Tissue sodium excess is not hypertonic and reflects extracellular volume expansion

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Our understanding of Na\(^+\) homeostasis has recently been reshaped by the notion of skin as a depot for Na\(^+\) accumulation in multiple cardiovascular diseases and risk factors. The proposed water-independent nature of tissue Na\(^+\) could induce local pathogenic changes, but lacks firm demonstration. Here, we show that tissue Na\(^+\) excess upon high Na\(^+\) intake is a systemic, rather than skin-specific, phenomenon reflecting architectural changes, i.e. a shift in the extracellular-to-intracellular compartments, due to a reduction of the intracellular or accumulation of water-paralleled Na\(^+\) in the extracellular space. We also demonstrate that this accumulation is unlikely to justify the observed development of experimental hypertension if it were water-independent. Finally, we show that this isotonic skin Na\(^+\) excess, reflecting subclinical oedema, occurs in hypertensive patients and in association with aging. The implications of our findings, questioning previous assumptions but also reinforcing the importance of tissue Na\(^+\) excess, are both mechanistic and clinical.

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In the last decade, the concept of tissue Na\(^+\) shifted the traditionally nephrocentric view of Na\(^+\) homeostasis to the interstitium\(^{1-3}\), where the negatively charged glycaminoglycans and a TonEBP/NFAT5-VEGF-c-mediated expansion of the lymphatic network serve as a depot and draining system for its accumulation, respectively\(^{4-5}\). The proposed water-independent nature of this phenomenon\(^{6-8}\) marked its novelty\(^{9,10}\) and its potential implications in the pathogenesis of hypertension and cardiovascular disease at large, as independent studies reported a boosted activation of pathogenic immune-inflammatory cells upon culture in supraphysiological concentrations of Na\(^+\)\(^{11-13}\). The translational value of this evidence relies on the presence of hypertonic (HT) microenvironments in tissues. In humans, increased tissue Na\(^+\) concentration has been identified in association with aging and hypertension\(^{14}\), diabetes\(^{15}\), chronic kidney disease\(^{16}\) and acute heart failure\(^{17}\) in skin (where the accumulation was initially described as specific in rodent models\(^{18,19}\) and/or skeletal muscle, as well as in sclerodermic\(^{20}\) or infected skin\(^{21}\), by means of \(\text{Na}\) MRS. Excess Na\(^+\) accumulation upon salt loading, with some sex specificities, was also found in healthy subjects by direct skin chemical analysis\(^{22}\). Importantly, both methods measure Na\(^+\) in the whole tissue. We have previously suggested that tissue architecture per se can markedly impact on Na\(^+\) content and concentration (Fig. 1)\(^{22}\). Such histochemical\(^{23,24}\) considerations are key to conclude on the HT nature of this Na\(^+\) excess, which currently lacks demonstration in humans. In fact, even in rodent models, attempts to isolate hyperosmolar interstitial fluid or lymph proved unsuccessful\(^{25}\). Moreover, the dual osmotically active and inactive nature of interstitial Na\(^+\), driving TonEBP-mediated signalling while simultaneously eluding parallel and commensurate water accrual, appears equivocal. On these premises, we sought to test the existence, distribution and putative correlates of HT tissue Na\(^+\) accumulation by probative and disprobative approaches (i.e., by verifying and by assuming its occurrence, respectively) in preclinical models of and real-life patients with arterial hypertension.

Our study offers a reappraisal of the tissue Na\(^+\) theory, disproves its water-independence in both experimental salt-sensitive hypertension and hypertensive subjects and suggests systemic isotonic (IT) Na\(^+\) excess as an important player in the pathogenesis of cardiovascular disease, particularly in association with the process of aging.

**Results**

**Tissue Na\(^+\) accumulation is systemic and isotonic.** Male and female stroke-prone spontaneously hypertensive (SHRSP) and control Wistar–Kyoto (WKY) rats (12 weeks old) were treated with 1% NaCl in drinking water (high salt, HS) or normal tap water (normal salt, NS) for 3 weeks. In males, baseline blood pressure (BP) was higher, irrespective of strain differences, and salt sensitivity of BP was confined to SHRSPs (Supplementary Fig. 1). Skin Na\(^+\) content (mmol g\(^{-1}\) of dry weight (DW)) was increased in SHRSP-HS (+20.7%), as expected; however, we observed similar increases in tissue Na\(^+\) also in liver (+10.3%), lungs (+14.8%) and skeletal muscle (+23.6%), but not in myocardium (Fig. 2a and Supplementary Data 1). Apart from skeletal muscle, they were consistently paralleled by increases in tissue water (skin: +14.1%; liver: +4.8%; lungs: +9.0%) with similar trends observed for Na\(^+\) concentrations (i.e., normalised for water content, [Na\(^+\)], mmol L\(^{-1}\); Supplementary Data 1). These changes upon HS were not observed in WKY rats. A sub-analysis for males and females showed almost identical patterns (Supplementary Data 1). Different experimental results for tissue Na\(^+\) across organs fit well with a prediction model based on the intracellular/extracellular volume proportion (ECV/ICV) typical of each tissue (Supplementary Fig. 2a)\(^{22,26}\). Lower water and Na\(^+\) between control (NS) strains in skin and liver reflect differences in fat content that limit the volume of distribution (and total content, accordingly) of both (Supplementary Fig. 3). Importantly, K\(^+\) concentration ([K\(^+\)]) showed opposite trends to [Na\(^+\)] (Supplementary Data 1). In physiological conditions, the sum of [K\(^+\)] and [Na\(^+\)] in both ECV and ICV is similar, although with opposite predominance of Na\(^+\) and K\(^+\), respectively; a mixture of ECV and ICV in any proportion would still result in a solution of \(\approx 140-160 \text{Na}^+ + \text{K}^+ \text{mmol L}^{-1}\). In all tissues, [Na\(^+\) + K\(^+\)] consistently fell within this range, with no differences between strains or NS/HS allocation (Fig. 2a). Overall, rather than any HT phenomenon, this points to a shift in ECV/ICV ratio and, along with the concomitant increase observed for tissue water, to systemic oedema. The difference in the proportional changes of Na\(^+\) and water does not reflect different amounts, as previously suggested, but different sensitivities to oedema detection (Supplementary Fig. 4). For skeletal muscle where the water-independent ECV/ICV shift is likely due to HS-induced sarcopenia\(^{22}\), the lack of oedema in skeletal muscle and myocardium also reflects the lower physical...
compliance of these tissues\textsuperscript{28}; of note, in similar experiments conducted in NS-fed but aged rats (52 weeks old), myocardial oedema was detectable and accompanied by extracellular matrix remodelling with an increase in glycosaminoglycans (Supplementary Fig. 5).

The currently proposed mechanistic framework indicates that water-independent tissue Na\textsuperscript{+} excess is responsible for a VEGF-c mediated signalling via TonEBP/NFAT\textsuperscript{53,4}. Although traditionally associated with the response to osmotic stress\textsuperscript{29}, we observed an increase in TonEBP/NFAT\textsuperscript{5} expression in skin, lungs and
**Fig. 3 Impact of in vivo and ex vivo Na⁺ excess on vascular function of resistance arteries.** Panels a, b contractile response to KCl, by strain and experimental treatment; box-and-whisker plots (Tukey: centre line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range; points, outliers); WKY-NS: n = 15 and HS n = 18; SHRSP-NS: n = 14 and HS n = 17; two-way ANOVA results at bottom and predeﬁned or post hoc (inter-strain) comparisons on top. Panel a the response to the ﬁrst KCl stimulation was higher in SHRSP than WKY but was unaffected by in vivo treatment; panel b the response to a second stimulation, expressed as percentage of ﬁrst peak response, was reduced in SHRSP, particularly upon HS. Panels c, d contraction and relaxation dose-response curves to U46619 (thromboxane A₂ receptor agonist; n = 11-17/group, please see Supplementary Table 1a, b) and SNP (endothelium-independent nitric oxide donor; n = 9–16/group, please see Supplementary Table 1a, b), respectively; mean ± SEM. Differences in maximal responses and half maximal effective/inhibitory concentrations (EC₅₀ and IC₅₀, respectively) across groups are shown by brackets; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, at Extra sum-of-squares F test upon non-linear regression by least-square method. c Effect of 1% NaCl in drinking water (HS): HS did not worsen the hypercontractile phenotype observed in SHRSP rats (max response to U46619: SHRSP-NS vs. WKY-NS, p = 0.0001; SHRSP-NS vs. SHRSP-NS, p = 0.892; LogEC₅₀ to U46619: SHRSP-NS vs. WKY-NS, p = 0.017; SHRSP-NS vs. SHRSP-NS, p = 0.142), but induced oversensitivity to NO-mediated relaxation (max response to SNP: SHRSP-NS vs. SHRSP-NS, p = 0.041; LogIC₅₀ to SNP: SHRSP-NS vs. SHRSP-NS, p < 0.0001); no signiﬁcant effect was observed in WKY rats. d Effect of ex vivo 5 h incubation in a hypertonic (+15 mmol/l NaCl, HT) vs. physiological solution on vessels from NS rats: ex vivo hypertonic Na⁺ excess did not affect contractile responses (max response to U46619: SHRSP-HT vs. SHRSP, p = 0.328; WKY-HT vs. WKY, p = 0.382; LogEC₅₀ to U46619: SHRSP-HT vs. SHRSP, p = 0.856; WKY-HT vs. WKY, p = 0.516), but induced oversensitivity to NO in both strains, opposite to in vivo HS (LogIC₅₀ to U46619: SHRSP vs. WKY, p < 0.0001; SHRSP-HT vs. SHRSP, p < 0.0001; WKY-HT vs. WKY, p = 0.007); none of these ex vivo effects would justify an increase in peripheral vascular resistance. Please see Supplementary Table 1a, b for all comparisons; source data are provided as a Source Data file.

Extracellular hypertonic Na⁺ does not induce hypertensive vascular dysfunction. Blockade of lymphangiogenesis induces development of salt-sensitive hypertension. If the resulting excess tissue Na⁺ were independent of water (i.e., volume), the expectation is that it would impact on the other determinant of hypertension, i.e., peripheral resistance. To test this hypothesis, we investigated the vascular function of peripheral resistance arterioles from our NS/HS rats. HS treatment did not result in hypercontractile responses to U46619 (a thromboxane-receptor agonist) or KCl but reduced the latter upon repeated stimuli and the sensitivity to the vasorelaxant effect of nitric oxide (NO) in SHRSP (Fig. 3 and Supplementary Table 1). Intriguingly, the concept that vascular swelling could determine increased peripheral resistance and vascular stiffness (to both contraction and relaxation) in the pathogenesis of hypertension dates back to the 1950s; although it is supported by subsequent evidence, which extends to related diseases and includes preliminary data from our rats (Supplementary Fig. 6), it still lacks firm demonstration. To test whether an HT environment could directly induce similar changes, arterioles from NS rats were also preincubated in an IT or HT (+15 mmol L⁻¹ NaCl) solution. Akin to HS in vivo, HT ex vivo incubation did not affect contractile responses; however, it induced an opposite shift toward an earlier (borderline significant) liver in SHRSP-HS, as well as in myocardium of aged SHRSP, despite the lack of hypertonicity (Fig. 2b and Supplementary Fig. 5). As recently demonstrated for VSMCs, we speculate that biomechanical (rather than osmotic) stress due to oedema accumulation is responsible for the activation of the lymphangiogenic signal cascade.
NO-induced loss of pre-constriction tone in both WKY and SHRSP (Fig. 3 and Supplementary Table 2).

This is not reminiscent of classic hypertensive vascular phenotypes and appears more consistent with the neuromuscular signs of hypernatremia (e.g., lethargy and muscle weakness). Overall, these data suggest that impaired drainage of tissue Na\(^+\) is unlikely to induce hypertension by directly and adversely modulating peripheral vascular function in a water-independent manner.

**Skin Na\(^+\) accumulation in hypertensive patients.** To translate our preclinical findings to humans, we performed a chemical analysis of arm skin punch biopsies of 76 hypertensive subjects (Supplementary Table 3). The two anatomically distinct layers of epidermis/superficial dermis (ESD) and deep dermis (DD) were analysed separately: as our histochemical approach would predict, [Na\(^+\)] and [K\(^+\)] were lower and higher in the ESD compared to DD, respectively (Fig. 4a). In virtually no patients, and therefore in none of the specified different clinical subgroups, did [Na\(^+\)+K\(^+\)] exceed the physiological value of 155 mmol L\(^{-1}\) in either layer (Fig. 4b), thus ruling out any HT accumulation. In both ESD and DD, water content was positively and negatively correlated with [Na\(^+\)] and [K\(^+\)], respectively (Fig. 4c); in DD, water, Na\(^+\) and K\(^+\) contents, but not concentrations, were all positively and highly correlated (Fig. 4c), primarily reflecting their volume of distribution which excluded dermal fat. Accordingly, male DD contained more water, Na\(^+\) and K\(^+\) than female dermis, which is known to be richer in subcutaneous fat (Supplementary Fig. 7); no such differences were seen in the ESD (Supplementary Table 4). Identical histochemical sex differences were observed in gluteal biopsies from young healthy volunteers (Supplementary Table 5); notably, fluid-retentive\(^{36}\) progestinic states were characterised by higher water and Na\(^+\) content, but unchanged [Na\(^+\)+K\(^+\)] (Supplementary Fig. 7). In the hypertensive cohort, age was independently associated with an increase in ESD water and [Na\(^+\)], and a decrease in [K\(^+\)] in both layers (Fig. 4d and Supplementary Fig. 8); this likely indicates excess fluid accumulation in the context of reduced tissue cellularity, traditionally accepted as a hallmark of skin aging (Fig. 4d). Salt intake predicted epidermal water content, but not Na\(^+\) or K\(^+\), independently of age, sex and BMI (Supplementary Fig. 8); at variance

![Fig. 4](https://example.com/fig4.png)

**Fig. 4** Histochemical analysis of skin from hypertensive patients and clinical correlates. Panel a bars show mean ± 95% CI, with individual points (overlay); Na\(^+\) and K\(^+\) concentrations and water content in the epidermis/superficial dermis (ESD) and deep dermis (DD) layers, reflecting different architecture and cellularity. Panel b skin Na\(^+\)+K\(^+\) concentration in ESD and DD; red X = automatically detected outliers (ROUT, Q = 1%); in virtually no patient, including n = 3 cases with primary aldosteronism, did [Na\(^+\)+K\(^+\)] exceed physiological values (grey). Panel c correlations of histochemical parameters in ESD and DD; Pearson \(\rho\) and statistical significance are colour coded as per legend. Panel d association of ESD water, [Na\(^+\)] and [K\(^+\)] with age, reflecting the development of oedema and the shift in ECV/ICV ratio; red X = automatically detected outlier; bottom: representative pictures of age-related reduction in skin cellularity; immunofluorescence, DAPI staining; scale bars (bottom right) = 200 \(\mu\)m. Panel e relationships between skin histochemical parameters, age, BMI, Na intake and pulse pressure (PP), plasma NT-proBNP and transepidermal water loss (TEWL), by skin layer. Data are presented as standardised B regression coefficients (95% CI); for skin histochemical parameters, coefficients for all patients are visualised in black, for males in blue, for females in pink. PP and NT-proBNP positive associations with ESD water accumulation were mostly driven by females; the association with Na\(^+\)/K\(^+\) ratio was overall generalised to both layers and sexes. No significant associations were observed with TEWL, making relative local water deficit an unlikely explanation for any Na\(^+\) hypertonic excess. All panels refer to n = 76 samples from independent subjects; missing data due to technical issues or unavailable samples were ≤8 for all variables/correlations; source data are provided as a Source Data file.
with aging, the lack of any significant shift in the Na/K ratio in relation to salt intake would suggest a fluid accumulation evenly distributed between ICV and ECV. Office diastolic BP was inversely associated with ESD water content (ρ = −0.250; p = 0.03) and a surrogate of vascular stiffness, pulse pressure (PP), was positively correlated with Na+/K+ ratios (Fig. 4e); in ESD, the ratio was higher in patients with uncontrolled BP, independent of other covariates. No significant interaction with medications was observed. NT-proBNP levels, positively associated with age (ρ = 0.526; p < 0.001), estimated Na+ intake (ρ = −0.260; p = 0.03) and systolic BP (ρ = 0.288; p = 0.01) and negatively with diastolic BP (ρ = −0.277; p = 0.02), mirrored all measures of skin oedema accumulation (Fig. 4e). Finally, the lack of correlations between histochemical skin data and transepidermal water loss (TEWL)37 (Fig. 4e) stood against the predefined hypothesis that local regulatory mechanisms could make skin an exception to the IT nature of systemic tissue Na+ excess/accumulation via a relative deficit of water.

**Discussion**

Our study has epistemological, mechanistic, diagnostic and therapeutic implications. First, while we cannot unconditionally exclude an HT Na+ accumulation in any tissue milieu or any clinical/experimental condition, our findings prompt reconsideration of excess tissue Na+ epidemic in light of a simpler histochemical deductive approach34. Nunnquam est ponenda pluraltas sine necessitate38 (plurality is not to be posited without necessity). Over 80 years ago, Simms and Stolman made cadastral observations consistent with our conclusions39. Second, this necessitates38 (plurality is not to be posited without necessity). Over 80 years ago, Simms and Stolman made cadastral (HS) or normal drinking water (NS) for 3 weeks (12 weeks), rats (littermates) were randomised to 1% NaCl (rat and mouse No. 1 maintenance diet; Special Biological Chemical) using HNO3– and MilliQ– carefully washed glassware. Before photometric measurements, digested samples were further diluted to fall within the range of 0–5 ppm. MilliQ was used as the diluent for both standards and samples after Na+ and K+ concentration in the digested sample solutions were calculated against the linear regression line obtained from the calibration standards. At the calibration used, reported CV% for reproducibility is 2% (i.e., <0.005 mmol/l for both Na and K in the measured sample); in our hands CV% was 1.5 intra-sample and 3% inter-sample (from the same original tissue). Concordance correlation coefficient for replicated (technical) measurements was 0.98 (Supplementary Fig. 9a); random duplicates from the same stored tissues showed similarly good reproducibility (Supplementary Fig. 9b, c). All samples from the same type of tissue were analysed in a batch on the same day to minimise technical variability; therefore, few samples irreversibly affected by experimental issues (e.g., accidentally dropped sample temperature, assay contamination, volume shortage) had to be excluded and are reported as blank cells in the Source Data File. During measurements, blank (diluent) and calibration standards were checked after every block of approximately eight samples to control for drift. Na+ and K+ concentrations in the analysed solutions were used to back-calculate their total content in the digested tissues and normalised by DW for Na+ and K+ tissue content (mmol/DW), or by tissue water for Na+ and K+ tissue concentration (mmol/l). During flame photometry analysis, operators were blind to group allocation of samples.

**Fat content analysis.** After careful removal of the solubilised material used for Na+/K+ quantification, total fat was extracted from the acid-insoluble residue of skin and liver tissues in 200 µl of a solvent mixture (chloroform/methanol, 2:1 v/v; protocol adapted from Lowry et al.24 and Folch et al.35); extraction of the total fat content was quantified by flame photometry (Sherwood scientific, model 410 C). Na+ and K+ calibration standards were prepared from 0–5 ppm (mg/l) using (milliQ, Fisher) for >40 h, to a stable DW. Water content was measured as (WW – DW)/DW and expressed as mg water/mg DW, or as water percentage (W% = (WW – DW) × 100%/WW). Dried samples were digested at 65 °C in a Thermostir (Eppendorf), for >40 h, to a stable DW. Myocardium (left ventricle), lungs, liver, descending aorta and samples from the epicardial layer (Histoclear) and progressive rehydration to constant weight through repeated SpeedVac cycles. Total fat content in tissues were maintained on standard diet and normal tap water ad libitum until 20 and 52 weeks of age (Sigma). Tissue harvesting and processing. At the end of the 3 weeks of experimental treatment (main experiment) or at the appropriate age (additional experiment) rats were euthanized by exsanguination under general terminal isoflurane anesthesia. Myocardium (left ventricle), lungs, liver, descending aorta and samples from subepicardial region (n = 25 animals) and subepicardium (n = 12 animals, immediately below the epicardial layer) with an Olympus BX41 microscope and dedicated image capture software. sGAG were quantified as %AlineB− stained area after standardised

**Histology.** Sulphated glycosaminoglycans (sGAG) myocardial content was estimated by Alcian Blue staining of available paraffin-embedded transverse midventricular 5 µm sections from the 20- or 52-week-old WKY and SHRSK male rats (n = 4–5/group). After deparaffinization (Histoclear) and progressive rehydration to distilled water, slides were incubated in Alcian Blue solution (1% Alcian Blue 88PB, pH 2.5; 0.3% acetic acid, with 0.02% Na2SO3, 2.25 g/l NaCl) for 20 min, washed in running tap water for 5 min, rinsed in distilled water and counterstained in Nuclear Fast Red solution (Vector), washed again in running tap water for 1 min and mounted following dehydration.

Random, non-competitive pictures were taken at ×40 from midmyocardium (n ≥ 25/animal) and subepicardium (n ≥ 12/animal, immediately below the epicardial layer) with an Olympus BX41 microscope and dedicated image capture software. sGAG were quantified as %AlcianB− stained area after standardised metabolism and drug development. In many relevant clinical scenarios beyond sole cardiovascular disease. Finally, the effect of novel natriuretic agents in both prevention and treatment of major cardiovascular events40,41 prompts better identification of those patients who will benefit most. Considering the demonstrated potential of these agents in reducing tissue Na+ content39 and the above functional considerations, it is tempting to speculate that this quest should take into account systemic and/or organ-specific tissue Na+ analysis.

**Methods**

**Animals.** All protocols were performed in accordance with the United Kingdom Animals Scientific Procedures Act 1986 (Project Licence 70/9021 held by Delyth Graham) and ARRIVE Guidelines and approved by the institutional ethics review committee (University Animal Welfare and Ethics Review Board, University of Glasgow, Glasgow, UK). We used SHRSP and WKY rats from colonies inbred at the University of Glasgow since 1991; animals were housed under controlled environmental temperatures (21 ± 3 °C) and lighting (12-h light–dark cycles). In the main set of experiments, male and female SHRSP and WKY were maintained on standard rat diet (rat and mouse No. 1 maintenance diet; Special Diet Services, Grangemouth, United Kingdom) and provided tap water ad libitum until 11 weeks of age. At 12 weeks, rats (littermates) were randomised to 1% NaCl (HS) or normal drinking water (NS) for 3 weeks (n = 8 males/group and n = 10 females/group, except n = 9 female WKY-NS). Systolic BP was measured at 11 weeks of age (baseline) and then monitored weekly by tail-cuff plethysmography, in an operator-blind fashion whenever possible. In another set of experiments, male SHRSP and WKY were maintained on standard diet and normal tap water ad libitum until 20 and 52 weeks of age (WKY 20 weeks n = 10; WKY 52 weeks = 9; SHRSP 20 weeks = 6; SHRSP 52 weeks = 9).
thresholding across pictures, by use of an in-house developed macro (Supplementary Note 1) for ImageJ.

**TonEBP gene expression analysis.** For all tissues, total RNA extraction was performed using Qiagen (Qiagen) and RNAeasy mini-column kit (Qiagen) according to manufacturer’s guide. For qRT-PCR, CDNA was prepared using the High Capacity CDNA reverse transcription kit (Applied Biosystems) and analysed using Taqman fast advanced master mix with specific Taqman gene expression assay probes for TonEBP (Rn01462487 g1), GAPDH (Rn01426621 g1), beta-actin (Rn00667069 m1). The expression levels were normalised to either GAPDH (myocardium) or beta-actin (other tissues; housekeeping gene was selected upon evidence of Ct consistency across groups) and were compared and presented as delta Ct values (inversely proportional to gene expression).

**Rat vascular function studies.** After organ harvesting for tissue chemical/molecular analysis, mesenteries were also dissected from NS/HS WKY and SHRSP rats and small resistance arteries were isolated as previously described. Briefly, arterial segments were mounted on isometric wire myographs (Danish Myo Technology, Denmark) filled with 5 ml of physiological saline solution (PSS; 119.0 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4·7H2O, 24.9 mM NaHCO3, 1.2 mM KH2PO4, 2.5 mM CaCl2 and 11.1 mM glucose) and continuously gassed with a mixture of 95% O2 and 5% CO2 while being maintained at a constant temperature of 37 ± 0.5 °C. LabChart (ADInstruments) was used for data recording. Following 30 min of equilibration, baseline tension was normalised as per DMT recommendations and internal diameter was estimated (https://www.dmt.dk/uploads/5/6/5/8/6589239/dmt_normalization_guide.pdf). Vessels with an internal diameter ≥ 500 μm were excluded from further analysis. After normalisation, the vessels conducted in duplicate with the Declaration of Helsinki. After consent was obtained, normotension confirmed and comorbidities ruled-out during a preliminary screening visit, young (19–35 years old) participants recruited among MVLS students attended a morning visit (starting at 8.00–9.30) after fasting from midnight. For female participants, the date of the main visit was arranged in the menstrual cycle phase of their spontaneous cycle (SNP; 0.1 μmol/L), following pre-constriction with the U64619 dose that produced 75% of the maximal contractile response. Other vessels from NT-treated animals were incubated for 5 h in PSS or in NaCl-supplemented-PSS (+15 mM NaCl, HT) to test the impact of environmental hypertonicity on vascular function with an ex vivo approach. Duration and toxicity of the incubations were based on previously reported tissue changes with HS diet and effects of HT culture conditions on rat VSMCs hypertrophy.

**Human hypertensive subjects.** The protocol for the cross-sectional SALT (Skin Sodium Accumulation and water balance in hypertension) study was approved by the West of Scotland Research Ethics Committee 3 (ref. 18/WS/0238) and Greater Glasgow and Clyde NHS Research and Development (ref. GN18CA634). The study was conducted in compliance with the Declaration of Helsinki. Adult, non-pregnant patients were recruited from the High BP clinic, Queen Elizabeth University Hospital Glasgow, between March and June 2019. On the day of their scheduled clinic appointment (9.00 a.m. to 4.30 p.m.), non-pregnant patients willing to take part gave written, informed consent and had anthropometric (body height and weight) and routine office BP measures taken as per current guidelines. PP was calculated as systolic BP minus diastolic BP. Relevant comorbidities and ongoing medications were recorded. On the same occasion we administered a short questionnaire to estimate sodium intake, we measured TEWL and we collected a skin biopsy, serum and EDTA-plasma (for p-Na, p-Urea and p-Creatinine and for NT-proBNP, respectively) and a random urine sample (for albuminuria).

**Questionnaire.** A short, validated questionnaire was administered to patients while waiting for their scheduled visit (Supplementary Note 2). Calculation was made as reported. Briefly, it included 42 food items with six possible consumption responses: never; one to three times per week; four to six times per week; once a day; twice a day; and three plus times a day. Predefined absolute amounts of sodium per serving size per specific item, according to MRC Food Composition Table, were multiplied by the consumption frequency factor that each individual reported, and summed up to a total weekly Na+ intake for each subject, later divided by seven to estimate daily intake. For simplicity, the absolute amounts of Na per serving for each food category were divided by 50 mg Na+ units and rounded to the nearest integer.

**Human skin samples analysis.** Frozen skin samples were macroscopically transversally cut into a superficial layer, including the epidermis and the immediately adjacent superficial dermis (ESD), and DD layer in a cold room (please see Supplementary Fig. 7). Tissue processing was identical to what described for rat tissues, with the exception of a five decimal (0.00001 g) scale (Ohaus, DV214CD) used for weight measurements due to small size of the samples. Water and Na+/K+ contents were measured as described for rat tissues. The few missing values secondary to technical issues or insufficient sample were excluded.

**Statistical.** All data were collected in Microsoft Excel spreadsheets. Statistical analysis was performed using Prism (GraphPad Software) and SPSS (IBM).

Categorical variables are presented as absolute numbers and percentages and compared by χ2 test. The effect of two factors (e.g., strain and salt load) on different quantitative response variables in the experimental groups was tested by two-way ANOVA. For predefined comparisons (e.g., the effect of HS vs. NS) we used Fisher least-significant-difference test (animals) or Student’s t test (humans) for normally distributed variables (presented as mean ± SD or, graphically, as mean [95% CI]) or Mann–Whitney test for non-normally distributed variables (presented as median [interquartile range] or, graphically, as median [95% CI]). For post hoc comparisons (e.g., between strains or sexes), labelled as such in the relative tables, Holm–Bonferroni multiple comparisons (e.g., between strains or sexes), labelled as such in the relative tables, Holm–Bonferroni multiple comparisons (e.g., between strains or sexes), labelled as such in the relative tables, Holm–Bonferroni multiple comparisons (e.g., between strains or sexes), labelled as such in the relative tables, Holm–Bonferroni multiple comparisons (e.g., between strains or sexes), labelled as such in the relative
figures. Regression curves were derived by least-square method and compared by
Extra sum-of-squares F test; for vascular function analysis, maximal contraction/ relaxation and (Log)EC50/IC50 were independently compared. Correlations were
ascertained by Pearson test, upon appropriate transformation of skewed variables
to attain normal distribution. Univariable and multivariable (including age, sex,
BMI and estimated Na intake) linear regression models were used and results were
presented as standardised B coefficients (95% CI). The α level was set at 0.05 and all
statistical tests were two tailed (\(p < 0.05\), \(*p < 0.01\), \(**p < 0.001\), \(***p < 0.0001\)).

**Reporting summary.** Further information on research design is available in the Nature
Research Reporting Summary linked to this article.

**Data availability**

The data authors declare that all data supporting the findings of this study are available
within the paper and its supplementary information. Source data are provided with this.

Received: 7 January 2020; Accepted: 17 July 2020;
Published online: 24 August 2020

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cells (95% CI). The **p** values of this study are available
within the paper. Source data are provided with this.

**Acknowledgements**

We gratefully acknowledge the support from Dr. Katrina Brooksbank (University of
Glasgow, UoG) for protocol optimisation and ethical/NHS R&D approvals; from Adam
Harvey for the rat studies; from Michael Beglan, Holly Yu and Prof. Samuel Jackson’s
team (UoG, Chemistry) for the chemical analysis of samples; from Jackie Thomson and
Eleanor Nethercot (UoG) for their technical assistance and processing of plasma and
urine samples; from Kayley Scott (UoG), Laura Haddow and John McAbney (MyoCORE
facility, UoG) for the animal protocols and microscopy experiments; from all the con-
sultants in the Glasgow High Blood Pressure Clinic (Queen Elizabeth University
Hospital) for the recruitment of participants. We are also grateful to Dr. Helen Taylor,
Environmental Services (UK distributors), Couragie & Khazaka products, for
facilitating our access to Tewl equipment and for her technical and scientific support.
None of the individuals/companies acknowledged above received compensation for their
contributions to this study. This work was supported by the British Heart Foundation (BHF)
Centre of Research Excellence Awards, RE/13/5/30177 and RE/18/6/34217 to R.M.T., C.D. and G.R.; the Academy of Medical Sciences-Newton International fellowship to S.M.; a Carnegie Trust Undergraduate Vacation Scholarship, VAC008890 to J.Y.C. and University of Glasgow Head of College Scholars’ List Scheme Summer Studentship Award 2017/18 to K.S.C. R.M.T. is funded through a BHF Chair award (CH/4/29762). A.C.M. is supported by a Walton Fellowship (University of Glasgow).

Author contributions
G.R. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design: G.R. and C.D.; experiment conduction and interpretation: G.R., S.M., J.Y.C., P.W., A.C.M., R.M.T. and C.D.; human study design, recruitment of participants samples/data collection: G.R., J.Y.C., K.S.C., R.M.T. and C.D.; performance, analysis and interpretation of myography data: G.R., K.B.N., R.L.A. and R.M.T.; analysis and interpretation of biochemical data: G.R., P.W. and C.D.; drafting of the paper: G.R. and J.Y.C.; critical revision of the paper for important intellectual content: G.R., S.M., J.Y.C., P.W., M.C.P., R.M.T. and C.D.; statistical analysis: G.R.; supervision: R.M.T., M.C.P. and C.D.; funding: G.R., S.M., J.Y.C., K.S.C., R.M.T. and C.D.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-17820-2.