatory cells accordingly, kidneys could be protected, and BP lowered. Two weeks of treatment with nitro-l-arginine methyl ester hydrochloride (L-NAME), followed or not by 4% high salt diet to recapitulate a salt-independent and SSHTN, respectively, increased renal lymphatic density and the immune infiltrate. However, the renal-specific overexpression of VEGF-D reduced renal immune cell accumulation and, most importantly, prevented HTN [181]. Such evidence was also confirmed in Ang II-induced HTN, in both male and female mice [182]. The relevance of the local renal immunomodulation for these results has already been discussed [30], but we recently found that conditional VEGF-D overexpression attenuates BP in established HTN regardless of immune cell infiltration [183]. In fact, it promoted natriuresis in models of L-NAME HTN and of chronic, but not acute, sodium challenge. The BP lowering and the renoprotective effects are in keeping with independent reports of salt-loaded mice treated with subcutaneous VEGF-C [184], or its targeted delivery to the kidney with nanoparticles [185]. Although data on correlate RIHP changes are missing and whether an expanded lymphatic network results in an increased total lymph flow [186] remains to be established, it is safe to conclude that targeting VEGFR-3 in the kidney opposes renal sodium retention and decreases BP.

Of note, and at variance with established tubule-vascular crosstalk involving other VEGF factors [187], the potential significance of VEGF-C and VEGF-D-induced VEGFR-3 signaling in tubular homeostasis and function, including reabsorption of sodium to the "hybrid" AVRs [145], is largely unknown. A recent study explored the impact of a global VEGFR-3 loss of function in murine

kidneys. While homozygous missense mutations were lethal, heterozygosity of the VEGFR-3 mutation reduced renal lymphatic volume, length, and density but did not appear to affect systemic BP, renal filtration, or albuminuria or worsen low-dose cisplatin-mediated chronic kidney disease [188]. The authors advocated a possible increase in venous and/or urinary efflux of fluid to compensate for reduced lymphatic density and prevent interstitial edema, in line with the early ligation studies [164–170], but experimental confirmation is lacking. Alternatively, low lymphatic density could suffice to meet drainage demands under normal conditions, but not under renal stress, *i.e.*, salt loading. This would be in keeping with our observation that induced lymphangiogenesis did not alter renal sodium handling at baseline but facilitated the maintenance of sodium homeostasis during conditions of renal salt-loading and retention [183], with Wilcox's results [170] (Figure 3) and, eventually, with the concept of lymphatic reserve [64].

Compared to the currently available tools to target lymphangiogenesis, our capacity to modulate renal lymphatic function beyond vascular network expansion is rudimental, at most. Recent work shed some light on the topic: a dependence of renal lymphatic vessel contractility on NO and preload up to a critical point [189], as already shown for other vascular beds [72], and an optimum preload pressure range under which lymphatics can most effectively pump were demonstrated. Additionally, drugs that are commonly used to target impaired interstitial clearance, *i.e.*, loop diuretics, were paradoxically shown to induce a dose-dependent decrease of magnitude and frequency of spontaneous contractions, thereby reducing global lymph propulsion [189]. Subsequent studies from the same group suggested that kidney injury increases tissue sodium and water content (as assessed by ^{23}Na-MRI) but diminishes lymphatic vessel pumping efficiency [190]. Translation of these recent mechanistic findings to HTN is still awaited.

Lymphatics of the Heart in Hypertension

We have known for decades that one-kidney one-clip hypertensive dogs are characterized by higher myocardial interstitial fluid and pressure, and cardiac lymph flow rate compared to controls; this is likely explained by an increase in microvascular permeability [46]. More recently, in a model of cardiac venous HTN, Nielsen and colleagues found similar cardiac edema that resulted over time in myocardial dysfunction and epicardial fibrosis; cardiac lymphatics appeared to compensate by exhibiting both an acute dilatory and a chronic growth response [191]. These findings help interpreting other studies, conducted with more traditional HTN models.

SHR fed an 8% salt diet were shown to exhibit a further increase in BP, decrease in left ventricular (LV) function, increase in perivascular and interstitial fibrosis in the LV; in keeping with the seminal findings in the skin [84,86], the authors found also an upregulation of TonEBP, increased macrophage tissue infiltration, and an increase in cardiac lymphatics, *i.e.* SAL [192,193]. Treatment with a VEGF-C mutant was able to significantly increase lymphatic density and this was associated with decreased cardiac fibrosis and macrophage infiltration, improved cardiac function, and slightly decreased BP [192]; conversely, a VEGF-trap exacerbated HTN, LV remodeling, and cardiac fibrosis [192]. Others have used high levels of Ang II for 4 weeks to mimic hypertensive cardiac dysfunction and eventually heart failure. Zhang and colleagues demonstrated that mice with sirtuin-3 overexpression, which exhibit increased lymphangiogenesis, had decreased cardiac injury and fibrosis compared to WT mice administered Ang II for the same period of time [194]. Another group reported that lymphatic vessel markers were significantly decreased in human hearts that had experienced heart failure [195], while mice treated with Ang II and a lymphangiogenic VEGF-C mutant specific for VEGFR-3 had improved fluid clearance in the heart, with decreased cardiac dysfunction, fibrosis, and inflammation after 1 week, and decreased inflammation and BP after 4 weeks compared to Ang II-only treated mice [195].

Another model of cardiac pressure overload and failure is the transverse aortic constriction (TAC) surgery. After an initial lymphatic expansion, corresponding to the phase of adaptive hypertrophy,

TAC has been reported to decrease cardiac lymphatics [196–198]. This was suggested to reflect a decrease in VEGFR-3 signaling, leading to cardiac edema, hypertrophy, fibrosis, inflammation, and dysfunction, which was exacerbated in mice lacking VEGFR-3 [198]. However, other investigators who used a VEGFR3-blocking antibody did observe an accelerated development of LV dilation and dysfunction after TAC, but no further increase in cardiac edema or interstitial fibrosis [197]. Conversely, cardiac fibrosis, ventricular dilatation and systolic dysfunction were reduced by prevention of negative lymphatic remodeling, via inhibition of specific monocyte subsets [196] or via pre- and post-TAC treatment with a VEGF-C mutant [198]. The latter dose-dependently decreased cardiac edema, as assessed by gravimetry and MRI. Overall, these preclinical findings suggest that a reduced cardiac lymphangiogenesis response to pressure overload may contribute, in part, to the transition from HTN to heart failure, and that augmenting cardiac lymphangiogenesis may help reduce this progression.

Unfortunately, translation to humans is not straightforward. Hypertensive patients who developed heart failure with preserved ejection fraction demonstrated reduced functional reserve of their peripheral lymphatic vessels, reduced lymphatic markers and terminal lymphatic density in the skin, with the remaining vessels showing larger diameters [64]. In cardiac biopsies, lymphatic vessel markers were similarly decreased in patients who had experienced chronic heart failure [195], but vessels were increased and smaller in primary hypertrophic and dilated cardiomyopathies, respectively [197]. These discrepancies may reflect different diseases or timings, and the phenotype specific to human hypertensive heart disease remains unclear.

Lymphatics of other organs in Hypertension: Brain, Lung, Liver and Gonads

While HTN induces end organ disturbances throughout the body, the brain, lung, and liver are notable because of their pressure feedback and organ-specific HTN conditions. Fluid transport and

pressure in the brain through glymphatic and lymphatic pathways are increasingly appreciated in many aspects of intracranial physiology. What roles lymphatics play in the interstitial fluid balance in pulmonary and hepatic responses to HTN, however, remain largely unexplored.

The brain has a bona fide lymphatic network in the meningeal layer (meningeal lymphatics) and another pathway of fluid balance in glymphatics. Since the characterization of the meningeal lymphatics about a decade ago, their role in central nervous system help has largely been identified as that of immune regulation and antigen trafficking rather than fluid balance [199]. Cerebral spinal fluid does drain via these lymphatic vessels, but their absence or increased density through lymphangiogenesis, does not appear to directly impact intracranial pressure [58,200]. Their critical role in macromolecule clearance (*i.e.*, extracellular tau or beta-amyloid) may help to explain the link between HTN and neuroinflammation with cognitive decline or neurodegenerative disease [201,202]. Cerebrospinal fluid generation, balance, and pressure appear to be more linked to glymphatic flow. The glymphatic network – the space between brain endothelium and the surrounding glial cells – and glymphatic flow have been surveyed over a range of physiological and pathological conditions and found to be highly responsive to subtle changes in pressures. These impacts on localized fluid balance have also linked glymphatics to idiopathic intracranial HTN and idiopathic normal pressure hydrocephalus with glymphatic flow changes measurable in these conditions by MRI [203,204]. Glymphatic fluid is created by vascular extravasation, and thus changes with increased sodium loading or HTN that impact brain vascular perfusion, dilation, pulsatility and permeability would result in altered glymphatic fluid balance [61,205–208]. Specifically, glymphatic transport was identified to be significantly reduced in SHR by contrast MRI [62]. Though brain lymphatics are an exciting and increasingly explored area of research for interstitial biology, whether alterations in central lymphatic or glymphatic functions are merely reflective of HTN or potentially modulate the disease remains to be fully characterized.

The interactions of sodium, the interstitium, and lymphatics in the liver in HTN is even less clear. High salt diet independently increases liver inflammation and fibrosis and worsens existing conditions of liver disease [209,210]. This salt-dependent inflammation has been linked in part to changes in the microbiome and subsequent macrophage activation by bacterial translocation [211]. Gao and colleagues demonstrated that even with high salt diet withdrawal and reduced BP, SIRT3-dependent liver inflammation remained, with liver cytokines continuing to spill over into the systemic circulation [212]. Sustained liver inflammation, as in NAFLD, is strongly associated with HTN and may be a source of vascular modulating factors [213]. Lymphatic roles in inflammation resolution may therefore be less responsive to hepatic injury with salt loading. It is more clear, however, that lymph flow is impacted in portal HTN wherein extensive liver damage has already occurred that drive the phenomenon [214]. Increased thoracic duct lymph flow has been measured in dogs, rats, and patients with liver cirrhosis [215–217]. Ascites formation in portal HTN is a peritoneal imbalance of lymphatic uptake being insufficient to normalize extravasated fluid.

Similar to the liver, high salt levels exacerbate lung injury in part through increased macrophage activation [218]. Interestingly, high salt feeding increases sodium and water content, and ultimately tissue mass, of the lungs and livers of rats with SSHTN but not in those who are salt-resistant [107,219]. This appeared to be at least in part through "enlargement of the organs" [219], or subclinical tissue edema as we later suggested [107]. In fact, pulmonary lymphatics are very efficient at maintaining normal fluid; only in mice with significantly impaired pulmonary lymphangiogenesis has tissue edema been consistently observed, with the edema more typically caused by generalized inflammation or infection and blood-side changes [220,221]. Increased venous pressures, for example, increase pulmonary lymph flow [222]. Lung lymphatics, lymphatic growth factors, and lymph flow increases have been better characterized in pulmonary HTN and congenital heart disease [223–226].

Finally, the gonads are another organ affected by sodium intake and HTN. Infertility and sexual dysfunction are associated with HTN [227]. We observed that testes from male and ovaries from

female mice with SSHTN exhibited an imbalance toward proinflammatory polarization of gonadal macrophages, inflammation, lymphangiogenesis, and dysfunction [228]. How sodium induces these changes in the gonads and how altering gonadal lymphatics may affect gonadal function have yet to be determined.

Conclusions

With a teleological evolutionary perspective, 60 years ago Mayerson suggested that the lymphatic vessels appeared in mammals as "*phylogenetic late-comers*", "*as soon as a high-pressure, closed cardiovascular system became necessary to ensure an adequate supply of oxygen to the tissues*" [165]. In other words, for blood circulation to properly operate, lymphatics are non-dispensable. This must have echoed in Mekarsky's mind when he first proposed that *"in principle, hypertension could develop*" to compensate for "*lymphatic system aberrations*" and guarantee adequate tissue supply, if "*without lymph flow the tissue pressure increases*" and "*capillary filtrate is a function of differences in hydrostatic and colloidal pressure acting across the capillary wall* (…)" [18].

While the pathogenesis of HTN, and particularly salt-related HTN, has proven to be far more complicated over the years [2], the hydraulic perspective retains elements of validity. Our interpretation of *lymphatic aberrations* is now based on much better understanding of molecular and structural plasticity of lymphatic vessels and on the concept of lymphatic reserve, which reflects the capacity of the system to respond to any local inflammatory or hydraulic load, and provide drainage accordingly. We have learned about the changes that lymphatics undergo upon different hydrodynamic and sodium-related conditions, similar to inflammation, and the idea of reduced lymphatic reserve has already been associated with the pathogenesis of human heart failure [64,65]. However, how and when this reserve becomes reduced at earlier stages of disease, particularly in relation to renal pathophysiology, remains to be established. Early waves of enthusiasm

following the reports of excess skin sodium accumulation being "drained" (along with the associated HTN) by an expanded terminal lymphatic network [84–86] were recently dampened by contrasting findings, suggesting no effect of hypoplastic or even absent dermal lymphatic vessels on BP or tissue sodium content with high salt diet [88]. This has made clear that extent of anatomical lymphatic density does not necessarily equal function, which still awaits validated practical tools for its assessment in humans but was convincingly enhanced in animals by salt loading and interstitial expansion. On the other hand, despite inconsistent results in the skin, preclinical VEGF-based approaches for lymphatic expansion in other organs have demonstrated promising beneficial effects in SSHTN treatment and prevention of renal and cardiac damage; in this regard, with the recent advancements in the strategies of pharmacological delivery [229], a future translation to humans appears at closer reach.

In conclusion, the role of lymphatics in salt-related HTN is a re-emerging area of exciting research. Compared to seminal physiological studies, we are now endowed with molecular tools to better understand the plasticity and, ultimately, function of these sophisticated vessels. Future investigations should focus on human disease, to elude differences with other species that could be particularly relevant for the pathophysiology of lymphatics, and bias conclusions. Attention should also be paid to the other systems integrated (*e.g.* muscular tissue pump) or reciprocally operating (*e.g.* in renal "three-way" physiology) with lymphatics for the maintenance of interstitial homeostasis, which can be variably affected even in different forms or stages of the same disease [(companion review)]. Eventually, this may lead to include lymphatic targeting in the armamentarium of precision medicine for HTN.

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None

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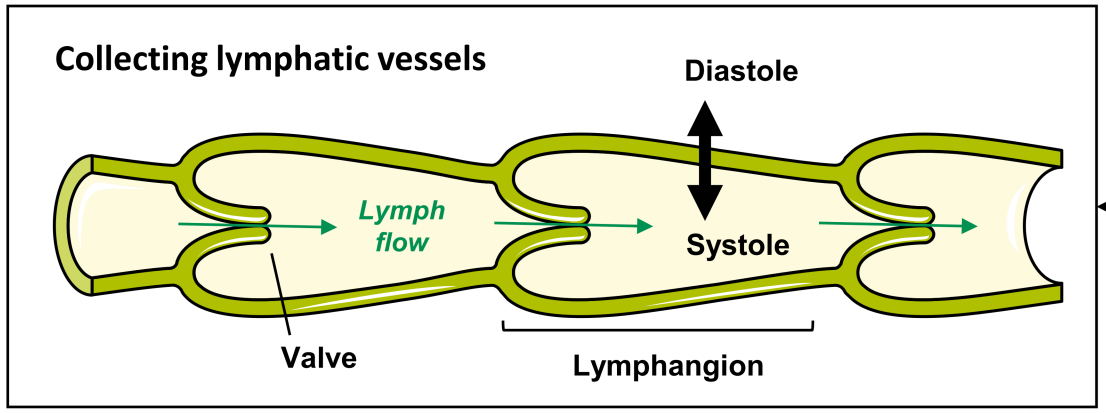
Figures legends

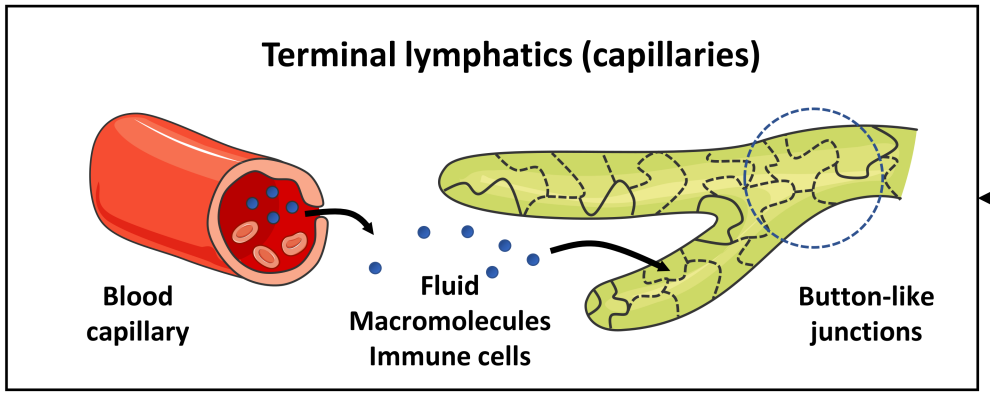
Figure 1. Lymphatic vasculature in the cardiovascular system. By draining extravasated fluids and solutes from the interstitium, lymphatic vessels are crucial for the maintenance of fluid homeostasis. Fluid uptake from terminal lymphatics (or lymphatic capillaries) is driven by capillary filtration (black arrows) and interstitial pressure (Pi), acting as a "*vis a tergo*". Filtration depends on microvascular permeability and the net result of hydraulic and osmotic pressure gradients across the endothelial glycocalyx and vascular wall [40]. Interstitial pressure is a function of interstitial fluid volume (IFV) and tissue compliance, determined by matrix constituents and their anchoring to resident cells [50]; at euvolemia, steep changes in interstitial pressure occur with even minimal changes in volume or capillary filtration rate [230]. All these determinants may be affected in hypertension. Once lymph has entered larger collectors, propulsion for its transport back to the blood circulation against gravity and central venous pressure is provided by the intrinsic pumping activity of each lymphangion, separated from the others by one-way valves, and by extrinsic forces ("tissue pump") including skeletal muscle contraction, intestinal peristalsis, respiration and arterial pulsations.

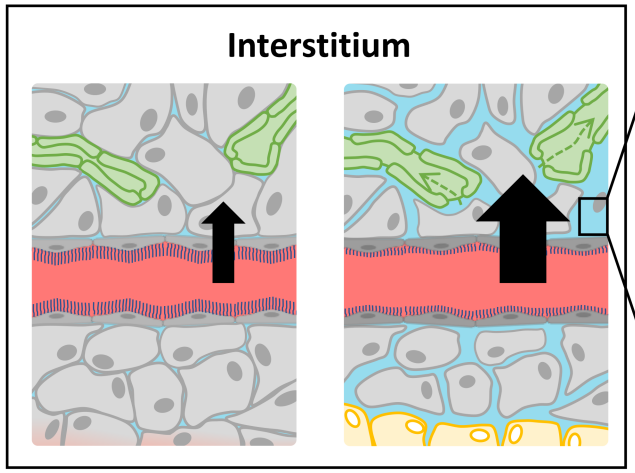
Figure 2. Renal lymphatic vessels. *Panels A and B*: functional anatomy or renal lymphatics (green). Cortical lymphatic capillaries drain toward a capsular lymphatic plexus or into arcuate, interlobar, and progressively larger hilar lymphatics that run in parallel with arteries and veins. Renal medulla is relatively devoid of lymphatic capillaries, while the local drainage of interstitial fluid and solutes is guaranteed by the ascending vasa recta, characterized by a hybrid nature combining hemodynamic, structural and molecular features of lymphatic and blood vessels (green glow). *Panel C*: early functional studies revealed a three-way system, whereby lymphatics would provide a route for fluid drainage alternative to the ureteral (amber) and venous (blue) routes. Experimental ligation/obstruction of either route (depicted in rows #1, #2 and #3) leads to an

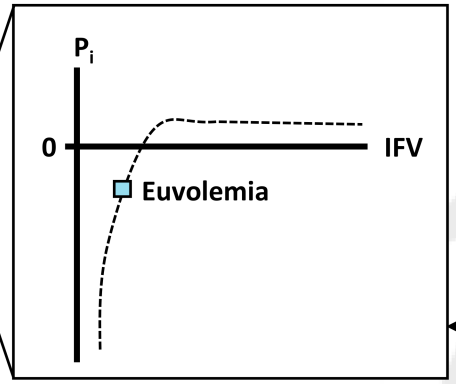
increase in renal interstitial pressure (I, background color as per renal parenchyma) and a compensatory flow increase in the others (please see text for details and references). Ligation of hilar and capsular lymphatics, which could model renal lymphatic dysfunction, led to interstitial edema and isosthenuria, with loss of solutes including sodium; however, under conditions of salt loading, whole renal balance reflected reduced natriuresis (uNaV) and sodium retention (red arrow), similar to ureteral obstruction and renal venous hypertension/ligation (see also figure 3).

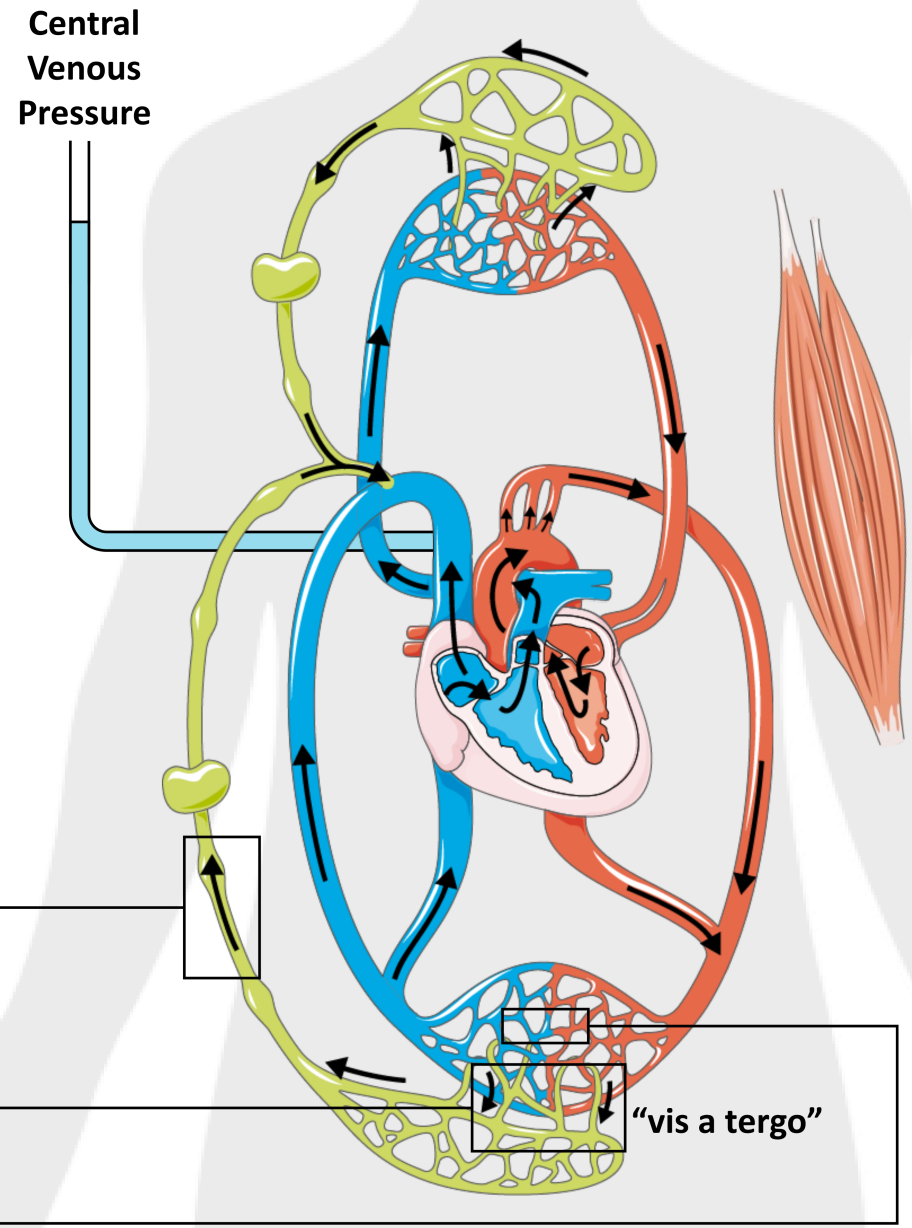
Figure 3. Effects of renal lymphatic ligation on intrarenal pressure and the excretion of water, sodium and potassium. Drawn from Am J Physiol. 1984 Aug;247(2 Pt 2):F344-51 with permission. *Panel A*: Renal interstitial hydraulic pressure (measured as subcapsular pressure; P) was higher in the lymphatic-ligated (Lig) than in Sham-operated kidney both at baseline (B) and after volume expansion with saline (S). *Panel B*: comparison of Sham or actively (Lig) instrumented kidneys with contralateral kidneys as controls (C), at baseline and after saline; data presented as mean±SEM. At baseline, lymphatic ligation induced an increase in urinary flow (V) and sodium excretion (uNaV). However, the excess diuresis induced by salt loading was not paralleled by natriuresis, but excess potassium wasting; on the contrary, the contralateral uninstrumented kidney showed an anti-natriuretic response that overall reduced total uNaV.

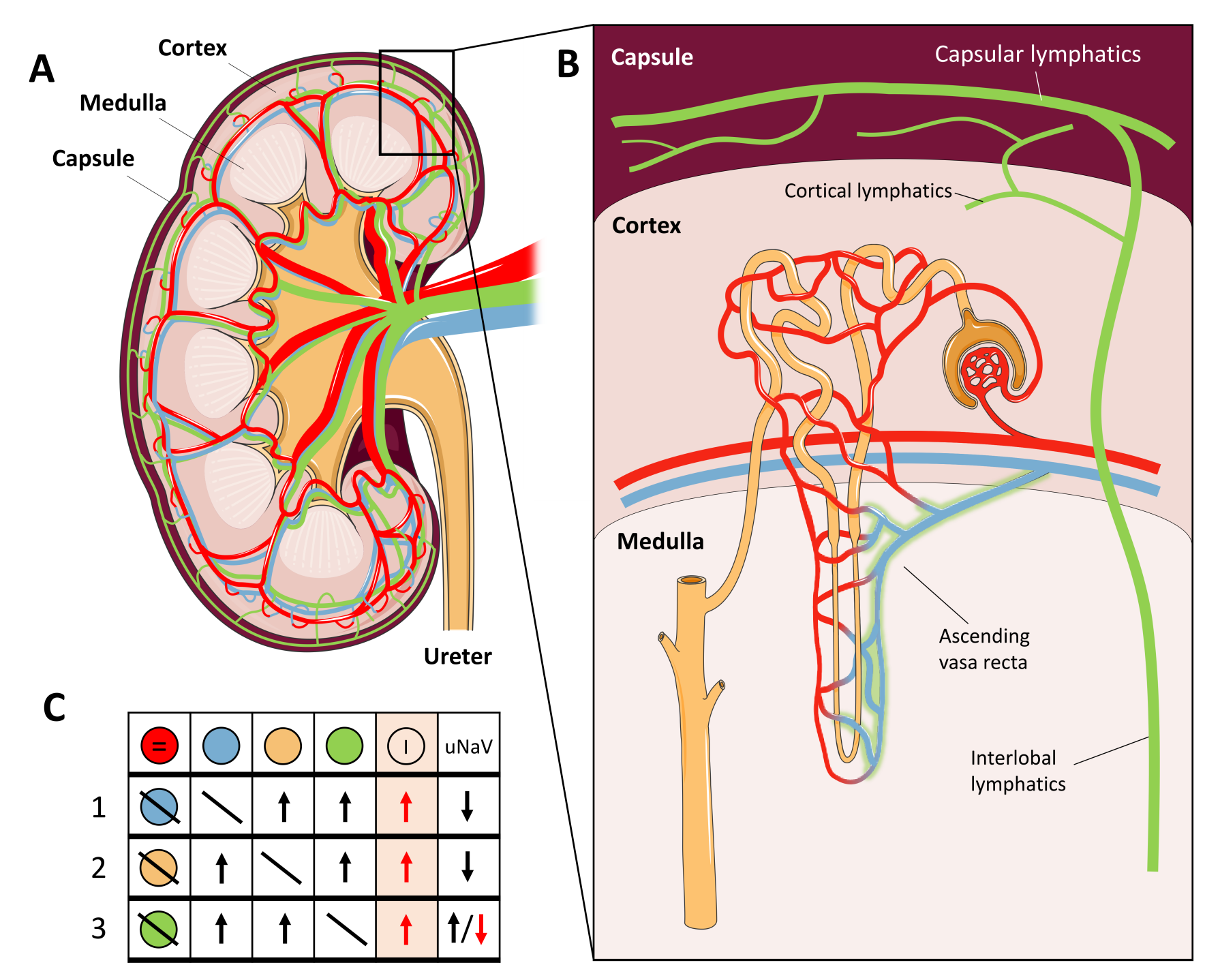

Collecting lymphatic vessels
Diastole
Lymph flow
Systole
Valve
Lymphangion


Terminal lymphatics (capillaries)
Blood capillary
Fluid
Macromolecules
Immune cells
Button-like junctions


Interstitium


P_i
0
IFV
Euvolemia


Central Venous Pressure
Tissue pump
"vis a tergo"


A
Cortex
Medulla
Capsule
Ureter
B
Capsule
Capsular lymphatics
Cortical lymphatics
Cortex
Medulla
Ascending
vasa recta
Interlobal
lymphatics
C
=
I
uNaV
1
2
3

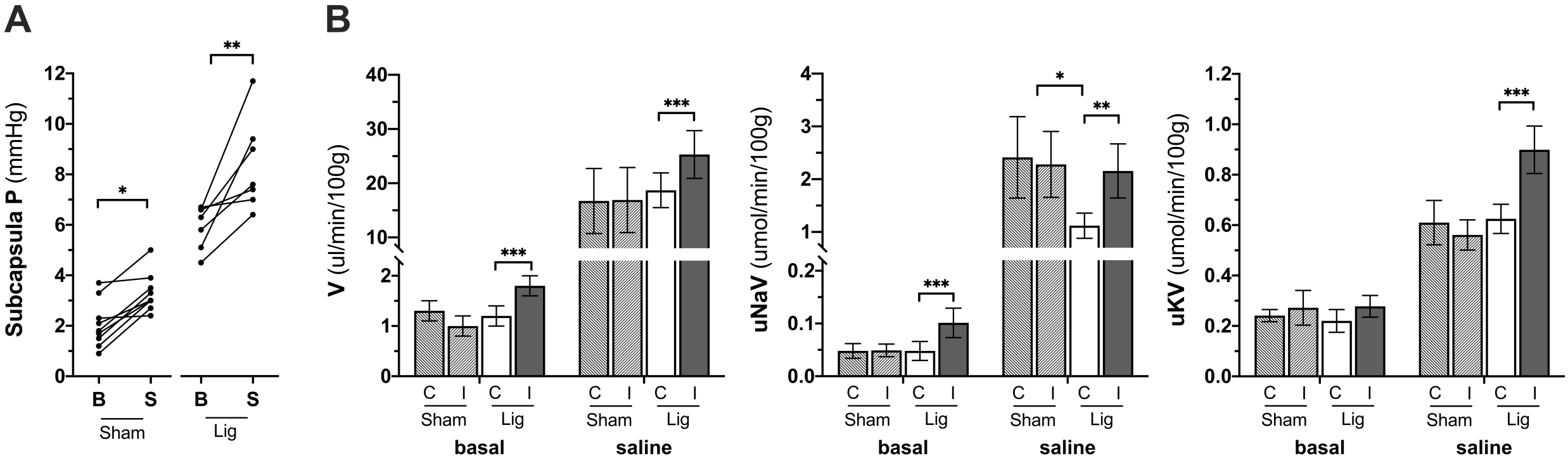

A
Subcapsula P (mmHg)
12
10
8
6
4
2
0
*
**
B
S
B
S
Sham
Lig
B
V (ul/min/100g)
40
30
20
10
2
1
0
***
***
C
I
C
I
C
I
C
I
Sham
Lig
Sham
Lig
basal
saline
uNaV (umol/min/100g)
4
3
2
1
0.2
0.1
0.0
***
*
**
C
I
C
I
C
I
C
I
Sham
Lig
Sham
Lig
basal
saline
uKV (umol/min/100g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
C
I
C
I
C
I
C
I
Sham
Lig
Sham
Lig
basal
saline

Table 1. Molecular signature of Lymphatic Endothelial Cells.

Marker	Molecular function/characteristics	LEC	BEC	References
Prox1	Transcription factor crucial for lymphatic phenotypic identity. Nuclear expression.	+++	-	[s1,s2]
LYVE1	Type I integral membrane glycoprotein and receptor for hyaluronan, promoting dendritic cells entry into terminal lymphatic vessels. Its expression is reduced or absent in collecting vessels LECs.	+++	- (+) [a]	[s3,s4]
VEGFR-3	Receptor tyrosine kinase mediator of lymphangiogenesis, lymphatic growth/survival control, lymphatic pump stimulation	+++	- (+) [b]	[s5,s6]
VEGFR-2	Receptor tyrosine kinase crucial for blood vascular angiogenesis; it can form heterodimers with VEGFR3 in LECs in response to VEGF-C	+	+++	[s7]
Podoplanin (PDPN)	Mucin-like transmembrane protein that prevents postnatal blood filling of the lymphatic system, contributes to efficient migration of dendritic cells to lymph nodes and mediates the attachment of tumour-associated macrophages to the lymphatic endothelium. Also expressed on some epithelial cells and glomerular podocytes.	++	-	[s8,s9]
CD31	Also known as platelet endothelial cell adhesion molecule (PECAM-1), is a transmembrane glycoprotein that takes part in the majority of the intercellular junctions of endothelial cells. Weakly expressed also on some LECs.	+/-	+++	[s10]
VE-cadherin	Adhesion molecule, important for cell-cell adhesion and interaction, transmembrane signal transduction, permeability, maintenance of distinct lymphatic beds, development of intraluminal lymphatic valves. The organization of the cell–cell junctions differs between terminal and collecting LECs.	+	+	[s11]
FOXC2	Transcription factor, master regulator of lymphatic valve morphogenesis in collecting vessels LECs. Upregulated by the mechanosensitive GATA2, in response to shear stress.	++	-	[s12,s13]

LEC = Lymphatic endothelial cells. BEC = blood endothelial cells. Additional regulators and markers of lymphatic identity are reviewed elsewhere [s2]. a) LYVE1 expression was found in embryonic blood vessels [s14] ; b) VEGFR3 is expressed in BECs during embryonic development, becoming later essentially restricted to LECs, and pathological angiogenic processes [s15,s16]. References for this table are made available as supplementary material.

Table 2. GWAS association between lymphedema-related genes and blood pressure or hypertensive organ damage

Gene	Lymphedema phenotype	Role	SNP	Risk allele	p-value	RAF	Trait	β	Reference
VEGFC	Congenital primary lymphedema of Gordon	Lymphangiogenesis, lymphatic vessel development and permeability	rs7660760	T	$4x10^{-7}$	0.5476	Left ventricle wall thickness	0.009 unit decrease	[s17]
FLT4	Milroy disease (ORPHA79452)	Encodes for VEGFR3; involved in lymphangiogenesis and maintenance of lymphatic endothelium and pumping	rs112967731	G	$6x10^{-7}$	0.9118	Carotid artery intima media thickness	0.023 unit decrease	[s18]
FOXC2	Lymphedema-distichiasis syndrome (ORPHA33001)	Lymphatic vascular and valvular formation and maturation under shear stress	rs7199751	T	$3x10^{-6}$	0.2246	White matter hyperintensity volume	0.034 unit increase	[s19]
					$3x10^{-7}$		White matter hyperintensity volume	0.037 unit increase	
GATA2	Deafness-lymphedema-leukemia syndrome (Emberger syndrome, ORPHA3326)	Lymphatic vessel morphogenesis and maintenance	rs55914222	C	$3x10^{-8}$	0.3828	SBP	0.038 unit increase	[s20]
					$9x10^{-9}$		DBP	0.510 mmHg decrease	[s21]
					$5x10^{-14}$		PP	0.048 unit increase	[s20]
			rs62270945	C	$1x10^{-10}$	NR	SBP	0.608 unit decrease	[s22]
					$2x10^{-6}$		SBP	0.534 unit decrease	[s22]
				T	$2x10^{-13}$	0.0270	PP	0.669 mmHg increase	[s21]
					$2x10^{-9}$		PP	0.607 unit increase	[s23]
ADAMTS3	Hennekam syndrome (ORPHA2136)	Activates VEGF-C, inducing lymphangiogenesis and lymphatic network expansion	rs13104467	NR	$4x10^{-8}$	NR	BP (DBP x SBP)	0.071 unit increase	[s24]
			rs788908	T	$2x10^{-11}$	0.6182	DBP	0.094 mmHg decrease	[s25]
			rs7697556	T	$1x10^{-10}$	0.4781	DBP	0.088 mmHg increase	[s25]
EPHB4	Noonan syndrome	Lymphatic vessel integrity and remodelling and formation of valves during development	rs221005	A	$3x10^{-9}$	NR	PP	0.163 unit increase	[s26]

Based on search of https://www.ebi.ac.uk/gwas, performed on 26/04/2023. SNP: single-nucleotide polymorphism; RAF: risk allele frequency. References for this table are made available as supplementary material.