Glucocorticoids affect metabolic but not muscle microvascular insulin sensitivity following high versus low salt intake

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**Background** Salt-sensitive hypertension is often accompanied by insulin resistance in obese individuals, but the underlying mechanisms are obscure. Microvascular function is known to affect both salt-sensitivity of blood pressure and metabolic insulin sensitivity. We hypothesized that excessive salt intake increases blood pressure and decreases insulin-mediated glucose disposal, at least in part by impairing insulin-mediated muscle microvascular recruitment (IMMR).

**Methods** In 20 lean and 20 abdominally obese individuals, we assessed mean arterial pressure (MAP; 24h ABPM), insulin-mediated whole body glucose disposal (M/I-value; hyperinsulinemic, euglycemic clamp technique), IMMR (contrast enhanced ultrasound), osmolyte and water balance, and excretion of mineralocorticoids, glucocorticoids, and amino and organic acids after a low and high salt diet during seven days in a randomized double-blind cross-over design.

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The authors have declared that no conflict of interest exists.
Abstract

Background Salt-sensitive hypertension is often accompanied by insulin resistance in obese individuals, but the underlying mechanisms are obscure. Microvascular function is known to affect both salt-sensitivity of blood pressure and metabolic insulin sensitivity. We hypothesized that excessive salt intake increases blood pressure and decreases insulin-mediated glucose disposal, at least in part by impairing insulin-mediated muscle microvascular recruitment (IMMR).

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Conclusion Our findings imply that hemodynamic and metabolic changes resulting from alterations in salt intake are not necessarily associated. Moreover, they are consistent with the concept that a high salt intake increases muscle glucose uptake as a response to high-salt-induced, glucocorticoid-drive muscle catabolism to stimulate urea production and thereby renal water conservation.

Clinical Trial Registration Number: NCT02068781
Introduction

In obesity, an increased susceptibility to the hypertensive effects of salt (‘salt sensitivity’) is often seen in parallel with impaired insulin-mediated glucose disposal (insulin resistance) (1-6). The exact underlying mechanisms for the association of salt-sensitive hypertension with insulin resistance in obese individuals have not been clarified, although several explanations have been proposed, including inappropriate activation of the renin-angiotensin-aldosterone and sympathetic nervous systems, sodium-induced elevation of circulating free fatty acids, and insulin-mediated sodium retention (2,7,8).

We and others (9-13) have proposed that impairment of microvascular function may contribute to the detrimental effects of salt on blood pressure and insulin sensitivity, particularly in obesity. First, if excess salt impairs microvascular dilatation, the resulting increase in peripheral resistance, other things being equal, will increase blood pressure. Indeed, (skin) capillary recruitment capacity during reactive hyperemia has been shown to be inversely associated with salt sensitivity of blood pressure in normotensive and hypertensive individuals (10). In addition, salt loading was found to impede skin postocclusive reactive hyperemia in healthy women (9). Conversely, a modest reduction in salt intake increased basal and maximal skin capillary density in mildly hypertensive individuals (11), while a larger decrease in sodium intake resulted in a higher bulbar conjunctival arteriolar density in essential hypertensive individuals, compared to controls (14). Second, microvascular dysfunction can impair insulin-stimulated glucose disposal. An important physiological function of insulin in muscle is to dilate arterioles and recruit capillaries, thus enhancing its own access and that of glucose to myocytes, and increasing muscle glucose uptake (15). In addition, these microvascular actions of insulin may affect blood pressure by reducing peripheral vascular resistance (16,17). As a consequence, impairment of insulin-mediated microvascular dilatation and capillary recruitment, as often observed in obese individuals, may hinder insulin-stimulated glucose disposal and increase peripheral vascular resistance, thereby contributing to the development of, and linking, insulin resistance and hypertension (12,13). However, it is not known whether excess salt intake impairs insulin’s microvascular effects.

We hypothesized that excess salt intake can impair insulin-mediated microvascular recruitment by interfering with nitric oxide (NO) availability, and thus contribute to salt-induced increases in blood pressure and decreases in insulin-mediated glucose disposal, especially in obesity.
Indeed, in lean rats, a high salt diet impaired both insulin-stimulated microvascular recruitment and glucose uptake in muscle (18), whereas in obese rats, salt restriction prevented the development of hypertension and insulin resistance (19). Data in humans, however, are lacking.

In view of these considerations, we studied, in lean and abdominally obese individuals, insulin-mediated muscle microvascular recruitment and its associations with 24h ambulatory blood pressure and whole-body insulin-mediated glucose disposal after salt loading and salt restriction. We expected blood pressure to decrease, and insulin’s metabolic and microvascular actions to improve on a low, compared to a high salt diet. To investigate underlying mechanisms of potential changes in blood pressure and metabolic and microvascular insulin sensitivity, we also assessed urinary excretion of osmolytes, water excretion and mineralo- and glucocorticoids. Because changes in water excretion might result from alterations in urea synthesis, which in turn could affect insulin-mediated glucose disposal via increased AMPK-levels due to an energy deficit in skeletal muscle (20-22), we measured amino acid and organic acid excretion as well.
Results

General characteristics (Figure 1 and Table 1)

Twenty-one lean and 26 abdominally obese individuals were randomized to start with either the low or the high salt intervention, and ultimately 20 lean and 20 abdominally obese individuals completed the study. Eleven lean and 10 abdominally obese participants used a low salt diet prior to the first set of measurements; the remaining participants started with a high salt diet. Insulin levels during the hyperinsulinemic clamp after a low salt diet were lacking in one lean participant due to hemolysis of blood samples; insulin-mediated muscle microvascular recruitment (IMMR) data after a high salt diet were unavailable in one abdominally obese individual for technical reasons. Lean, compared to abdominally obese, participants had significantly lower systolic and mean arterial pressure (MAP), expected creatinine excretion, and LDL cholesterol and triglyceride levels, while HDL cholesterol concentration was higher. The numbers of pre- and postmenopausal women were comparable in both groups. Urinary sodium excretion showed adequate compliance to both the low and the high salt diets.

Mean arterial pressure, M/I-value and IMMR on a low, as compared to a high salt diet in the total study population

On a low, as compared to a high salt diet, MAP was lower, M/I-value was lower and IMMR was greater (low vs. high salt: MAP 96±11 vs. 100±11 mmHg, p < 0.01; M/I-value: 8.8 [5.4 – 12.6] vs. 10.2 [6.1 – 14.5] ((mg/kg/min per mU/L)*100), p < 0.01; IMMR: 58 [23 – 71] vs. 17 [7 – 54]%, p = 0.03; Figure 2). Similar conclusions were reached after adjustment for group, age and sex (low vs. high salt: MAP: 96±10 vs. 100±11 mm Hg, p < 0.01; M/I-value: 8.8 [5.6 – 12.9] vs. 10.1 [7.0 – 15.2] ((mg/kg/min per mU/L)*100), p < 0.01; IMMR: 38 [32 – 60] vs. 19 [14 – 41]%, p = 0.03.

Changes in MAP, M/I-value and IMMR during low and high salt intake were not statistically significantly different between lean and abdominally obese individuals (p for interaction all > 0.26) and were not affected by pre- vs. postmenopausal status.
Mean arterial pressure, M/I-value and IMMR in lean vs. abdominally obese individuals on a low and high salt diet

In lean, as compared to abdominally obese individuals, and on both a low and a high salt diet, MAP was lower, M/I-value was higher, and IMMR was not statistically significantly different (Table 2).

Adjustment for age and sex gave comparable findings with regard to MAP and M/I-value, but IMMR was significantly greater in abdominally obese, compared to lean individuals, on a low salt diet (lean vs. abdominally obese: MAP: low salt 91±10 vs. 100±10 mm Hg, p = 0.01; high salt 97±11 vs. 103±11 mm Hg, p = 0.08; M/I-value: low salt 12.9 [11.0 – 14.4] vs. 5.9 [4.7 – 6.4] ((mg/kg/min per mU/L)*100), p < 0.01; high salt 15.2 [12.6 – 16.9] vs. 7.0 [5.6 – 8.0] ((mg/kg/min per mU/L)*100), p < 0.01; and IMMR: low salt: 34 [20 – 38] vs. 60 [41 – 69]%), p = 0.03; high salt: 19 [-1 – 19] vs. 41 [15 – 43]%, p = 0.22; Table 3).

The salt sensitivity index was comparable between lean and abdominally obese participants (lean: 5.8 [3.0 – 9.2]%, abdominally obese: 4.6 [-0.3 – 7.2]%, p = 0.124; difference lean vs. abdominally obese adjusted for age and sex: 2.8 (-0.9 to 6.5)%.

Carry-over effects were not detected in any of the above analyses (p-values all > 0.11).

Associations of Ln IMMR with MAP and M/I-value on a low and a high salt diet (Table 4)

On a low salt diet, Ln IMMR was not associated with MAP or Ln M/I-value in the total study population, either without or with adjustment for potential confounders (Table 4). Interaction analyses, however, showed a significant and independent inverse association of Ln IMMR with MAP in lean participants (crude: standardized β = -0.592 (-0.820 to -0.203), p = 0.006; age- and sex-adjusted: standardized β = -0.511 (-0.778 to -0.088, p = 0.013)), while there was no association in abdominally obese participants (crude: standardized β = 0.071 (-0.384 to 0.498), p = 0.767; age- and sex-adjusted: standardized β = 0.149 (-0.314 to 0.555), p = 0.535; p for interaction 0.084) (Table 4 and Figure 3).

On a high salt diet, Ln IMMR was not associated with MAP or Ln M/I-value in the study population as a whole (Table 4), or in lean and abdominally obese individuals separately (MAP: p for interaction = 0.986; Ln M/I-value: p for interaction = 0.831).
Urinary excretion of osmolytes, mineralo- and glucocorticoids, and amino and organic acids on a high and a low salt diet (Tables 5 and 6)

Copeptin levels on a high salt diet were lacking in one lean participant, due to a laboratory error.

On a high, as compared to a low salt diet, 24h urinary sodium and total osmolyte excretion increased (UNaV, 70±30 vs. 240±68 mmol/24h; p < 0.001; U(2Na2KUrea)V, 595±186 vs. 928±277 mmol/24h; p < 0.001), but urine volume was unchanged. Thus, free water clearance decreased (-14±1055 vs. -1287±1072 mL/24h; p < 0.001). Urea excretion and serum copeptin levels were comparable on a low and high salt diet.

On a high, as compared to a low salt diet, urinary aldosterone excretion decreased (8002 [6272–10561] vs. 2199 [1262–4073] ng/24h; p < 0.001), whereas urinary cortisol and cortisone excretion increased (cortisol, 16673 [12432–24829] vs. 16673 [12432–24829] ng/24h; p < 0.001; cortisone, 68666 [54969–80669] vs. 83444 [70035–104639] ng/24h; p = 0.019).

Results of the analyses above were not different between lean and abdominally obese individuals (p all > 0.107). Carry-over effects were not detected (p-values all > 0.08).

As a reduced free water clearance and higher glucocorticoid levels on a high salt diet might reflect increased urea generation for renal medullary water reabsorption (20), we also measured amino acid and organic acid excretion (Table 6). On a high, as compared to a low salt diet, there were increases in the excretion of threonine, methionine, alanine and tyrosine, which serve as nitrogen donors for ureagenesis; of ornithine, which is an early reactant in the urea cycle and a by-product of urea synthesis; and of citrulline, which is an early product in the urea cycle. Urinary excretion of other by-products of urea generation, i.e. glutamine and proline, also increased on a high salt diet. Excretion of serine (another nitrogen donor), glutamic acid (as a measure of glutamate, the amino source for urea synthesis), and of late reactants and products (aspartic acid (as a measure of aspartate), argininosuccinate, arginine and fumarate) was not statistically significantly different between the low and high salt diets. Pyruvate excretion, which is used to generate alanine (20,23) was also unchanged.
It has been suggested that a high salt diet induces ketogenesis to reprioritize energy expenditure in favor of urea production (20). However, both on a low and a high salt diet, ketone bodies were only demonstrable in a few participants, and on a high salt diet, ketone body excretion was not significantly increased.

We and others hypothesized that salt-induced glucocorticoid synthesis might induce a catabolic state, and, consequently, an energy deficit in skeletal muscle (20), resulting in increased insulin-mediated glucose disposal (21,22). Indeed, cortisol excretion was directly associated with M/I-value on a high salt diet (crude: standardized β = 0.416 (0.120 to 0.644), p = 0.008; group-, age- and sex-adjusted: standardized β = 0.256 (-0.060 to 0.523), p = 0.051; Figure 4). This association was similar in lean and abdominally obese individuals (p for interaction 0.384). Cortisol excretion was not associated with M/I-value on a low salt diet in the study population as a whole (crude: standardized β = 0.088 (-0.234 to 0.393), p = 0.592; group-, age- and sex-adjusted: standardized β = 0.035 (-0.284 to 0.347), p = 0.770 (Figure 4), or in lean and abdominally obese individuals separately (p for interaction 0.270), and was not associated with MAP or IMMR on either diet (data not shown).
The present study demonstrates that on a low, compared to a high salt diet, blood pressure decreases, whole-body insulin-mediated glucose disposal decreases, and microvascular insulin sensitivity increases in both lean and abdominally obese individuals. In addition, greater insulin-mediated muscle microvascular recruitment is associated with lower mean arterial pressure on a low salt diet in lean, but not in abdominally obese participants, and is not associated with whole-body insulin-mediated glucose disposal on either a low or a high salt diet.

A major finding is the improvement of insulin-mediated muscle microvascular recruitment in humans following salt restriction, similar to earlier observations in rats (18), and in line with previous observations of improved microvascular function and structure in normotensive and hypertensive humans in other vascular beds (skin, conjunctiva) and in response to other stimuli (post-occlusive reactive hyperemia, venous congestion) (9,11,24). Increased salt intake may interfere with insulin-mediated endothelial NO production and thus insulin-stimulated microvascular dilatation at several levels, notably by reducing eNOS protein expression and activation, by accelerating NO degradation through superoxide generation by NADH oxidase and eNOS when tetrahydrobiopterin availability is reduced, and by inducing superoxide dismutase deficiency, which further increases oxidative stress (25). It logically follows that reducing salt intake improves microvascular insulin signaling via opposite mechanisms. Whether basal (as opposed to insulin-stimulated) muscle microvascular perfusion is also diminished after a high salt diet cannot be derived from our data, as the ultrasound method we used has a large inter-individual variation under basal circumstances (26,27). However, it is likely that functional muscle microvascular density under basal circumstances is also diminished after a high salt diet, given the fact that an elevation in blood pressure occurring during high salt intake is eventually the result of increased peripheral resistance (28,29), which is determined largely at the microvascular level (12). The rise in (muscle) microvascular resistance can be ascribed to failure of the microvasculature to dilate in response to an initial expansion of the cardiac output induced by increasing salt intake, and/or to direct microvascular actions of salt (25,28).

After a low salt diet, IMMR was greater in abdominally obese than in lean participants, while after a high salt diet IMMR was more or less equally diminished, and of similar magnitude, in lean and abdominally obese individuals.
In a previous study, we have demonstrated impaired IMMR in obese, compared to lean men under ad-libitum salt intake (26). As the net effect of variation in salt intake on IMMR will be determined by changes in both basal functional muscle microvascular density and microvascular insulin signaling, the discrepancy in responses of IMMR to low, ad-libitum and high salt diets between lean and abdominally obese individuals may be related to differences in the relative contributions of both factors. Earlier observations by us and others indicate that under unspecified or ad libitum salt-intake, both functional muscle capillary density under basal circumstances and IMMR are diminished in (abdominally) obese, compared to lean individuals (26,30,31), due to increased levels of free fatty acids and inflammatory cytokines, and changes in adipokine signaling in obese individuals (12,32). An explanation for our current findings may be that also under low salt circumstances and through similar mechanisms, basal functional muscle microvascular density is diminished in abdominally obese versus lean individuals, while salt restriction improves microvascular insulin signaling in both lean and abdominally obese participants, thus resulting in greater IMMR in the abdominally obese participants. Vice versa, the intrinsic capacity of the muscle microvasculature to dilate in response to salt loading might be greater in lean than abdominally obese individuals. Therefore, deterioration of microvascular insulin sensitivity, which is already impaired under ad-libitum salt intake in the abdominally obese individuals, will ultimately lead to a comparable IMMR after a high salt diet in both groups (Figure 5).

Contrary to expectation, insulin-mediated whole-body glucose disposal decreased after seven days of low, compared to seven days of high salt intake. Controlled experiments in healthy and Dahl salt-sensitive animals have demonstrated that salt loading impairs insulin-mediated glucose disposal (3,33-35). Similar findings were obtained using other measures of insulin sensitivity in hypertensive and obese rats (36,37), while salt restriction has been shown to improve the HOMA index (which reflects both hepatic and muscle insulin sensitivity) in obese rats (19). However, controlled experiments in humans are limited. Nevertheless, there are several reports of decreased insulin-mediated glucose disposal, as assessed by the hyperinsulinemic, euglycemic clamp technique, in healthy (38,39), hypertensive (40), and hypertension-prone (41) individuals after both moderate and more extreme salt restriction. Comparable results have been acquired with the HOMA index (42,43).
We speculate that increased insulin-mediated glucose disposal on a high salt diet may be explained by salt-induced, glucocorticoid-driven muscle catabolism to increase urea production and thereby renal water conservation (20,44). In skeletal muscle of mice fed a high salt diet, AMPK levels increased (20), which in turn may promote whole-body glucose disposal (21,22,45,46). Indeed, we observed that on a high, compared to a low salt diet, free water clearance was reduced, which occurred independently of changes in ADH (anti-diuretic hormone) secretion (as reflected by similar copeptin levels on the high and low salt diets), and glucocorticoid excretion increased. In addition, urinary excretion of amino acids serving as nitrogen donors for urea synthesis, and of early reactants and (by-)products in the urea cycle were higher on a high vs. a low salt diet, suggesting increased urea production. Excretion of later products and reactants in the urea cycle, i.e. of products of reactions involving argininosuccinate synthetase, which is the rate limiting enzyme in urea synthesis (47), and of reactants and products after this part of the cycle (23) was not statistically significantly increased on a high salt diet. It is likely that measurement of urinary excretion is a relatively insensitive method of detecting changes in amino and organic acid fluxes, particularly in the later part of the urea cycle, which have been demonstrated previously directly in murine muscle and liver (20). Although a higher salt intake during at least 4 weeks increased plasma urea concentration in mice, and decreased urinary urea excretion in both mice and healthy men (20,44) we did not observe differences in serum urea levels or urinary urea excretion between the low and high salt diets. However, noticeable changes in circulating or urine urea levels might only occur after several weeks, as urea has been shown to be actively transported to and accumulate in the renal medulla to increase water reabsorption on a high salt diet (20), and it may take some time before a steady state has been reached. Cortisol excretion was directly associated with insulin-mediated glucose disposal on a high salt diet, but not on a low salt diet, which we interpret as consistent with the concept that, under circumstances of high salt intake, greater insulin-mediated glucose disposal is a compensating mechanism for the glucocorticoid-induced energy deficit in skeletal muscle.

To the best of our knowledge, this is the first study investigating the association of IMMR with whole-body insulin-mediated glucose disposal on a low versus a high salt diet. At ‘usual’ or nonspecified salt intake, IMMR has been demonstrated to be a direct determinant of whole-body insulin-mediated glucose disposal (26,30,48), but this seems not to be the case at a very low or a very high salt intake (Figure 5).
Dissociated effects of low and high salt diets on vascular and metabolic insulin signaling have been reported previously in rats and healthy humans, although in these studies, insulin-induced vasodilatation was assessed in larger vessels (39, 49, 50).

Thus, impairment of microvascular function on a high salt diet is not sufficient to impair insulin-mediated glucose disposal, possibly because the energy deficit in skeletal muscle, caused by salt-induced glucocorticoid synthesis, is a greater stimulus for (insulin-mediated) muscle glucose uptake. Although higher AMPK levels resulting from glucocorticoid-induced muscle catabolism have also been demonstrated to promote microvascular insulin signaling in animals and humans (51-53), this effect is presumably offset by direct interference of salt with (insulin-mediated) vasodilatation.

As expected, and in agreement with previous studies performed in normotensive, hypertensive and obese individuals, salt reduction lowered blood pressure in lean and abdominally obese participants (54, 55), although to a similar extent in both groups, indicating a comparable degree of salt sensitivity. Previous investigations have shown greater salt sensitivity in obese, compared to lean Zucker rats (56) and human adolescents (6), and in Chinese non-diabetic individuals with versus without the metabolic syndrome (5). These seemingly contradictory findings might be explained by differences in degree of obesity and ethnicity between the current and other study populations, as the mean BMI in the obese adolescent population was 33.6 kg/m\(^2\), vs. 31.3 kg/m\(^2\) in our abdominally obese population, and Asian individuals tend to be more salt-sensitive (57) and generally consume diets with a higher salt content than Caucasian individuals (58-60).

Although a low salt diet reduced blood pressure and improved insulin-mediated muscle microvascular recruitment in both lean and abdominally obese individuals, a higher IMMR was associated with lower blood pressure under low salt circumstances in the lean individuals only, which may be ascribed to a contribution of insulin’s microvascular actions to decreased peripheral vascular resistance (17). In the abdominally obese participants, mean arterial pressure was higher than in the lean participants after a low salt diet, probably due to interaction of several factors, including overactivity of the renin-angiotensin-aldosterone and sympathetic nervous systems, and physical compression of the kidneys (61), and these might overrule the contribution of an improvement in IMMR following salt restriction to blood pressure regulation.
A limitation of the present study is the fact that insulin-mediated muscle microvascular recruitment and whole-body insulin-induced glucose disposal were not measured under ad-libitum salt ingestion, which is ~ 140 mmol per day in the Netherlands (59).

In addition, the underlying mechanisms of the improvement in microvascular insulin signaling, which was presumed to involve enhanced NO availability, were not identified in the current investigation. Lastly, water intake was not registered, which would have allowed estimation of water balance. An important strength of this study is its randomized, placebo-controlled, blinded design, with a wash-out period between the low and high salt diets. In addition, we assessed insulin-mediated microvascular function directly in skeletal muscle, which is the main site of peripheral glucose uptake, and we used the gold standard for the determination of metabolic insulin sensitivity.

In conclusion, a low, as compared to a high salt diet during seven days reduces blood pressure, impairs insulin-mediated glucose disposal but improves insulin mediated-muscle microvascular recruitment in both lean and abdominally obese participants. In addition, the enhancement of IMMR was associated with decreased mean arterial pressure, but only in lean individuals. The higher insulin-mediated glucose disposal on a high salt diet may reflect an energy deficit in skeletal muscle caused by salt-induced glucocorticoid synthesis with the goal of increasing urea production and thereby renal water conservation.

An important question is whether the observed increase in insulin-mediated glucose disposal on a high salt diet should be regarded as a beneficial effect of salt, or merely a compensating mechanism. This could be investigated by exposing individuals to a high salt diet with and without increasing water intake and comparing insulin-mediated glucose disposal between these conditions. In addition, the mechanisms underlying the improvement of insulin-mediated muscle microvascular recruitment on a low salt diet require further elucidation. Nevertheless, our findings indicate that determinants of insulin-mediated glucose disposal are dynamic, i.e. are affected by salt status. Moreover, hemodynamic benefits of reductions in salt intake are not necessarily paralleled by metabolic advantages.
Materials and Methods

Study population

Lean and abdominally obese individuals were recruited at the Maastricht University Medical Center, Maastricht, the Netherlands, between September 2014 and August 2016 via advertisements in local newspapers and among participants in previous investigations. A sample size of 20 individuals per group was calculated to be sufficient for detecting a mean difference of 5 mm Hg in MAP, and of 1 ((mg/kg/min per mU/L) * 100) in M/I-value between the low and high salt diets with a power (1 – β) of 0.80 and α = 0.95, and a mean difference of 7 mm Hg in MAP and 4 ((mg/kg/min per mU/L) * 100) in M/I-value between lean and abdominally obese individuals with the same power and α. Although data on relevant differences in and variation of IMMR were limited, we also expected this sample size to be large enough to demonstrate a difference in IMMR, as previous investigators have observed an average difference in IMMR of 40% between 10 lean and 11 abdominally obese participants (30). Thus, we aimed at 20 lean and 20 abdominally obese Caucasian individuals to complete this randomized double-blind cross-over trial with masked analyses. Participants were 18-65 years of age, non-smoking, nondiabetic and free of cardiovascular disease, and had a waist circumference below 80 cm (lean women)/94 cm (lean men) or above 88 cm (abdominally obese women)/102 cm (abdominally obese men). Exclusion criteria were fasting plasma glucose > 6.1 mmol/L, office blood pressure > 180/110 mmHg, unstable or severe pulmonary or thyroid disease, a recent history of malignancy, inflammatory diseases, impairment of renal or hepatic function, pregnancy or lactation, and use of glucose-lowering medication, nonsteroidal anti-inflammatory drugs or corticosteroids. Four abdominally obese participants were taking antihypertensive medication at the time of inclusion (calcium channel blocker: n=1; angiotensin receptor blocker in combination with a thiazide diuretic: n=1; angiotensin converting enzyme (ACE) inhibitor combined with a β-blocker: n=1; ACE-inhibitor combined with a thiazide-like diuretic; n=1). Antihypertensives were discontinued three weeks before measurements; statin use was not interrupted (n=1 (abdominally obese man)).

Women on oral contraceptives were instructed to continue using them throughout the study period (n=2 (abdominally obese women)).
Measurements were performed in either the follicular or the luteal phase of the menstrual cycle, if applicable, with the exception of two lean women (in one, the first study day took place in the follicular phase and the second in the luteal phase; in the other, vice versa). Data on the menstrual cycle phase were unavailable in 3 lean and 2 abdominally obese women, due to the presence of a hormonal IUD without bleedings (n=4) or a very irregular cycle (n=1).

Study design and general procedures

Prior to the first and second sets of measurements, participants adhered to a diet aimed at either a high (250 mmol NaCl/24h) or a low (50 mmol NaCl/24h) salt intake for seven days in randomized order in a 1:1 ratio, with a washout period of 14 days. Randomization was performed by an independent investigator using block randomization with variable block sizes. Every individual participant was provided with a personalized diet by a dietician, which was used during both the low and high salt phases. This diet contained 50 mmol NaCl and 70-80 mmol K+ per day, and the same daily amount of calories that he or she ingested before the intervention started (and in the wash-out period). It was supplemented with sodium capsules in the high salt week (9 per day, containing 1.3 g (22.2 mmol) NaCl per capsule (BasicPharma, Geleen, The Netherlands)), and with matched placebo capsules (BasicPharma, Geleen, The Netherlands) in the same amount in the low salt week, to reach a salt intake of 14.6 and 2.9 g salt/day, respectively. Thus, the energy content and energy sources, although different for every individual, were kept constant throughout the intervention periods. To prevent side effects, capsules with delayed release properties were used (DRcaps, Capsugel; Morristown, New Jersey, USA). The containers with capsules were labeled in accordance with the randomization numbers and handed over to the participants by a member of the research team; both were unaware of the treatment allocation. Study data were deblinded only upon completion of all analyses by an independent investigator.

On the seventh day of both the low salt and high salt weeks, 24h urine was collected for assessment of sodium, potassium and creatinine excretion, and 24h ambulatory blood pressure measurements (ABPM) were performed (Mobilograph (New Generation), I.E.M., Stolberg, Germany) at the non-dominant arm with appropriately sized cuffs at 15-min intervals from 8 a.m. to 11 p.m. and at 30-min intervals from 11 p.m. to 8 a.m.
Mean arterial blood pressure values collected with ambulatory blood pressure monitoring during the low- and high-salt diets were used to compute the salt sensitivity index (SSI). The SSI is the difference in MAP between the low and high salt diet divided by MAP during the low salt diet (62).

Assessments of whole-body insulin-stimulated glucose disposal and insulin-mediated muscle microvascular recruitment were conducted in a temperature-controlled room (T = 24°C ± 0.5°C) after a 12-hour overnight fast with participants in the supine position. Individuals were instructed to refrain from alcohol and meals rich in lipids for a period of 24 hours prior to each study day, and strenuous physical exercise for a period of 48 hours prior to each study day. After insertion of two intravenous catheters and a 30-minute acclimatization period with the participants in the supine position, we took blood samples for determination of glucose and creatinine levels.

Assessment of whole-body insulin-mediated glucose disposal

We determined metabolic insulin sensitivity by means of a modified version of the hyperinsulinemic, euglycemic clamp technique as described by DeFronzo et al. (63). Briefly, insulin (Insuman Rapid, Sanofi, Paris, France) was administered in a primed continuous manner at a rate of 1 mU/kg/min during 180 minutes. Isoglycemia was maintained by adjusting the rate of a 20% D-glucose infusion based on plasma glucose measurements performed at 5-minute intervals. Whole-body glucose disposal (M-value) was estimated from the steady-state glucose infusion rate between 90 and 150 minutes after initiation of insulin administration. M was expressed per kilogram body weight per unit of plasma insulin concentration (M/I-value), thus correcting for variation in steady-state insulin concentrations. For convenience, the M/I ratio was multiplied by 100.

Assessment of insulin-mediated muscle microvascular recruitment

Insulin-mediated muscle microvascular recruitment was assessed with contrast enhanced ultrasound as described previously (26). Briefly, microvascular blood volume (MBV) of forearm skeletal muscle was measured before and during hyperinsulinemia with a Toshiba Aplio XG ultrasound system (Toshiba, Otawara, Japan) during continuous i.v. administration of sulfur hexafluoride gas-filled microbubbles (SonoVue, Bracco diagnostics, Amsterdam, The Netherlands).
After steady state microbubble concentration was achieved (3 minutes), five real-time replenishment
curves of 30 seconds were acquired. These replenishment curves were stored and analyzed offline in a
blinded fashion after completion of the trial using the CHI-Q software (Toshiba, Otawara, Japan).
The replenishment curves were fitted to the exponential function \( y = A(1 - e^{-\beta t}) \) where \( t \) is time since
high mechanical index pulse, \( y \) is the video intensity at any given \( t \), \( A \) is the plateau video intensity
(representing MBV), and \( \beta \) is the microvascular flow velocity. Insulin-mediated muscle microvascular
recruitment (IMMR) was calculated as the relative increase in muscle microvascular blood volume during
hyperinsulinemia.

Blood and urine measurements
Plasma glucose was determined with a YSI2300 glucose analyzer (YSI, Yellow Springs, OH, USA). Blood
samples were analyzed for total cholesterol, HDL cholesterol and triglycerides (enzymatic colourimetric
method; Roche Diagnostics, Mannheim, Germany). LDL cholesterol was calculated with the Friedewald
formula (64). Sodium and potassium in serum and urine were determined with the ion-selective electrode
(ISE) method (Roche Diagnostics, Mannheim, Germany); creatinine and urea in serum and urine were
measured with enzymatic assays (Roche Diagnostics, Mannheim, Germany). Estimated glomerular
filtration rate (eGFR) was calculated using the CKD Epidemiology Collaboration equation (65). Expected
creatinine excretion was computed as \( 879.89 + 12.51 \times \text{weight (kg)} - 6.19 \times \text{age} - 379.42 \) if female,
as proposed by Ix et al. (66), and the creatinine index, i.e. the ratio of observed vs. expected 24h urinary
creatinine excretion, was used to assess the completeness of the 24h urine collection (67). Serum insulin
levels before and during the hyperinsulinemic clamp were measured with a sandwich immunoassay
(MSD, Rockville, MD, USA; intra-assay CV = 4.2%, inter-assay CV = 5.4%).
Copeptin concentration was determined with an automated immunofluorescent assay (B•R•A•H•M•S,
Hennigsdorf, Germany; intra-assay CV < 15%, inter-assay CV < 18%). Osmolality in serum and urine was
determined by assessment of freezing point depression. Free water clearance was calculated as: \( \text{urine volume} \times (1 - \text{urine osmolality/plasma osmolality}) \).
Urinary aldosterone, cortisol and cortisone excretion were measured by Supported Liquid Extraction (SLE) followed by LC tandem MS detection (LC-MS/MS) (intra-assay CV: aldosterone, 6%; cortisol, 5%; cortisone, 7%; inter-assay CV: aldosterone, 7%; cortisol, 6%; cortisone, 8%).

To assess amino acid excretion, urine samples were diluted to a creatinine concentration of ~1 mmol/l.

For analysis, 10 µl urine sample was diluted in 1500 µl of 0.5 mM tridecafluorohexanoic acid in ultrapure water (buffer A). Five µl of the diluted sample was analysed. Quantitation was performed using ultra performance high pressure liquid chromatography – tandem mass spectrometry on a configuration of an Acquity UHPLC and a Micromass Quattro Premier XE Tandem Mass Spectrometer (Waters, Milford, MA) equipped with an Acquity UHPLC BEH C18, 1.7 µm, 2.1 x 100 mm column. The mobile phase consisted of buffer A and 0.5 mM tridecafluoroheptanoic acid in acetonitrile. Mass spectrometry was performed in multiple reaction monitoring mode using electrospray ionisation in positive mode. Concentrations were determined using stable isotope-labelled internal standards and external calibration curves. For full details see (68). For the measurement of organic acid excretion, urine samples were diluted to a creatinine concentration of ~1 mmol/l. 25 µl of diluted urine sample was mixed with 25 µl of an internal standard mixture and 350 µl 0.1% v/v formic acid in ultra-pure water analysed using liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF/MS) on an LC-QTOF/MS configuration (Agilent Technologies, Amstelveen, the Netherlands) consisting of an Infinity II1290 UHPLC coupled to a 6550 iFunnel QTOF equipped with an electrospray ionization source. LC-separation was done on an Acquity C18 column UPLC HSS T3 1.8 µm 2.1 x 100 mm with Acquity VanGuard PreColumn UPLC HSS T3; 2.1 x 5 mm (Waters, Manchester, UK). The mobile phase buffers were 0.1% v/v formic acid in water and 0.1% formic acid in 95% acetonitril/5% water v/v. The mass spectrometer was tuned for low mass range (≤ 1700 m/z) at high resolution slicer mode + 2G Hz extended dynamic range, and run in the negative mode for full scan. Concentrations were determined using stable isotope-labelled internal standards and external calibration curves. For full details see (69).
Normally distributed variables were expressed as mean ± SD; variables with a skewed distribution were displayed as median and interquartile range, and natural logarithmic transformation was performed before further analyses where appropriate (triglycerides, HDL cholesterol, insulin, HOMA, M/I-value, IMMR, copeptin, aldosterone, cortisol, cortisone). Because IMMR results were partially negative, natural logarithmic transformation could only be performed after adding 40 to each value (lowest value, –38).

We used two-tailed independent samples T-tests to compare general characteristics between lean and abdominally obese individuals and two-tailed paired samples T-tests to compare 24h ambulatory blood pressure, M/I-value and IMMR between the low and high salt diets in the study population as a whole. Next, we used repeated measures ANCOVA to adjust these comparisons for group (lean or abdominally obese), age and sex. We then compared 24h ambulatory blood pressure, M/I-value and IMMR on the low and high salt diets between lean and abdominally obese individuals with two-tailed independent sample T-tests, followed by repeated measures ANCOVA with adjustment for age and sex. We performed interaction analyses (group * low or high salt condition) to investigate whether effects of low and high salt conditions were different between lean and abdominally obese individuals. Where appropriate, stratified analyses are presented. To establish whether IMMR was a potential determinant of MAP and M/I-value under low and/or high salt conditions, we used multiple linear regression analysis with IMMR as independent variable and MAP or M/I-value as dependent variables, adjusted for group, age and sex; we performed interaction analyses to study whether these associations differed between lean and abdominally obese individuals. We compared osmolyte excretion, copeptin levels, free water clearance, and mineralocorticoid and glucocorticoid excretion between the low and high salt diets with repeated measures ANCOVA with adjustment for group, age and sex, and we performed interaction analyses to establish whether results differed between lean and abdominally obese individuals. We analyzed differences in amino acid and organic acid excretion between the low and high salt conditions with two-tailed paired-sample T tests or Wilcoxon signed-rank tests, where appropriate. Lastly, to investigate whether cortisol was a potential determinant of M/I-value, IMMR and MAP on a low and/or high salt diet, we used multiple linear regression analysis with cortisol as independent variable and M/I-value, IMMR or MAP as dependent variables, adjusted for group, age, and sex.
We also carried out interaction analyses to determine whether associations were different between lean and abdominally obese individuals. Analyses were performed using the SPSS statistical software package (IBM SPSS Statistics version 20, Chicago, IL). Two-tailed p-values of < 0.05 and < 0.10 were considered statistically significant in the main and interaction analyses, respectively.
All participants gave written informed consent. The study was approved by the local ethics committee, performed in accordance with the Declaration of Helsinki, and registered at clinicaltrials.gov (NCT02068781).

**Author contributions**

MTJS, YHAMK, AJMH, PWdL, and CDAS designed the study. MTJS, HEN, JotR, JLJMS and MPvdW performed the measurements included in this article, and MTJS and YHAMK performed the analyses. MTJS and CDAS wrote the manuscript. All authors critically read and commented on the manuscript.

**Acknowledgements**

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Clinical Trial Registration Number: NCT02068781
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Figure 1: Enrollment, randomization and drop-out of participants

Enrollment

Assessed for eligibility (n=134)

Randomized (n=47; 21 lean, 26 obese)

Excluded (n=65)
- Not meeting inclusion criteria (n=41)
- Declined to participate (n=22)
- Other reasons (n=2)

WEEK 1

Allocated to low salt intervention first (n=35; 14 lean, 14 obese)
- Received allocated intervention (n=25)
- Did not receive allocated intervention (n=10)
- Drop-out (n=2; 2 failure to insert intravenous catheters, 1 illness, 1 discontinued after week 1)

Allocated to high salt intervention first (n=22; 10 lean, 12 obese)
- Received allocated intervention (n=22)
- Did not receive allocated intervention (n=0)
- Drop-out (n=0; 1 failure to insert intravenous catheters, 1 non-compliance)

Lost to follow-up (n=6)
- Discontinued intervention (n=0)
- Drop-out (n=1; failure to insert intravenous catheters)

WEEK 4

Analysed (n=20; 10 lean, 10 obese)
- Excluded from analysis (n=0)

Analysed (n=20; 10 lean, 10 obese)
- Excluded from analysis (n=0)
Figure 2: Mean arterial pressure, M/I-value, and insulin-mediated muscle microvascular recruitment (IMMR) on a low, as compared to a high salt diet in the total study population.
Data are presented as median (black line), 1st and 3rd quartiles (box edges), and minimum and maximum (whiskers).

Data were analyzed with repeated measures ANCOVA, adjusted for group, age and sex.

A: Mean arterial pressure; low salt: n=40 (20 lean and 20 abdominally obese individuals) vs. high salt: n=40 (20 lean and 20 abdominally obese individuals)

B: M/I-value; low salt: n=39 (19 lean (no insulin levels available in 1 participant) and 20 abdominally obese individuals) vs. high salt: n=40 (20 lean and 20 abdominally obese individuals)

C: Insulin-mediated muscle microvascular recruitment (IMMR); low salt: n=40 (20 lean and 20 abdominally obese individuals) vs. high salt: n=39 (20 lean and 19 abdominally obese individuals (IMMR data unavailable in 1 participant))

Mean arterial pressure was assessed with 24h ABPM, M/I-value with a hyperinsulinemic euglycemic clamp, and IMMR with contrast-enhanced ultrasound before and during hyperinsulinemia, on both a low (50 mmol/24h) and high (250 mmol/24h) salt diet during 7 days in randomized order.
Figure 3: Association of Ln IMMR with mean arterial pressure on a low salt diet in lean and abdominally obese individuals

Standardized regression coefficients ($s\beta$; derived from multiple linear regression analyses) are adjusted for age and sex; $p$ for interaction lean vs. abdominally obese = 0.084.

Lean (○): n=20; abdominally obese (●): n=20

Mean arterial pressure was assessed with 24h ABPM, and IMMR (=insulin-mediated muscle microvascular recruitment) with contrast-enhanced ultrasound before and during hyperinsulinemia, on a low salt diet (50 mmol/24h) during 7 days.
Figure 4: Association of Ln urine cortisol with Ln M/I-value on a low and a high salt diet

Standardized regression coefficients (sβ; derived from multiple linear regression analyses) are adjusted for group (lean/obese), age and sex.

Low salt (●): n=39; high salt (○): n=40

Urinary cortisol excretion was measured by Supported Liquid Extraction (SLE+) followed by LC tandem MS detection (LC-MS/MS), and M/I-value was assessed with a hyperinsulinemic euglycemic clamp, on both a low (50 mmol/24h) and high (250 mmol/24h) salt diet during 7 days in randomized order.

Low salt: sβ = 0.035, p = 0.77
High salt: sβ = 0.256, p = 0.05
Figure 5: Schematic proposal of basal functional muscle microvascular density, insulin-mediated muscle microvascular recruitment (IMMR; i.e. microvascular insulin sensitivity) and the association of IMMR with M/I-value (i.e. metabolic insulin sensitivity) during low, ad-libitum and high salt intake in lean compared to abdominally obese individuals.

The continuous line represents the insulin-mediated increase of muscle microvascular density, relative to basal density (represented with the interrupted line).

* The continuous line represents the insulin-mediated increase of muscle microvascular density, relative to basal density (represented with the interrupted line).
Table 1: General characteristics of the lean and abdominally obese participants

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>Abdominally obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Sex (n of men/women)</td>
<td>7/13</td>
<td>6/14</td>
</tr>
<tr>
<td>Age (years)</td>
<td>49±10</td>
<td>50±11</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.5±2.0</td>
<td>31.3±3.9</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>84±5</td>
<td>114±8</td>
</tr>
<tr>
<td>Women</td>
<td>74±4</td>
<td>101±12</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/L)</td>
<td>5.11a±0.47</td>
<td>5.31±0.42</td>
</tr>
<tr>
<td>Office SBP/DBP (mmHg)</td>
<td>119a±15/74±12</td>
<td>130±17/81±9</td>
</tr>
<tr>
<td>Use of antihypertensive medication (n)</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Use of statins (n)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Hormonal status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal (n)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Postmenopausal (n)</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Use of oral contraceptives (n)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Hormonal IUD (n)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td>86±12</td>
<td>82±13</td>
</tr>
<tr>
<td>Expected creatinine excretion (mmol/24h)</td>
<td>10.3±2.6</td>
<td>13.0±2.6</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.1±1.0</td>
<td>5.4±1.0</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.67a [0.59 – 0.94]</td>
<td>1.04 [0.93 – 1.54]</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.92a [1.75 – 2.33]</td>
<td>1.55 [1.32 – 1.77]</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.90a±0.87</td>
<td>3.62±0.96</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD or medians [interquartile ranges].

General characteristics between lean and abdominally obese individuals were compared with two-tailed independent sample T-tests.

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, IUD: intrauterine device

* Lean vs. abdominally obese, p < 0.05
Table 2: Blood pressure, insulin-mediated whole-body glucose disposal and insulin-mediated muscle microvascular recruitment on a low and a high salt intake in lean and abdominally obese individuals

<table>
<thead>
<tr>
<th></th>
<th>Lean (n=20)</th>
<th>Abdominally obese (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low salt</td>
<td>High salt</td>
</tr>
<tr>
<td>24h SBP/DBP (mmHg)</td>
<td>113±8/73±9</td>
<td>120±8/77±7</td>
</tr>
<tr>
<td></td>
<td>125±15/79±11</td>
<td>130±17/81±12</td>
</tr>
<tr>
<td>24h MAP (mmHg)</td>
<td>92±8ab</td>
<td>97±7</td>
</tr>
<tr>
<td></td>
<td>100±12b</td>
<td>103±14</td>
</tr>
<tr>
<td>Salt sensitivity index (%)</td>
<td>5.8 [3.0 – 9.2]%</td>
<td>4.6 [-0.3 – 7.2]%</td>
</tr>
<tr>
<td>24h heart rate (bpm)</td>
<td>66±8</td>
<td>66±8</td>
</tr>
<tr>
<td></td>
<td>69±7</td>
<td>67±8</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/L)</td>
<td>4.88±0.33a</td>
<td>4.70±0.31</td>
</tr>
<tr>
<td></td>
<td>5.06±0.44b</td>
<td>4.93±0.43</td>
</tr>
<tr>
<td>Fasting serum insulin (mU/L)</td>
<td>2.26 [1.67 – 2.65]c</td>
<td>1.92 [1.29 – 2.90]d</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.48 [0.37 – 0.60]c</td>
<td>0.39 [0.24 – 0.60]d</td>
</tr>
<tr>
<td></td>
<td>1.23 [0.89 – 1.57]</td>
<td>1.07 [0.75 – 1.24]</td>
</tr>
<tr>
<td>M-value (mg/kg/min)</td>
<td>7.5±3.1c</td>
<td>7.8±3.7d</td>
</tr>
<tr>
<td></td>
<td>4.4±1.5</td>
<td>4.3±0.96</td>
</tr>
<tr>
<td>M/I-value ((mg/kg/min per mU/L) * 100)</td>
<td>11.4 [10.1 – 17.7]abc</td>
<td>13.9 [10.7 – 18.2]d</td>
</tr>
<tr>
<td></td>
<td>5.5 [4.7 – 7.8]</td>
<td>6.3 [4.7 – 9.8]</td>
</tr>
<tr>
<td>IMMR (%)</td>
<td>45 [13 – 64]</td>
<td>10 [2 – 54]</td>
</tr>
<tr>
<td></td>
<td>68 [28 – 74]</td>
<td>18 [14 – 71]</td>
</tr>
<tr>
<td>Urinary sodium excretion (mmol/24h)</td>
<td>67±32a</td>
<td>241±61</td>
</tr>
<tr>
<td></td>
<td>73±28b</td>
<td>239±76</td>
</tr>
<tr>
<td>Urinary potassium excretion (mmol/24h)</td>
<td>54±22</td>
<td>56±28</td>
</tr>
<tr>
<td></td>
<td>55±18</td>
<td>58±20</td>
</tr>
<tr>
<td>Urinary creatinine excretion (mmol/24h)</td>
<td>11.2±4.2</td>
<td>11.8±4.1</td>
</tr>
<tr>
<td></td>
<td>12.7±3.1</td>
<td>13.2±3.4</td>
</tr>
<tr>
<td>Creatinine index</td>
<td>1.08±0.24</td>
<td>1.14±0.20</td>
</tr>
<tr>
<td></td>
<td>0.99±0.21</td>
<td>1.03±0.22</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD or medians [interquartile ranges].

Differences between low and salt diets in lean and abdominally obese individuals separately were compared with paired samples T-tests; differences between lean and abdominally obese individuals on either a low or a high salt diet were compared with two-tailed independent sample T-tests.

SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial pressure, IMRR: insulin-mediated muscle microvascular recruitment

\(a\) Lean: low vs. high salt, \(p < 0.05\)  
\(b\) Abdominally obese: low vs. high salt, \(p < 0.05\)  
\(c\) Lean vs. abdominally obese under low salt circumstances, \(p < 0.05\)  
\(d\) Lean vs. abdominally obese under high salt circumstances, \(p < 0.05\)


Table 3: Age- and sex-adjusted blood pressure, insulin-mediated whole-body glucose disposal and insulin-mediated muscle microvascular recruitment on a low and a high salt intake in lean versus abdominally obese individuals

Data are presented as mean ± SD or medians [interquartile ranges].

<table>
<thead>
<tr>
<th></th>
<th>Lean (n=20)</th>
<th>Abdominally obese (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low salt</td>
<td>High salt</td>
</tr>
<tr>
<td>24h MAP (mmHg)</td>
<td>91±10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97±11</td>
</tr>
<tr>
<td>M/I-value ((mg/kg/min per mU/L) * 100)</td>
<td>12.9 [11.0 – 14.4]&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.2 [12.6 – 16.9]&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IMMR (%)</td>
<td>34 [20 – 38]&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19 [-1 – 19]</td>
</tr>
</tbody>
</table>

Age- and sex-adjusted differences between lean and abdominally obese individuals on a low and high salt diet were compared with repeated measures ANCOVA.

MAP: mean arterial pressure, IMRR: insulin-mediated muscle microvascular recruitment

<sup>a</sup> Lean vs. abdominally obese under low salt circumstances, p < 0.05; <sup>b</sup> Lean vs. abdominally obese under high salt circumstances, p < 0.05
Table 4: Associations of Ln IMMR with MAP and M/I value on a low and on a high salt diet

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Dependent variable: 24h MAP (mm Hg)</th>
<th>Dependent variable: Ln M/I-value ((mg/kg/min per mU/L) * 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standardized β</td>
<td>95% CIs</td>
</tr>
<tr>
<td><strong>Low salt (n=40)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ln IMMR (%, overall)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude analysis</td>
<td>-0.052</td>
<td>-0.358 to 0.264</td>
</tr>
<tr>
<td>Model 1: adjusted for group</td>
<td>-0.135</td>
<td>-0.428 to 0.184</td>
</tr>
<tr>
<td>Model 2: model 1 plus age and sex</td>
<td>-0.056a</td>
<td>-0.361 to 0.260</td>
</tr>
<tr>
<td>Ln IMMR (%, lean) (n=20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude analysis</td>
<td>-0.592</td>
<td>-0.820 to -0.203</td>
</tr>
<tr>
<td>Model 1: adjusted for age and sex</td>
<td>-0.511</td>
<td>-0.778 to -0.088</td>
</tr>
<tr>
<td>Ln IMMR (%, abdominally obese) (n=20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude analysis</td>
<td>0.071</td>
<td>-0.384 to 0.498</td>
</tr>
<tr>
<td>Model 1: adjusted for age and sex</td>
<td>0.149</td>
<td>-0.314 to 0.555</td>
</tr>
<tr>
<td><strong>High salt (n=39)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ln IMMR (%, overall)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude analysis</td>
<td>-0.024</td>
<td>-0.337 to 0.294</td>
</tr>
<tr>
<td>Model 1: adjusted for group</td>
<td>-0.096</td>
<td>-0.399 to 0.226</td>
</tr>
<tr>
<td>Model 2: model 1 plus age and sex</td>
<td>-0.090c</td>
<td>-0.394 to 0.232</td>
</tr>
<tr>
<td>Ln IMMR (%, lean) (n=20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude analysis</td>
<td>-0.216</td>
<td>-0.601 to 0.250</td>
</tr>
<tr>
<td>Model 1: adjusted for age and sex</td>
<td>-0.226</td>
<td>-0.608 to 0.241</td>
</tr>
<tr>
<td>Ln IMMR (%, abdominally obese) (n=19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude analysis</td>
<td>-0.027</td>
<td>-0.475 to 0.433</td>
</tr>
<tr>
<td>Model 1: adjusted for age and sex</td>
<td>-0.016</td>
<td>-0.467 to 0.441</td>
</tr>
</tbody>
</table>

Data are standardized βs (derived from multiple linear regression analyses), i.e. for every SD increase in Ln IMMR, the dependent variable increases with β SDs.

IMMR=insulin-mediated muscle microvascular recruitment, MAP=mean arterial pressure

\(^a\) Lean vs. abdominally obese, p for interaction = 0.084
\(^b\) Lean vs. abdominally obese, p for interaction = 0.323
\(^c\) Lean vs. abdominally obese, p for interaction = 0.986
\(^d\) Lean vs. abdominally obese, p for interaction = 0.831
Table 5: Urinary osmolyte excretion, serum copeptin levels, free water clearance, and urinary mineralocorticoid and glucocorticoid excretion on a low vs. a high salt diet

<table>
<thead>
<tr>
<th></th>
<th>Low salt (n=40)</th>
<th>High salt (n=40)</th>
<th>p difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lean (n=20)</td>
<td>64.8±9.0</td>
<td>65.8±8.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Abdominally obese (n=20)</td>
<td>91.3±13.9</td>
<td>92.4±13.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Serum [Na⁺] (mmol/L)</td>
<td>139.8±1.5</td>
<td>141.1±1.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Serum [K⁺] (mmol/L)</td>
<td>4.1±0.3</td>
<td>3.9±0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Serum [urea] (mmol/L)</td>
<td>4.8±1.2</td>
<td>4.4±1.5</td>
<td>0.039</td>
</tr>
<tr>
<td>Serum osmolality (mosm/kg)</td>
<td>277.6±4.6</td>
<td>277.9±3.3</td>
<td>0.710</td>
</tr>
<tr>
<td>Serum copeptin (pmol/L)</td>
<td>5.23 [3.66 – 8.17]</td>
<td>5.15 [4.08 – 6.75]</td>
<td>0.979</td>
</tr>
<tr>
<td>Urine volume (mL/24h)</td>
<td>2211±1118</td>
<td>2057±794</td>
<td>0.204</td>
</tr>
<tr>
<td>Urine [Na⁺] (mmol/L)</td>
<td>38±29</td>
<td>134±57</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Urine [K⁺] (mmol/L)</td>
<td>29±16</td>
<td>31±16</td>
<td>0.258</td>
</tr>
<tr>
<td>Urine [urea] (mmol/L)</td>
<td>183±100</td>
<td>179±84</td>
<td>0.738</td>
</tr>
<tr>
<td>Urine osmolality (mosm/kg)</td>
<td>332±184</td>
<td>517±220</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>UNaV (mmol/24h)</td>
<td>70±30</td>
<td>240±68</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>UKV (mmol/24h)</td>
<td>54±20</td>
<td>57±24</td>
<td>0.276</td>
</tr>
<tr>
<td>UUreaV (mmol/24h)</td>
<td>346±127</td>
<td>334±144</td>
<td>0.514</td>
</tr>
<tr>
<td>U(2Na2KUrea)V (mmol/24h)</td>
<td>595±186</td>
<td>928±277</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Free water clearance (mL/24h)</td>
<td>-14±1055</td>
<td>-1287±1072</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Urine aldosterone (ng/24h)</td>
<td>8002 [6272–10561]</td>
<td>2199 [1262–4073]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Urine cortisol (ng/24h)</td>
<td>16673 [12432–24829]</td>
<td>31715 [21204–38783]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Urine cortisone (ng/24h)</td>
<td>68666 [54969–80669]</td>
<td>83444 [70035–104639]</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD or medians [interquartile ranges].
Data were analysed by repeated measures ANCOVA with adjustment for group (lean/obese), age, and sex.
Copeptin data are missing in one lean participant on a high salt diet.

UNaV = 24h urinary Na⁺ excretion; UKV = 24h urinary K⁺ excretion; UUreaV = 24h urinary urea excretion; U(2Na2KUrea)V = (2*24h urinary Na⁺ excretion)+(2*24h urinary K⁺ excretion)+24h urinary urea excretion.
Table 6: Urinary amino acid and organic acid excretion on a low vs. a high salt diet

<table>
<thead>
<tr>
<th>Amino Acid / Organic Acid</th>
<th>Low salt n = 40</th>
<th>High salt n = 40</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threonine (μmol/24h)</td>
<td>92 [66 – 123]</td>
<td>134 [93 – 216]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Methionine (μmol/24h)</td>
<td>5.39±2.69</td>
<td>6.26±4.00</td>
<td>0.092</td>
</tr>
<tr>
<td>Alanine (μmol/24h)</td>
<td>197 [142 – 296]</td>
<td>260 [179 – 382]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Tyrosine (μmol/24h)</td>
<td>71.1±32.5</td>
<td>97.1±58.9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Glutamine (μmol/24h)</td>
<td>337±111</td>
<td>507±270</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Aspartic acid (μmol/24h)</td>
<td>3.93 [2.65 – 5.34]</td>
<td>4.54 [3.12 – 8.31]</td>
<td>1.0</td>
</tr>
<tr>
<td>Arginine (μmol/24h)</td>
<td>25.4±9.7</td>
<td>29.5±22.2</td>
<td>0.217</td>
</tr>
<tr>
<td>Ornithine (μmol/24h)</td>
<td>5.48±3.79</td>
<td>9.80±7.57</td>
<td>0.002</td>
</tr>
<tr>
<td>Proline (μmol/24h)</td>
<td>6.54±2.37</td>
<td>9.05±6.93</td>
<td>0.012</td>
</tr>
<tr>
<td>Citrulline (μmol/24h)</td>
<td>2.11 [1.40 – 2.90]</td>
<td>3.05 [2.03 – 5.05]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Argininosuccinate (μmol/24h)</td>
<td>52.5±17.5</td>
<td>58.2±24.9</td>
<td>0.122</td>
</tr>
<tr>
<td>Fumarate (μmol/24h)</td>
<td>0.011 [0.000 – 0.015]</td>
<td>0.011 [0.000 – 0.016]</td>
<td>1.0</td>
</tr>
<tr>
<td>β-OH-butyrate (μmol/24h)</td>
<td>0.012 [0.009 – 0.015]</td>
<td>0.014 [0.011 – 0.839]</td>
<td>1.0</td>
</tr>
<tr>
<td>Acetoacetate (μmol/24h)</td>
<td>0 [0 – 0]</td>
<td>0.000 [0.000 – 0.011]</td>
<td>1.0</td>
</tr>
<tr>
<td>Pyruvate (μmol/24h)</td>
<td>43.5 [23.7 – 63.7]</td>
<td>39.3 [27.2 – 52.8]</td>
<td>0.741</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD or medians [interquartile ranges].
Data were analysed by two-tailed paired-sample T tests (normally distributed variables) or Wilcoxon signed-rank tests (variables with a skewed distribution).