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DISSERTATION

Sex-specific alterations in blood pressure and gene expression in Wistar rats on a high-salt diet

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1. ABSTRACT

1.1 German

Einleitung: Zahlreiche Experimente und klinische Studien betonen den Zusammenhang einer hohen Salzaufnahme und einem erhöhten Risiko für Herz-Kreislauf- und Nierenerkrankungen im Kontext eines erhöhten Blutdrucks. Allerdings ist nur wenig über die geschlechtsspezifischen Effekte einer hohen Salzaufnahme auf den Blutdruck und die Genexpression in wesentlichen Herz-Kreislauf-Organen wie Herz und Niere bekannt. In der hier vorgestellten Arbeit wurden in einem Tierexperiment die Effekte einer salzreichen Diät in verschiedenen Organe auf funktioneller, struktureller und genetischer Ebene untersucht. Darüberhinaus lag ein Fokus auf der Herausarbeitung geschlechtsspezifische Unterschiede in den jeweiligen Parametern sowie in den analysierten Genen.

Methoden: Männliche und weibliche Wistar-Ratten wurden auf zwei Gruppen aufgeteilt und erhielten jeweils entweder eine Standarddiät (NS, 0,2 % NaCl, n = 50) oder eine salzreiche Diät (HS, 4 % NaCl, n = 52). Die Fütterung der jeweiligen Diäten wurde zum Zeitpunkt des Absetzens vom Muttertier, im Alter von 21 Tagen, begonnen und bis zum Ende der Studie über sechs Monate fortgesetzt.

Ergebnisse: Die Tiere, welche eine salzreiche Diät erhielten, entwickelten einen höheren Blutdruck und ein größeres Organgewicht von Herz und Nieren im Vergleich zu den Ratten mit der Standarddiät. Die Gesamtprotein/Kreatinin-Ratio sowie die Natrium- und Chloridwerte im Urin waren in der Gruppe mit salzreicher Diät erhöht, während die Kaliumwerte im Urin verringert waren. Der Effekt der salzreichen Diät auf den Blutdruck war geschlechtsabhängig, da bei den weiblichen Tieren ein signifikant höherer Blutdruck im Vergleich zur Kontrollgruppe mit Standarddiät erst später auftrat als bei den männlichen Tieren. Auch die Gesamtprotein/Kreatinin-Ratio im Urin war bei den männlichen Tieren bedeutend höher als bei den weiblichen. Die Expression mehrerer Gene in der Niere und im Herzen war bei den Tieren mit der salzreichen Diät anders reguliert als bei den Tieren auf der Standarddiät. Auffallend war, dass sich in einigen Genen die Effekte der salzreichen Diät bei den weiblichen Ratten von denen bei den

männlichen Ratten unterschieden. Zu diesen Genen gehörten Clock-Gene wie das period circadian clock 1 (*Per1*), dem RAAS zuzuordnende Gene wie das promyelocytic leukemia zinc finger (*Zbtb16*) *Gen* und dem Vitamin-D-Metabolismus zugehörige Gene wie das cytochrome p450 family 27 subfamily b member 1 (*Cyp27b1*) und das cytochrome p450 family 24 subfamily a member 1 (*Cyp24a1*).

Fazit: Bei männlichen Wistar-Ratten traten als Reaktion auf die salzreiche Diät Bluthochdruck und eine niedrige Gesamtprotein/Kreatinin-Ratio im Urin früher auf als bei weiblichen Ratten. Diese Beobachtungen könnten im Zusammenhang mit den geschlechtsspezifisch unterschiedlich exprimierten Genen in der Niere und im Herzen stehen.

1.2 English

Introduction: Although numerous laboratory experiments and clinical studies have proven the strong correlation between high-salt intake and a higher risk of cardiovascular and kidney diseases through the influence of high-salt intake on blood pressure, little has been found out about how differences in high-salt intake impacts different sex in terms of blood pressure and gene expression in key cardiovascular organs such as the heart and kidney. Completely healthy Wistar rats were chosen here to specifically examine the effects of a high-salt diet on the functional, structural and genetic performance of different organs. We devoted ourselves to find out possible sex differences within the analyzed genes.

Methods: Male and female Wistar rats were divided into the following two groups: normal-salt diet rats (n=50), which were fed with 0.2% NaCl, and high-salt diet rats (n=52), which were fed with 4% NaCl, which is approximately comparable to the salt content of an average unhealthy diet. Feeding with the diets started directly after weaning at an age of 21 days and was continued until 6 months of age.

Results: Greater organ weights of the heart and kidneys were detected in rats fed with a high-salt diet compared to those on a normal-salt diet. In the high-salt group we saw higher urinary total protein-to-creatinine ratio, sodium and chloride as well as lower urinary potassium. The high salt diet had an effect on blood pressure, with significantly increased blood pressure levels in both male and female rats. However, increases in blood pressure were more pronounced and had an earlier onset in male rats. Similarly, the urinary total protein-to-creatinine ratio was significantly higher in male than in female animals, indicating sex-specific differences in the response to high salt intake. Moreover, the different sexes showed very different performance in the expression of several genes with regard to the kidney and heart in response to high-salt intake. The genes concerned included clock genes, e.g. period circadian clock 1 (*Per1*), RAAS associated genes. e.g. the promyelocytic leukemia zinc finger (*Zbtb16*) and vitamin D metabolism-associated genes, e.g. cytochrome P450 family 27 subfamily b member 1 (*Cyp27b1*) and cytochrome P450 family 24 subfamily a member 1 (*Cyp24a1)*.

Conclusion: A later onset of high blood pressure and lower urinary total protein-to-creatinine ratio were found in female Wistar rats compared to the male rats in response to high-salt intake. Sex-specific differentially expressed genes might be the cause of these phenomena.

2. INTRODUCTION

2.1 History of human salt intake

Before the discovery of sodium chloride as a food preservative about 10000 years ago (1)，the ancestors of humans consumed less than 0.69 g salt per day for millions of years. This small amount is comparable to the salt intake of other mammals (2). Utilization of sodium chloride as a preservative for fresh food during the cold months of the year became of great economic importance especially in highly urbanized societies (3). In contrast, today the average human daily consumption of sodium is about 4.9 grams (4). Human dietary habits may have changed over time, but the genetic adaption to today's environment and lifestyle might be limited (5). As a consequence, modern salt consumption patterns display several risk factors for common diseases.

2.2 Salt intake, hypertension and cardiovascular disease

The pathophysiological role of an increased dietary salt in the development of hypertension and cardiovascular disease has been acknowledged for a long time by scientists all over the world (6) and it is clinically supported by interventional studies showing that reduction of salt intake leads to a decrease in blood pressure (BP)(7, 8).

Increased BP is one of the major reasons for death in the world (3). This has been indicated by numerous studies, all of which support salt intake as a major factor in increasing blood pressure in the population. The diversity and intensity for that evidence are much more than other lifestyle factors, such as overweight, inadequate physical activity and low consumption of fruits and vegetables (3). The first study which showed an association between sodium intake and cardiovascular outcomes appeared in 1985 and was in Japanese men (9). Although it was reported that low-salt intake has no long-term beneficial effects regarding cardiovascular disorders including hypertension (10), various other experiments and clinical studies have underlined the role of high-salt (HS) intake for a greater risk of cardiovascular (11) and kidney disease (12).

2.2.1 Animal studies

Several animal studies have shown that salt intake can impact on BP. In almost all types of animal models, a HS intake caused the BP to rise. A study of chimpanzees (98.8% homologous to human genes) showed that a gradual increase of salt intake during 20 months from 0.5 g/day, which is close to the human evolutionary intake, to 10-15 g/day, which is comparable to our current salt intake, led to a gradual increase in BP (13). Studies using other species including rats (14-16), mice (17-19), dogs and lower primates (20) have shown that HS intake leads to elevated blood pressure.

2.2.2 Clinical studies

The epidemical INTERMAP study (International study of macro and micro-nutrients and BP) showed that salt intake played an important role in determining the levels of BP in the population and excess salt intake was associated with hypertension (21). Some studies have shown that the migration from an area with a low salt consumption to an environment with increasing salt intake is associated with a rise in BP. For example, migration from a rural Kenyan tribe to an urban environment with increased salt intake led to higher BP in comparison to the control group, which stayed in the rural environment(22). A study in the Pacific Islands reported that using seawater in cooking and in the household resulted in higher BP in subjects who were not used to a HS intake (23).

In the United States, it has been reported that a reduction in dietary salt intake by 3 g per day would reduce the annual number of new cases of stroke by 32,000 to 66,000, coronary heart disease by 60,000 to 120,000, and myocardial infarction by 54,000 to 99,000 and would to reduce the annual number of deaths from any cause by 44,000 to 92,000(24). In a British cohort from 2003 to 2011, salt reduction contributed to a decrease in BP by 3.0±0.33/1.4±0.20 mm Hg and in mortality from stroke by 42% and ischemic heart disease by 40%. After adjusting for age, sex, ethnic group, education, household income, alcohol consumption, fruit and vegetable intake and BMI, who did not use antihypertensive drugs, the BP was failed by 2.7±0.34/1.1±0.23 mm Hg(25).

2.2.3 Mechanisms of salt- induced hypertension

The body can detect an overabundance salt and responds with an increase in blood pressure. Earlier hypotheses focused mainly on raised plasma volume: sodium retention is thought to cause higher blood sodium, prompting increased thirst and plasma volume. Therefore, it leads to an increase in blood pressure. Although the cardiac index may initially rise under salt load conditions, it usually returns to normal levels whereas the total peripheral resistance (TPR) will rise and remain elevated (26). Another study reported that the cardiac index was not significantly, different while TPR was significantly elevated when the hemodynamics of normotensive and hypertensive individuals were compared (27). Therefore, the increase in blood pressure with chronic salt load appears to be the result of peripheral vasoconstriction where other hemodynamic parameters might remain unchanged.

Furthermore, plasma sodium may have a direct effect on the vascular system, and a slight increase in sodium concentration was reported to result in an increased stiffness of isolated human endothelial cells (28). Plasma sodium may also indirectly cause systemic vasoconstriction by altering the sympathetic nervous system outflow in animals and humans (28). Further indirect effects may occur through the participation of dietary sodium in steroidal hormonal pathways, which has been studied in rats and has been previously described (29). In addition, it has been shown salt loading stimulated the adrenal gland to release marinobufagenin (30), which is an endogenous steroidal α1-Na+K+-ATPase inhibitor, and resulted in peripheral vasoconstriction (31) and increased stroke volume (32).

2.2.4 Effects of high-salt intake beyond blood pressure

Hypertension is a traditional risk factor for cardiovascular disease and frequently associated with stroke, ischemic heart disease, ventricular hypertrophy, heart failure and chronic kidney disease (8, 33).

The understanding of hypertension as the sole culprit for consequences of a HS intake, however, has changed over the years. There is increasing evidence that the deleterious effects of HS intake may be mediated by mechanisms beyond blood pressure.

Pooled analysis of data from four studies showed that the association between salt intake and cardiovascular risk persisted even after adjustment for blood pressure (34). Other important factors may contribute to the risk of HS intake for cardiovascular and kidney disease, dependent on or independent of changes in blood pressure. Some animal and human studies indicated that HS intake may have a direct effect on stroke and left ventricular mass independent of blood pressure. *Polonia J et al.* performed a 7.2 year follow-up experiment (35) and *Perry IJ et al.* demonstrated an ecological analysis(36) showing that there was a significant positive correlation between urinary sodium excretion and stroke mortality. Left ventricular hypertrophy is an independent predictor of cardiovascular morbidity and mortality, and is considered to be associated with high-sodium intake(37). *Du Cailar G et al.* observed that in both hypertensive and normotensive individual, there was a positive correlation between left ventricular mass and 24-h urinary sodium excretion(38). This substantiated that dietary sodium intake may have a direct effect on left ventricular mass.

Studies in humans have demonstrated that salt intake is associated with protein or albumin excretion (39, 40). Albuminuria is an important risk factor for cardiovascular and kidney disease (41). Moreover, HS intake may be positively associated with albuminuria in diabetic patients (42, 43). This finding is associated with insulin resistance, suggesting that insulin resistance may contribute to increased sodium sensitivity, increased blood pressure and proteinuria (43).

The Renin–Angiotensin–Aldosterone system（RAAS）is important for maintaining the balance of sodium and water when reducing sodium or fluid intake(44). Physiological compensatory activation of the RAAS may occur during sodium limitation. Activation of the RAAS may be associated with increased cardiovascular morbidity and mortality. A meta-analysis study reported by *Graudal NA et al.* suggested that low sodium intake (<120 mmol / 24 h) was related to a significant increase in plasma renin and aldosterone, including a longer sodium limit study (≥4 weeks)(45). Therefore, severe sodium restriction in the diet may not be suitable for every case.

Moderate to severe sodium restriction causes a reduction in the plasma volume and an increase in the concentration of lipids in plasma. *Graudal NA et al.* demonstrated that

low density lipoprotein cholesterol increased significantly with short-term large reductions of in sodium intake (sodium reduction of >100 mmol/24 h for <4 weeks), and in a meta-analysis reported that with long-term moderate sodium restriction (mean sodium reduction of 75 mmol/24 h for >4 weeks), there was no significant change in low-density lipoprotein cholesterol (46).

Another study has suggested that endothelial dysfunction might be related to high-sodium intake in animal models and humans(47). Vascular endothelial dysfunction has been reported to increase the development of atherosclerosis, which plays an important role in the pathogenesis of the cardiovascular disease(47). In normotensive Sprague-Dawley rats, a HS diet was given for 4-5 weeks and their arterioles were less responsive to acetylcholine-induced vasodilation during high-sodium intake. The mechanism behind this finding could be that the HS diet caused the increased production of reactive oxygen species in the arteriolar wall in response to increased salt intake(48).

2.3 Sex-specific differences in the cardiovascular system and in kidney function

2.3.1 Sex-specific differences in the cardiovascular system

Although cardiovascular disease remains a major killer of both men and women, there are significant sex differences in the prevalence and burden of different cardiovascular diseases. A better understanding of the sex differences in the cardiovascular system is crucial for improving the ability to maintain a healthy population and for identifying and treating heart disease in both women and men.

In basic electrophysiology, women have longer QT intervals and a faster heart rate than men. These become most obvious after puberty and they decrease after menopause, although they still exist. PR and QRS intervals are shorter in women than in men. Not only because of the smaller size of the female heart, but also because sex hormones such as estrogen, progesterone, and androgens regulate various ion currents that have been reported to affect the duration of the electrocardiogram(49).

It has been reported that the incidence of atrioventricular nodal reentrant tachycardia (AVNRT) and inappropriate sinus tachycardia is higher in women than in men, whereas atrioventricular reentrant tachycardia (AVRT) is more common in men. *Gowd BM et al.*

demonstrated that AVNRT is more easily induced in premenopausal women at the beginning of a premenstrual or menstrual cycle(49).

According to a study, the absolute number of deaths from cardiovascular disease in women has surpassed that of men since 1984. However, when adjustments according for the differences in age distribution are made, the CVD-induced mortality in men was found to be significantly higher than in women(50).

Heart failure can be divided into heart failure with preserved ejection fraction (HFpEF) or heart failure with reduced ejection fraction (HFrEF). Most studies have found that patients with HFpEF are older, and more often hypertensive and obese in comparison to those with HFrEF.The most robust difference found is that HFpEF is more common in women and this may be associated with less coronary heart disease, increased concentric remodeling and age-related vascular stiffness(51). There is evidence that the outcomes of HFpEF differ between men and women; the I-PRESERVE trial (Irbesartan in Heart Failure with Preserved Ejection Fraction Study) had a high female enrollment rate of 60%, and showed a 20% reduction in the likelihood of death or hospitalization in women compared to men. Other studies of HFpEF have also shown similar findings(52).

HFrEF with an ischemic and non-ischemic etiology is more common in men than in women. Women with HFrEF are more likely to suffer from a non-ischaemic etiology while men are more likely to suffer from ischemic cardiomyopathy. In people with systolic heart failure, women have a longer survival rate than men, mainly because of significant differences in mortality among women and men with nonischemic cardiomyopathy. Mortality in women with ischemic cardiomyopathy may be slightly better than in men, but the difference is not as pronounced as in non-ischemic cardiomyopathy(53).

Mitral valve prolapse is more common in women, but compared to men, women tend to have a more benign course: fewer flail leaflets, lower regurgitation grades, higher ejection fractions, and smaller chamber dimensions. Clinically, women have shown fewer clinical manifestations, such as heart failure, atrial fibrillation, and stroke than men. However, with severe mitral regurgitation, referral to cardiac valve surgery is less likely, and long-term mortality is significantly higher in women than in men(54).

In patients with aortic stenosis who are at high risk and undergo transcatheter aortic valve replacement(TAVR), the incidence of postoperative complications in women is higher than that in men, such as major vascular complications, bleeding events and stroke, however, there is no difference in procedural or 30-day mortality. In addition, after TAVR women have less para-valvular aortic insufficiency and the late survival rate is significantly better than in men(55).

2.3.2 Sex-specific differences in kidney function

Traditionally, sex was seen as an important factor affecting the progression of kidney function and kidney disease. The biggest meta-analysis to date suggests that women with polycystic kidney disease, IgA nephropathy, membranous glomerulopathy, and 'chronic renal disease of unknown etiology' tend to progress to end-stage renal disease (ESRD) at a slower rate than men(56). The Prevention of Renal and Vascular End-stage Disease studies reported different results regarding the predictors of renal function in men and women: in both males and females high systolic blood pressure and plasma glucose were found to be independent predictors for a worse renal outcome. Moreover, in males urinary albumin excretion was considered the strongest independent predictor of renal function decline, and a better renal function outcome was associated with low waist circumference and cholesterol/HDL ratio. In women, on the other hand, better kidney outcomes were associated with low triglycerides(57).

Low nitric oxide (NO) production is commonly observed in chronic kidney disease（CKD）patients and has been suggested as one of the causes of CKD. In an animal study, *Hong Ji et al.* demonstrated that sex differences in renal NO production play a role in the progression of renal injury and reported that female rats have higher NO levels than males(58).

CKD appears to progress faster in men because of testosterone and in women CKD may progress more slowly because estrogen is protective. However, animal observations often conflict with human data. In animal models, endogenous estrogens have been found to show antifibrotic and antiapoptotic effects in the kidney(59). In addition, 17β-estradiol attenuated glomerulosclerosis and tubulointerstitial fibrosis and protected podocytes from

damage in animal models(60). Furthermore, orchiectomy attenuated glomerular and tubular damage, kidney fibrosis and proteinuria in animal models of kidney injury(61). However, the results of clinical studies are just the opposite. An observational study found an increased risk for microalbuminuria and cardiovascular disease in premenopausal oral contraceptive users and postmenopausal hormone replacement therapy users(62). However, a large case-control study found that androgen deprivation therapy increased the risk of acute kidney injury in men newly diagnosed with non-metastatic prostate cancer (63).

2.4 Sex-specific differences in response to a high-salt diet

One important aim of this experiment was to reveal possible sex specific differences of a HS diet. Several studies have suggested the presence of sex differences in BP regulation in response to a HS intake (64-68). After 3 weeks of a HS diet (8. 0% NaCl) , the blood pressure of both male and female Dahl salt-sensitive (DS) rats was significantly increased compared to that of rats on the corresponding low-salt diet (0. 3% NaCl), In addition, male rats fed with a HS diet had significantly higher blood pressure compared to females(64).

The kidney plays an important role in regulating blood pressure via alterations in sodium excretion. *Reckelhoff et al.* detailed that the administration of testosterone to ovariectomized female SHR increased blood pressure and changed the pressure-natriuretic relationship(69). In addition, there is evidence that female sex hormones may actually prevent salt-induced hypertension by increasing renal excretion of sodium. In DS rats, gonadectomy led to the accelerated development of salt-sensitive hypertension in females (70).

The impact of sex hormones on BP has been reported in many studies. *Pechère-Bertschi et al.* performed a series of studies to assess the effects of exogenous and endogenous female sex hormones on the systemic hemodynamic response to sodium intake changes in healthy young women with normal BP and in menopausal women(71). They found that the response of BP and heart rate to salt was comparable during the luteal and follicular phases of the normal menstrual cycle, and the pressure-natriuresis

relationship was steep in both phases. This indicated that these women were generally insensitive to salt. The administration of oral contraceptives did not change the pressure-natriuresis distribution, which suggest that no effect of exogenous female sex hormones on the BP in response to salt. In menopausal women, the pressure-natriuresis curve was shifted to the right, suggesting that menopausal women were sensitive to salt. In contrast to the BP respones, it was found that during the luteal phase of the menstrual cycle, control of the renal circulation is salt sensitive and endogenous female sex hormones act on glomerular hemodynamics either directly or indirectly due to a decrease in the fractional filtration after salt loading(72).

Menstrual cycles and menopause are characterized by dramatic changes in plasma progesterone, estrogen, aldosterone and plasma renin activity-hormone systems that are known to regulate sodium excretion in the proximal and distal renal tubules. Moreover, androgen receptors have been found in the proximal tubule segment of the nephron, indicating that testosterone may also affect renal sodium reabsorption. Endogenous lithium clearance technique is a method for investigating proximal segmental renal sodium handling in humans. When young normotensive men switched from a low-salt diet to a HS diet, there was a significant increase in lithium clearance and a decrease in distal reabsorption of sodium fraction, indicating a reduction of sodium proximal and distal reabsorption to maintain sodium balance(73). A similar investigation was performed in normotensive women and a significant difference in tubular response was observed between the follicle and the luteal phases(74).

There are also some genetic studies investigating sex differences in response to a HS intake. One study reported a blunted response to angiotensinogen II under HS conditions observed most commonly in males(75). *David Gerhold et al.*, using microarray profiling, found that the effects of a HS diet were completely different in the kidneys of male and female rats. In female Dahl Iwai(DI) rats, the HS diet regulated hundreds of genes, and only 13 genes were observed to be changed in male DI rats in response to the HS diet (76).

2.5 Aim of the study

Cardiovascular diseases and hypertension are on the rise around the world. Animal experiments, as well as epidemiological studies in humans, show sex-specific differences in their incidence and pathogenesis. A HS intake as a possible contributing factor to high blood pressure and cardiovascular diseases likewise may impact in a sex-specific manner. Therefore, this animal experiment was designed to investigate possible sex-specific differences and potential health consequences of a HS intake in male versus female Wistar rats.

To be able to characterize functional, morphological, and genotypical changes resulting from the HS intake, certain functional parameters such as blood pressure and plasma and urinary markers were analyzed and compared to data from rats with a normal-salt (NS) intake. Moreover, to investigate potential micromorphological differences of the kidney and the heart, standard histological methods were applied. In order to determine differences in the expression in these two organs at the mRNA level, quantitative Real Time-PCR (qRT-PCR) was performed. All results were then statistically compared between male and female rats.

3. MATERIALS

3.1 Technical devices

Device	Manufacturer
Pressure / pulse measurement	PowerLab,ADInstruments ,NSW,Australia
Magnetic stirrer MR 2000	Heidolph Instruments GmbH & Co. KG
Micropipettors(10µl - 1000µl)	Eppendorf, Hamburg, Deutschland
Power supply Biometra standard	Biometra GmbH, Göttingen, Germany
Precision balance Scaltec SBC22	Scaltec Instruments, Göttingen, Germany
Safelock Tubes	Eppendorf, Hamburg, Germany
ShakerWT-17	Biometra GmbH, Göttingen, Germany
Vortex mixer	Neo Lab, Heidelberg, Germany
Water purifier Milli Q	Mili-Q Purification System, Billerica, USA
Centrifuge Biofuge 13	Heraeus Sepatech GmbH, Osterode
Mx3000P thermal cycler	Stratagene, La Jolla, CA
Microtome RM2025	Leica Mikrosysteme Vertrieb GmbH
Digital Camera CFW-1310C	Leica Mikrosysteme Vertrieb GmbH
Bioanalyzer	Agilent Technologies, Santa Clara, CA, USA
ABI 7900HT Real-Time PCR System	ThermoFisher Sci., Carlsbad, CA, USA
Paraffin Embedding Center Microm EC-350	ThermoFisher Sci. Waltham, MA, USA

3.2 Chemicals

Chemicals	Manufacturer
Acetic acid	Merck, Darmstadt, Germany
Ethanol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Xylol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Paraffin	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sirius Red staining kit	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Hematoxylin-Eosin staining kit	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

Periodic Acid Schiff's staining kit	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
hydrochloric acid	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
RNeasy fibrous tissue kit	Qiagen, Hilden, Germany
RNase-free DNase I	ThermoFisher Sci. Waltham, MA, USA
ImProm II Reverse Transcription System	Promega Corp. Fitchburg, WI, USA
qPCR MasterMix Plus	Eurogentec, Liege, Belgium
Labeled Probes	Sigma Genosys， Darmstadt, Germany

3.3 Software

Software	**Developer**
Image J 1.37v	Shareware des National Institutes of Health, USA
Software SPSS Version 21.0	SPSS, USA
GraphPadprism Version 6	GraphPad Software Ltd, USA
Excel 2013	Microsoft, USA
Word 2013	Microsof, USA
Endnote 8	Thomson Corporation, USA
Primer 3	Whitehead Institute, Howard Hughes Medical Institute, USA

4. METHODOLOGY

4.1 Animals and protocol for salt loading

Sex differences in experimental animal models affect hormonal responses and organ function among other characteristics. Environmental factors may also have a significant impact and influence on such interactions.

In this study, the sex-dependence of physiological effects on heart and kidneys secondary to a HS diet were investigated. In order to clarify the molecular mechanisms of these interactions, biological samples were obtained from the organs of the experimental animals.

Male and female Wistar rats were obtained from Charles River Laboratories International, Inc. (Wilmington, MA, USA). Wistar rats were divided into two groups: normal-salt diet (NS, 0.2% NaCl, n=50, Altromin GmbH, Lage, Germany) or high-salt diet (HS, 4% NaCl, n=52, Altromin GmbH, Lage, Germany). The feeding of the respective diets started after weaning (21 days of age) and was continued throughout the study until adulthood (6 months of age). Both diets were composed of the same nutrients and differed only in the percentage of sodium chloride. All procedures were approved by the Institutional Animal Care Committee and followed the guidelines of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Proper accommodation and care were ensured as required by §2 of the Animal Welfare Act, including medical treatment. All experimental animals were kept under controlled environmental conditions at a room temperature of 20°C, with 46% humidity and a 12-hour light cycle. The animals were allowed food and water *ad libitum*. The pelleted feed provided was a standardized complete rat feed.

The animals were sacrificed under deep isoflurane-O_2 anesthesia. The heart and the kidneys were removed, washed in saline, dried and weighed. The organs were either fixed in 10% neutral-buffered formalin for 24 hours or snap frozen in liquid nitrogen and subsequently stored at -80 °C for further testing.

	Diet	Protein content [%]	Fat content[%]	Carbohydrate content [%]	NaCl content [%]
I)	**High salt**	**24**	**11**	**65**	**4**
II)	**Normal salt**	**24**	**11**	**65**	**0.2**

Table 1. Overview of the experimental diets used.

4.2 Plasma and urine collection

Twenty-four–hour urine samples were collected in metabolic cage experiments and plasma was drawn from the tail vein under anesthesia (2-3% isoflurane-O_2 inhalation anesthesia mixture) at weeks 6, 12. 18 and 24. Blood was removed with a hematocrit capillary and expelled with a syringe into a reaction vessel (1.5 ml and 0.5M EDTA coated). It was then centrifuged for five minutes at 3000 rpm and plasma samples were stored at -20°C until further processing.

4.3 Blood pressure measurement

Systolic blood pressure (SBP) and pulse were measured by non-invasive tail cuff plethysmography of the tail artery at 5, 9, 13, 17 and 21 weeks of age. Animals were fixed in size-adjustable plexiglas tubes. In order to properly place the occlusion tail cuff, the tail was put through the occlusion cuff and placed as close to the base of the tail as possible without using force. After threading the tail through the pulse sensor cuff, it was placed within two millimeters of the occlusion cuff. Computer-aided software continuously monitored the heart rate and mean SBP over a pressure curve. After 10 minutes of familiarization for the animal, constant heart rate recording was begun. At intervals of 30 seconds, at least four measurements were taken to obtain reliable means of heart rate and SBP. To get the animals used to this procedure, animals were trained before the actual measurement. The measurement was carried out with the PowerLab 4SP from AD Instruments and the associated Software Chart and Scope for Windows Version 4.1.

4.4 Morphological and histological analysis

4.4.1 Organ removal and fixation

The experimental animals were anesthetized with isoflurane at the age of 25 weeks, and at the onset of apnea, a cervical dislocation was performed for safe killing. Immediately thereafter, the organs were removed and weighed.

4.4.2 Embedding

Paraffin embedding of organs is used to achieve sufficient strength of the tissue so that the organs can be cut in the micrometer range. The prerequisite for the complete impregnation of the tissue with paraffin is the previous dehydration, which was carried out with an ascending alcohol series. The preparations were each dehydrated for one hour in 70%, 80%, twice in 96% and twice in 100% ethanol and then transferred twice for four hours in xylene. Xylene needs to be used as an intermediate because the residual alcohol in the tissue is not miscible with paraffin. Then the preparations were placed in pure molten paraffin at 56 °C for one hour and transferred to a second paraffin bath for an additional two hours. All of these steps were automatically performed overnight in the Shandon Citadel 1000 tissue machine from Thermo Electronics Corporation. The embedding in the histocassettes was then carried out on the paraffin machine Microm EC-350 from Thermo Scientific and the curing of the preparations on the cooling plate Microm EC-351.

Solvent	**Duration**
70% Ethanol	1 hour
80% Ethanol	1 hour
2x96% Ethanol	1 hour each
2x100% Ethanol	1 hour each
2x Xylene	4 hours
56 ° C molten paraffin	1 hour
Second paraffin bath	2 hours

Paraffin	until embedding

Table 2.Protocol for embedding

4.4.3 Preparation of the tissue sections

3 µm thick tissue sections were cut using a Jung RM 2025 microtome (Leica Biosystems, Wetzlar, Germany) and the tissue sections were placed in a water bath, and then transferred onto glass slides. Then the slides were placed in a warming cabinet for 30 minutes to dry.

4.4.4 Histological stains

The preparation of the histological tissue sections for the different stains was basically the same. First, the tissue sections were deparaffinized twice in xylene for each five minutes and then watered in a descending alcohol series for two minutes in 100%, 96%, 80%, and 70% ethanol, so that the tissue sections were accessible to staining with various water-soluble stains. After the respective staining step in which the desired structures were stained, the dehydration of the tissue sections in the ascending alcohol series was carried out in each case as follows: a brief immersion in 70% and 80% ethanol and two minutes in 96% and 100% ethanol with subsequent transfer into xylene. For the histological examination of the heart and the kidney, three stains were made:

1. Sirius Red staining to determine interstitial fibrosis, perivascular fibrosis in the heart and the kidney, and media-to-lumen-ratio in the kidney.
2. Hematoxylin-eosin staining (HE) to determine cardiomyocyte size in the heart.
3. Periodic Acid-Schiff staining (PAS) to determine glomerulosclerosis and glomerular size.

The different steps are listed in the following table.

Step	Instructions	Incubation time
Sirius red staining		
1	Prepare the Sirius red staining solution (0.1 % w/v) by dissolving Sirius red in saturated picric acid solution (1.3% picric acid in water)	

2	Stain the sections by immersing the slides in the Sirius red staining solution at room temperature away from direct light.	1 hour
3	Wash the slides in 0.01 M HCl	30 seconds
HE staining		
1	Incubate in hematoxylin solution.	6 minutes
2	Rinse in tap water.	10 seconds
3	Differentiate with hydrochloric acid 0.1 %	10 seconds
4	Rinse in flowing tap water.	6 minutes
5	Incubate in eosin solution.	30 seconds
6	Rinse in flowing tap water.	30 seconds
PAS staining		
1	Hydrolysis with periodic acid solution 1 %.	10 minutes
2	Rinse with tap water.	10 minutes
3	Rinse with distilled water.	2x2 minutes
4	Stain with Schiff's reagent (room temperature).	15 minutes
5	Rinse with warm tap water (35 °C minimum).	5 minutes
6	Rinse quickly with distilled water.	10 seconds
7	Counterstain with hemalum solution acc. to Mayer.	5 minutes
8	Blue in flowing tap water.	10 minutes

Table 3. Histology: Protocols for Sirius Red, HE and PAS staining of paraffin - embedded tissue slices.

Renal fibrosis was determined in sirius-red-stained tissue sections using computer-aided histomorphometry devices. The perivascular fibrosis of intrarenal arteries was evaluated in 30 randomly selected arteries of each sample using X200 magnification with the following scale: grade 0= no perivascular fibrosis; grade I=minor perivascular fibrosis; grade II=moderate perivascular fibrosis; grade III= strong perivascular fibrosis and grade IV= very strong perivascular fibrosis. Interstitial fibrosis was assessed by a macro for the image analysis software ImageJ, which was written to quantify the percentage of fibrosis as compared to the total area of the tissue section in approximately 30 randomly selected

pictures from each slide. For the assessment of media-to-lumen ratio, sirius red staining was used. Small arteries were photographed under light microscope (magnification x400) using a digital camera. Then the freehand selection tool of the ImageJ software was used to quantify the area of the media and the lumen and the media-to-lumen ratio was calculated. The glomerulosclerosis index was assessed in PAS-stained sections. The severity of glomerular lesions was graded according to the percentage of the glomeruli involved**:** 1% to 25% (grade I); 25% to 50% (grade II); 50% to 75% (grade III); and 75% to 100% (grade IV). Glomerular size was assessed in at least 50 glomeruli in each longitudinal PAS–stained kidney section using ImageJ software.

The perivascular and interstitial fibrosis of the heart were measured as described above. HE-stained tissue sections were used to quantify the cardiomyocytes' diameter. About 30 randomly chosen pictures from each slide were taken under a light microscope (magnification x400). Longitudinally cut cardiomyocytes with a visible and non-conspicuous nucleus from the left ventricular wall and the septum were photographed. The diameter was determined using the straight line selection tool of the ImageJ software.

4.5 Quantitative Real Time PCR

4.5.1 RNA extraction

Total RNA was extracted from frozen pulverized whole kidney and heart samples using RNeasy fibrous tissue kit (Qiagen, Hilden, Germany). Samples were cut with alcohol-sterilized scissors and were put into the homogenizing tube containing the lysis solution.4 ceramic beads were put into the tube and the tube was rotated for the homogenization of the tissues. Then the lysate was placed on ice and ethanol was added to the lysate to provide ideal binding conditions. The lysate was then loaded onto the RNeasy silica membrane. RNA bound into the membrane, and all contaminants were efficiently washed away. Pure, concentrated RNA was eluted in 30–100 µl water and checked for integrity on a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

4.5.2 qRT-PCR

In accordance with the standard protocol of the kit supplier, 1 µg of total RNA was first processed with RNase-free DNase I (Thermo Fisher Scientific. Waltham, MA, USA) for 15 minutes at room temperature for reverse transcription, and afterward reverse-transcribed utilizing of ImProm II Reverse Transcription System (Promega Corp. Fitchburg, WI, USA) in an aggregate response volume of 40 µL. Real-time polymerase chain reaction was managed using ABI Prism Sequence Detection System (Applied Biosystems ABI HT7900 Fast Real-Time PCR System, Carlsbad, CA, USA). Using a PCR mix containing Taq polymerase (qPCR MasterMix Plus, Eurogentec, Liege, Belgium) and primer sets with 6-FAM and TAMRA labeled probes (Sigma Genosys) in 384 multiwell plates for cDNA samples amplification. Reaction under standard conditions prescribed by thermocycler, cDNA samples (20 ng) were kept running in triplicates in response volumes of 20 µl (384 multiwell plates). By using Primer3 Plus to create TaqMan probe sets. Relative gene expression was calculated using the ddCt term (Applied Biosystems, User Bulletin No. 2) related to endogenous controls ribosomal protein L32 (RPL32). The specificity of the real time PCR primers was verified by reference gene sequences of the following NCBI GenBank accession numbers (Table 4).

Gene	Forward Primer	Backward Primer	Probe
Ace	GGAGTACACCTGGACACCAAA	AGTTGACGCGACTGGACTCT	CTCGTGCAGAAGGCTCCCTCCC
Acsl3	AAGTGCTTTCTGAGGCTGCT	GGGCTCAAACGAATTTTCAG	TCAGCAAGTCTGGAAAAGTTTGAAATCC
Adm	CGCAGTTCCGAAAGAAGTGG	CCCGTAGGGTAGCTGCTGGA	TAAGTGGGCGCTAAGTCGTGGGAAGAG
Agt	CACTTCCAAGGGAAGATGAGA	GTGCTGTTGTCCACCCAGA	CTTCTCCCAGCTGACTGGGCTCCAT
Agtr1a	GTGGACACTGCCATGCCCAT	CCCAGAAAGCCGTAAAACAG	ATAGCGTATTTTAACAACTGCCTGAACC
Agtr2	CTTATGTAGTTCCCCTTGTTTGG	AATGGTTCTGACATCTCGGAA	TATGGCTTGTCTGTCCTCATTGCCAACA
Alpl	TTTCACGTTTGGTGGCTACA	CTGTGAAGGGCTTCTTGTCC	TTTGGTCTGGCTCCCATGGTGA
Angpt2	CATCTGCAAGTGTTCCCAGA	TTCAAGTTGGAAGGACCACA	CTCACAGGAGGCTGGTGGTTCGAC
Apoa1	CAAGAACCACCCTACCCTGA	GGTTTGGCTTTCTCACCAAG	CATACCAAGGCCAGCGACCACC

Arntl	GAGACCCCAGGCTATCCCTA	TCTATCATGTCGATGCCTATGTG	TGATAGTTCGTCTATTCTTGGTGAGAACCC
Atp2a2	CCTTTGATGAGATCACAGCTATGACT	CCCGATTTCCGACTTCTTCA	TGTGAACGACGCGCCCGCT
Cdkn1A	CTCAGGGCCGAAAACG	CTTGCAGAAGACCAATCGG	CAGACCAGCCTAACAGATTTCTATCACTCCA
Chga	GAGGCGACTTTGAGGAAAAGAA	TGGCTGACAGGCTCTCTAGCT	AGGGCAGCGCCAACCGCA
Clock	CCAGCCACCACAACAGTTC	GGATGAGCTGTGTCGAAGGA	CAGACATCTCGGTTGCTCCATGG
Col1a1	GAAGCATGTCTGGTTTGGAGAGA	ATCGGAACCTTCGCTTCCAT	TGACCGATGGATTCCAGTTCGA
Cps1	TAAGATACCTCAGAAGGGCATT	AACTGCTCAGCAACACCAAG	CATCCAGCAATCATTCCGTCCAAG
Csad	AAGAAGGAGAGCCCAGATTACA	TCATGGTTCCCTTCTTCACC	CAGAGGCTGTCTCAGGTGGCCC
Cyp11b2	AGATGCTGCTCCTGCTTCAC	GATAGGCCATCTGCACATCC	TGCTGAAAACCTTCCAGGTGGAGA
Cyp17a1	GGGACCAGCCAGATCAGTT	TCTGCGTGGGTGTAATGAGA	CGCTTCTTAGATCCAACGGGAAGCC
Cyp24a1	GAAAGAATCCATGAGGCTTACC	CGTATTCACCCAGAACCGTT	CCATTTACAACTCGGACCCTTGACAAAC
Cyp27b1	CATCCATTGCAGCACAAACT	CTCACATGGTCAGGCAGAGA	CACCTGGTCTGCACCTGGTTGC
Dbh	TGCACTGTAACAAGACCTCTGC	CTGCGGAAGTGATCTTAGGC	TCCCGGGTAACTGGAACCTGCA
Ddc	TGTGCTGAGGGCAGAGAA	TTTTCTCTTCCCAGTTTTCG	CCGCTTCAGAGACCCAAAGTTGAA
Ddit4	CACCCTGGGAGTCTGCTAAG	TCTCTTCAAACACCACCTCGT	ATGTCGTCCCTGAGCCCAACCT
Dhcr24	GAGGCAGCTGGAGAAGTTTG	TCCTCCCGGTTCATATAGCA	AGCGTGCACGGGTTCCAGATGT
Dnmt3a	ACATCCTCTGGTGCACTGAA	TGGAGACGTCTGTGTAGTGGA	TGGAAAGGGTGTTTGGCTTCCC
Dnmt3b	ACGTTCTGTGGTGCACTGAG	CCATGTTGGACACGTCTGTG	TCGAAAGGATCTTCGGGTTCCCA
Fn1	GGGGTCACGTACCTCTTCAA	GTTGTGCTGTCAGAGGCTTG	TCTTTGCTGTGCATCAGGGCAG
Gdf15	ATCCAGTTCCCATTTGTATGTCCT	ACTCGCCCACGCACATG	CTAGGTTGGAGCGACTGGGTACTGT
Gsk3a	CCCCCTCTTCAACTTCAGTC	GGGATGAGAATGGCATTGAG	TGGTGAACTTTCCATCCAACCGTC
Gsk3b	GACTTTGGAAGTGCAAAGCAG	GGTGCCCTGTAGTACCGAGA	TCCGAGGAGAGCCCAATGTTTCATATATC
Havcr1	GGAATGGCACTGTGACATCCT	CTGCGGCTTCCTCAAAGG	AGGAGGCCTGGAATAATCACACTGTAAGA
Hdac2	AGTGGTGAGAAAACAGACACCA	TTGGGGAGTCAAATTCAAGG	AGGAGCCAAATCAGAACAACTCAACAA
Il1rl1	CCAAGATGGCATCTGTTGC	ACGACTCAAAAGTGTTTCAGGTC	TCCGTTGAGTGGCAAGGTGTGCT
Kdm1a	CTCAGCCAATCACTCCTGGT	TGCGGATTGTATGTTCTCCA	CCCCACAGCCAATTCCAAGACTC
Lcn2	CTGGGGAATATTCACAGCTACC	GCAAACTGGTCGTAGTCAGTGT	TCAGATACAGAGCTACGATGTGCAAGTGG
Lgals3	TGAGAGCGGCAAACCATTC	TTGACCGCAACCTTGAAGTG	AATACAGGTCCTGGTTGAAGCCGA
Lyve1	CTGGGTGTTTGCTACGTGAA	CCTTGGTTTCGATCATTTCC	TGTGAAGGCCTTCCCTTTCACAA

Mc2r	CCCTTTGTGCTCCATGTTCT	ATGCCATTGACCTGGAAGAG	TGCCCAAATAACCCTTACTGTGTTTGC
Mmp2	GTTTATTTGGCGGACAGTGACA	GGGAGCTCAGGCCAGAATG	CCCACAGGTCCCTTGCTGGTGG
Mmp9	GTAACCCTGGTCACCGGACTT	ATACGTTCCCGGCTGATCAG	CCGCGTCGTGGAGGGAAGG
Myh7	TGGAGAACGACAAGCAGCAG	CCTGGCGTTGAGTGCATTTA	TGGATGAGCGACTCAAAAAGAAGGACTTTG
Nos3	GCTACCACGAGGACATTTTCG	GCTGTCGCTCCTGCAAAGA	AGGTGACGAGCCGCATCCGC
Nppa	GGGGGTAGGATTGACAGGAT	TCGAGCAGATTTGGCTGTTA	CCCAGAGCGGACTAGGCTGCAA
Nppb	CGCCGCTGGGAGGTCACTCC	TCTCTTATCAGCTCCAGCAG	CTGGGAAGTCCTAGCCAGTCTCCAGA
Nr3c2	CTGCAGGCTCCAGAAATGCC	GAGGCCTTTTAACTTCCCCA	CATGAACTTAGGAGCTCGAAAGTCAAAGAA
Nr4a1	CTGCCTGTCTGCTCTGGTC	GGTTCTGCAGCTCCTCCA	ATCGACATGGCCTCCAGGACCC
Nr4a2	TCGACATTTCTGCCTTCTCC	CCACTCTCTTGGGTTCCTTG	TGCTGCCCTGGCTATGGTCACA
Nupr1	CAACCTGTAAACATAGAGGACGAA	CTCCACCGACGACATAAGAT	ATGGGATCCTGGATGAGTATGACCAGTACA
Per1	CACCTCAGCCAGCATCACT	CTCCAGCTTCAGCCTCTGA	ACAAGCAAGTACTTTGGCAGCATCGAC
Per2	CTACCGCCATCGACGTAAC	TCCTCATATGGCAGACAAAGG	TGTTTACTGTGAAAGTGAGGAGAAAGGCA
Ptgs2	TCAAAGACACTCAGGTAGACATGATCT	CGGCACCAGACCAAAGACTT	CACGTCCCTGAGCACCTGCGG
Ren	GCAGGACCTACACTCTCAGCA	GAGCCAGTATGCACAGGTCA	TGTGCAAAAGAATCCCTTCAGGAACG
Scarb1	GGTACTGCAAGAAGCCAAGC	GGTCTGACCAAGCTATCAGG	CCAGAAGACACCACGAGCCCCC
Serpine3	AAGATCCAGGGACTGATCACA	CCTTCCATTTGCCTTTAAAGTAG	CCTAGCTAAGAAGACATCCATGGTACTGGTGAAT
Serpine1	GGCCGACTTCACAAGTCTTTC	CGATCCTGACCTTTTGCAGT	ACCAAGAGCAGCTCTCTGTAGCACAGG
Slc51a	GCTTGCTCACCTCCCTATTC	GGTCTCCAGGATGAGCATGT	AAAATCAGGTCCCAAGTGATGAACTGTC
Sod2	AAGGAGCAAGGTCGCTTACA	AATCCCCAGCAGTGGAATAA	CCACTGCAAGGAACCACAGGCC
Spock2	AGGAGGCAGCCAAGAAGAAG	CTGCTCTGGTCACACTGCAT	TCATTCCGAGCTGTGATGAAGATGG
Spp1	AGCCATGACCACATGGACGA	GATTCGTCAGATTCATCCGAGT	AGACCATGCAGAGAGCGAGGATTCTGTG
Tgfb1	CTTTTGACGTCACTGGAGTTGT	CTGTCACAAGAGCAGTGAGC	CAGTGGCTGAACCAAGGAGACGGAAT
Th	TGTGTCCGAGAGCTTCAATG	CGGGTCAAACTTCACAGAGA	CGCCAAGGACAAGCTCAGGAAC
Timp1	CCGCAGCGAGGAGTTTCTC	GGCAGTGATGTGCAAATTTCC	TCGCGGGCCGTTTAAGGAA
Timp4	GAGAGCCTGAATCATCACTAC	ATGGTACACGGCACTGCATA	AACTGTGGCTGCCAAATCACCACTT
Tnfrsf11b	GCTGTGGAAGCATCAAAACA	CGTTGCACACTGCTTTCAC	CCAGGAAATGGTGAAGAAGATCATCCA
Tsc22d3	GGAGGGGATGTGGTTTCCGT	CTTGTTGTCTAGGGCCACCAC	AACTGGATAACAGTGCCTCTGGAGCCAGC
Vamp1	TGTGCCATCATCGTGGTAGT	CTTGGATGGCAATGGAGAA	TGAGAATGTGCCATCCCTTCCCTG

Zbtb16	GAGCAGCACAGGAAGCTG	CAGGAACCGTTTTCCACAG	ACAGTGGGATGAAGACATACGGATGCGA

Table 4. List of primers used for qRT‐PCR.

4.5.3 Computations

For the study of the tissue, the relative quantity (Q) was normalized to the expression of RPL32 using the following formula:

nQ = {2[minCt(gene of interest) ‐ Ct(gene of interest)]}/{2[minCt(RPL32) ‐ Ct(RPL32)]}

4.6 Statistical Analysis

All data are expressed as means ± standard error of the mean and using GraphPad Prism 5 software (La Jolla, CA, USA). Data for multiple observations over time were compared between groups by 2-way ANOVA. For comparing means of two or more groups of data, one-way ANOVA was applied when appropriate. Differences were considered statistically significant if $p<0.05$.

5. RESULTS

The following results are presented as mean ± standard error. Two experimental groups were analyzed, with the rats receiving either a NS diet or a HS diet until adulthood (24 weeks), and both groups were further subdivided by sex for a total of four treatment groups.

For all four groups, general data, such as body weight and blood pressure, and the organ weights were collected. The focus of this study was the investigation of sex specific differences in phenotype and genotype. Blood pressure was measured at 5, 9, 13, 17 and 21 weeks of age by tail-cuff system. Twenty-four–hour urine samples were collected in metabolic cage experiments and plasma was drawn from the tail vein under anesthesia at weeks 6, 12, 18, and 24.

5.1 Systolic blood pressure

The SBP was measured non-invasively using the tail-cuff method (see Section 4.3).

In male rats, there was no difference in SBP between the HS group and the NS group (NS) before week 5. Interestingly, from week 9 until the end of the study, the SBP was significantly elevated in HS males compared to NS males (week 9: NS=119±13, HS=144±18; week 13: NS=130±16, HS=159±24; week 17: NS=130±9, HS=149±24; week 21: NS=129±17, HS=153±25). In the female counterparts, the HS diet resulted in a significant elevation of the blood pressure only starting from week 13 of the experiment when compared to the NS fed females (week 13: NS=134±7, HS=147±17. Week 17: NS=132±11, HS=148±19. Week 21: NS=127±11, HS=150±18).

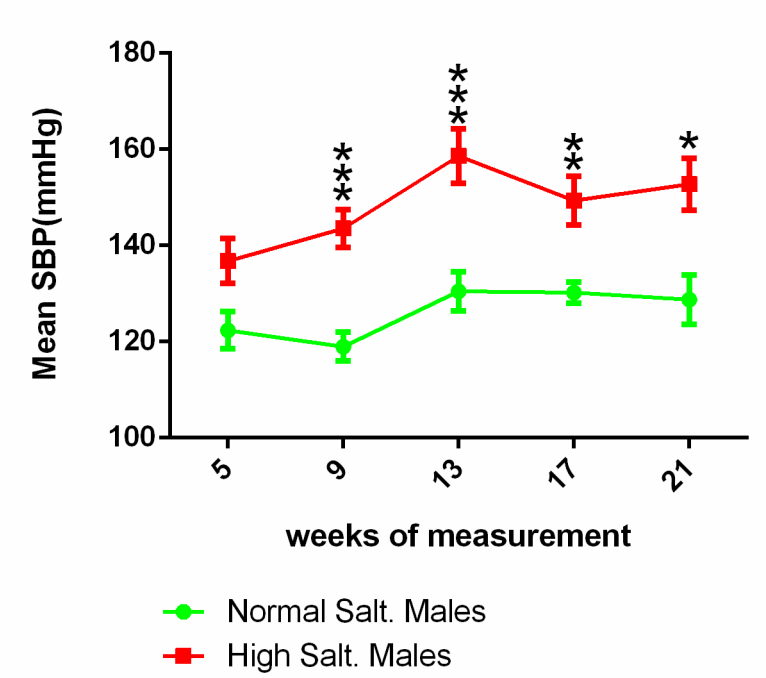

Figure 1. Mean systolic blood pressure of males. Values are given as mean ± SEM. ***P < 0.001, **P < 0.01,*P < 0.05.

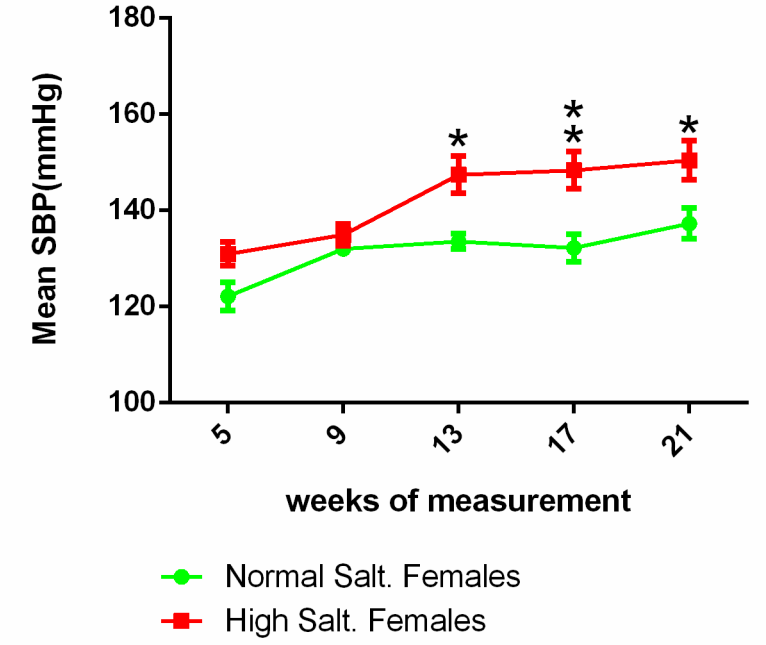

Figure 2. Mean systolic blood pressure of females. Values are given as mean ± SEM. ***P < 0.001, **P < 0.01,*P < 0.05.

5.2 Body weight

Growth curves were calculated using the body weights of the animals at different time points, as daily weighing was done from the first day of life until the 35th day of life, with

weighing twice weekly after the age of 35 to 168 days. There were no significant differences between NS and HS fed animals, either in males or in females.

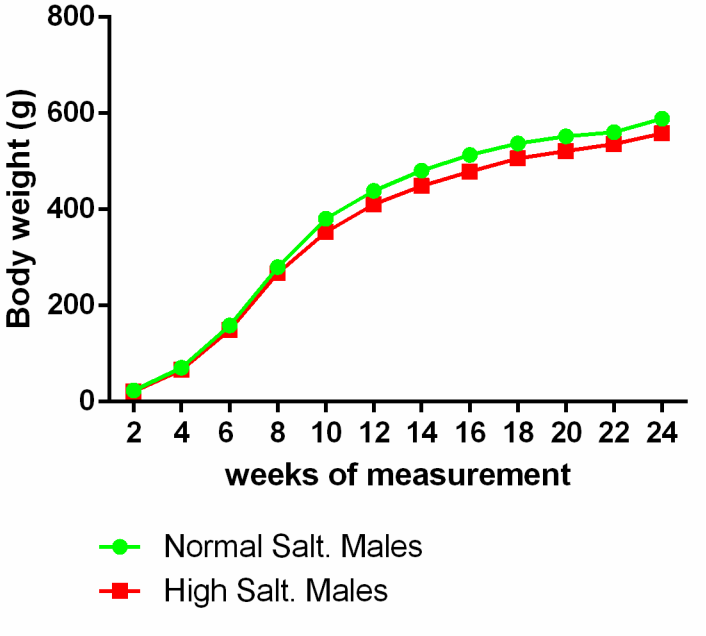

Figure 3.Body weights of males. Values are given as mean ± SEM.

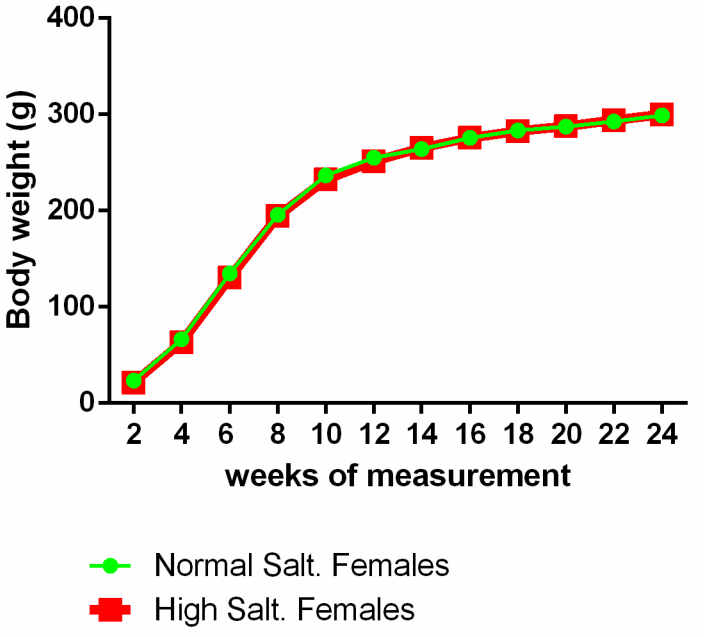

Figure 4. Body weights of females. Values are given as mean ± SEM.

5.3 Organ weight

The organ weights were measured after their removal and related to the body weight of rats.

5.3.1 Heart weight

The absolute heart weight (Figure 5) of HS males was significantly higher than the heart weight of NS males and HS females. The hearts of HS females weighed significantly more than hearts of NS females.

The relative heart weight (Figure 6) was higher in HS males than in NS males but lower than in HS females. HS females had a higher relative heart weight compared to NS females.

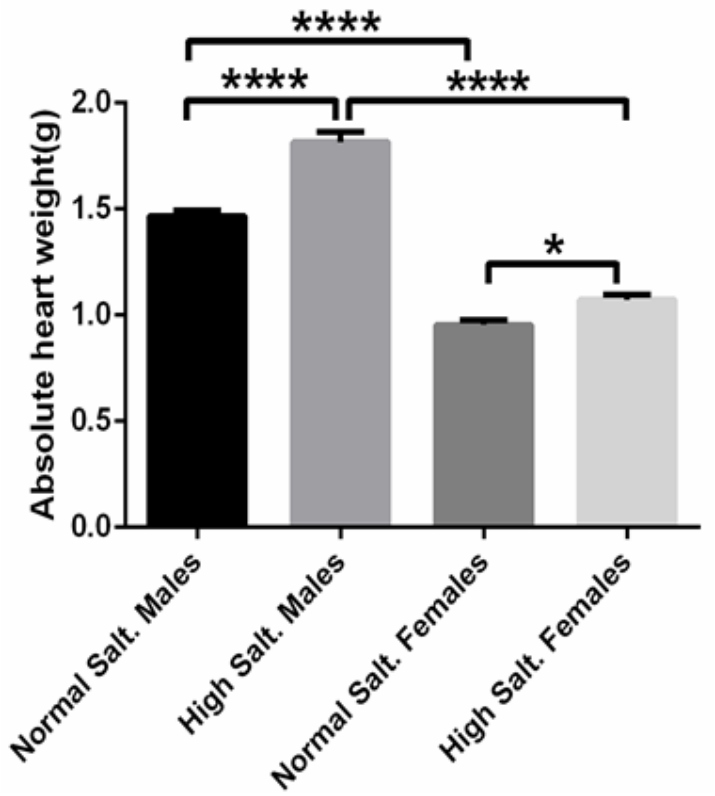

Figure 5.Absolute heart weights of males and females. Values are given as mean ± SEM. ****P < 0.0001, *P < 0.05. Data were found to be significantly different using anova.

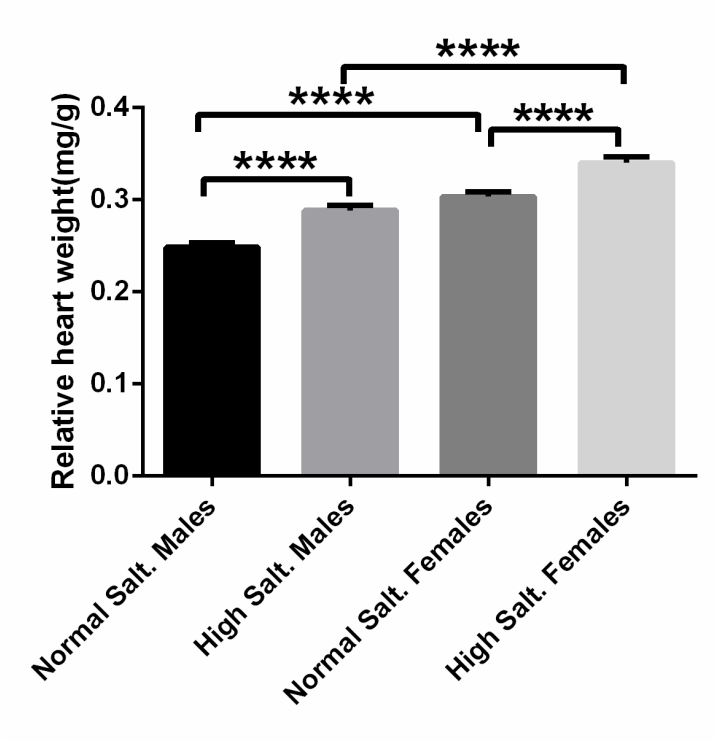

Figure 6. **Relative heart weights of males and females. Values are given as mean ± SEM. ****P < 0.0001. Data were found to be significantly different using anova.**

5.3.2 Kidney weight

The absolute kidney weight (Figure 7) of HS males was higher than the absolute kidney weight of NS males and HS females, whereas it was lower in NS females than in HS females and NS males.

The relative kidney weight (Figure 8) was higher in HS males than in NS males but lower than HS females. NS females had a lower relative kidney weight than HS females but a higher kidney weight than NS males.

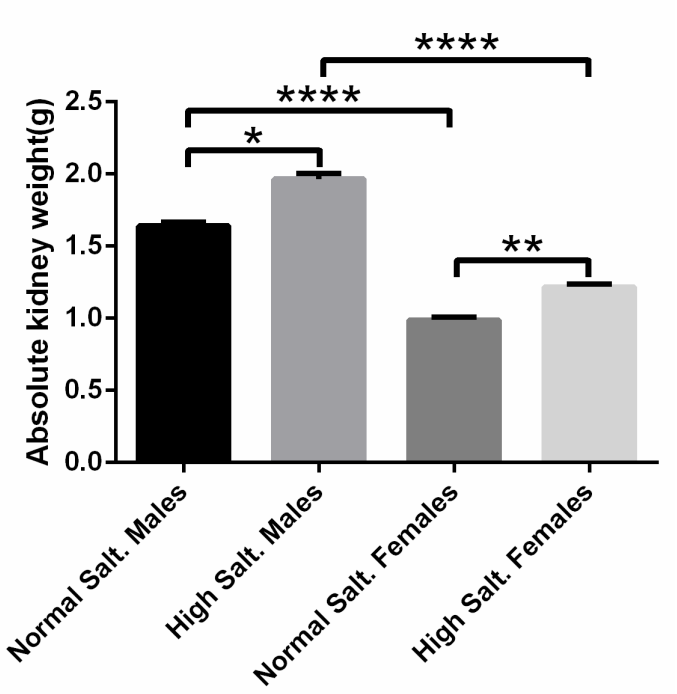

Figure 7. Absolute kidney weights of males and females. Values are given as mean ± SEM. ****P < 0.0001, **P < 0.01, *P < 0.05. Data were found to be significantly different using anova.

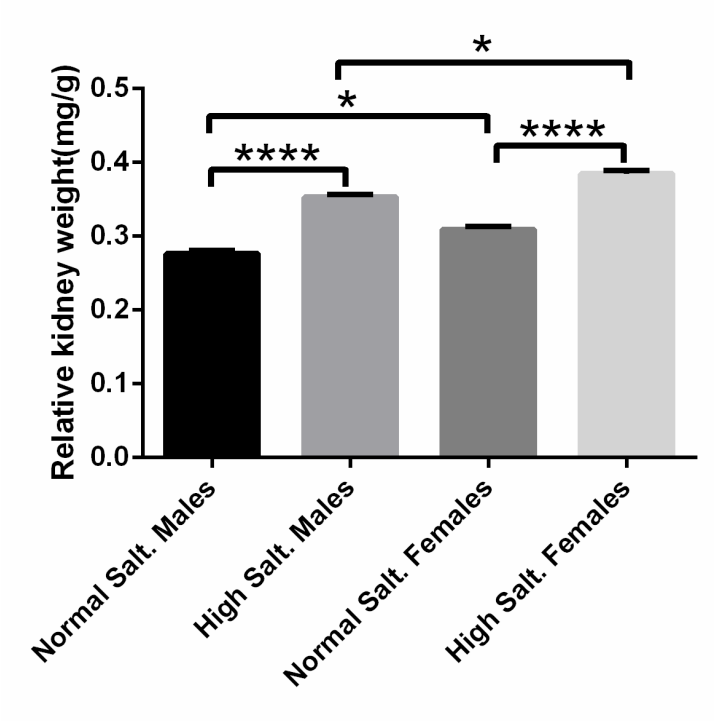

Figure 8. Relative kidney weights of males and females. Values are given as mean ± SEM. ****P < 0.0001, *P < 0.05. Data were found to be significantly different using anova.

5.4 Urinary / renal function and plasma hormone

Urinary chlorine and sodium levels were significantly higher, whereas the potassium was lower in both males and females of the HS fed groups compared to the NS fed groups. Adrenocorticotropic hormones did not show significant differences between the study groups. Corticosterone was higher in females compared to males in both in the NS and HS fed groups. The total protein-creatinine ratio was significantly higher in HS groups compared to the NS groups in both males and females. Females had lower levels than males in both the NS and the HS groups.

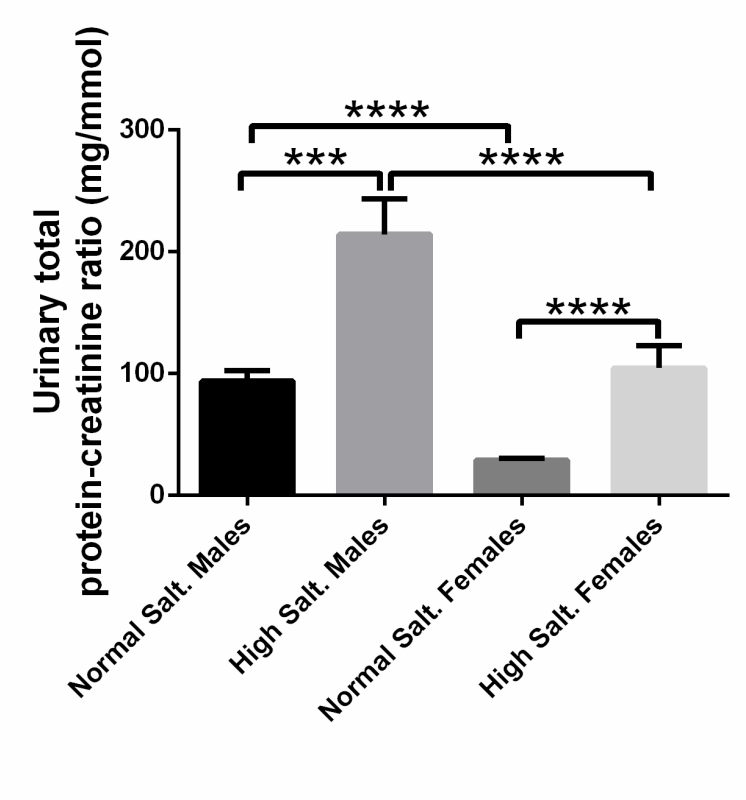

Figure 9. Urinary total protein-to-creatinine ratio in male and female rats. Values are given as mean ± SEM. ***p<0.001; ****P<0.0001. Data were found to be significantly different using anova.

Parameters	Normal-salt males	High-salt males	Normal-salt females	High-salt females
Urinary chlorine, (mmol/l)	68.44±11.76	167.38±13.52****	61.46±5.19	180.07±10.56^{aaaa}
Urinary potassium (mmol/l)	95.11±17.30	48.88±3.47**	84.07±7.46	47.29±3.00^{aa}

Urinary sodium (mmol/24h)	45.11±5.53	167.25±12.83****	46.46±3.61	176.50±9.87^{aaaa}
Urinary corticosterone (ng/mL)	18±5.05	16.38±2.74	14.11±2.18	11.01±1.26
Urinary total protein creatinine ratio (mg/mmol)	93.842±8.573	214.3±28.92***	29.04±1.28****	104.6±18.28^{aaaa,bbbb}

Table 5. Urinary parameters. Values are mean ±SEM. *p<0.05; **p<0.01; ***p<0.001; ****P<0.0001 versus normal salt males. ^{a}p<0.05; ^{aa}p<0.01; ^{aaa}p<0.001; ^{aaaa}P<0.0001 versus normal salt females. ^{b}p<0.05; ^{bb}p<0.01; ^{bbb}p<0.001; ^{bbbb}P<0.0001 versus high-salt males

Parameters	Normal-salt males	High-salt males	Normal-salt females	High-salt females
Corticosterone (ng/mL)	368.36±18.28	324.95±23.20****	600.16±46.96	485.21±44^{aaa}
Adrenocorticotropic hormone (pmol/L)	28.33±2.56	25.43±3.29	18.22±3.81	20.31±1.96

Table 6. Plasma hormone levels. Values are mean ±SEM. *p<0.05; **p<0.01; ***p<0.001; ****P<0.0001 versus normal salt males. ^{a}p<0.05; ^{aa}p<0.01; ^{aaa}p<0.001; ^{aaaa}P<0.0001 versus normal salt females.

5.5 Histology

5.5.1 Cardiac histology

Interstitial and perivascular fibrosis in cardiomyocytes was measured after Sirius red staining in six months old rats. Sirius red non-specifically binds extracellular matrix proteins, thus indicating the fibrotic state of the tissue. The role of the myocardial extracellular matrix is mainly to maintain muscle flexibility; its composition is therefore related to myocardial function. Likewise, the composition of the perivascular matrix plays an important role in the ability of a vessel to contract and dilate. Perivascular fibrosis and interstitial fibrosis were not impaired by the HS diet in males or females.

An increase in cardiac myocyte size may be the consequence of pathophysiological triggers such as hypertension or aortic stenosis; therefore, the cardiomyocyte diameter was assessed as an indicator for cardiac hypertrophy using HE-staining. However, no difference between groups was observed for this readout.

Parameters	Normal-salt males	High-salt Males	Normal-salt females	High-salt females
Heart perivascular fibrosis (%)	1.90±0.26	1.93±0.49	1.83±0.48	1.90±0.25
Heart interstitial fibrosis (%)	4.25±1.00	2.52±0.43	2.84±0.22	4.24±0.47
Heart cardiomyocyte Diameter (μm)	57.20±1.53	53.00±1.73	58.75±1.65	54.83±0.91

Table 7. Parameters of cardiac histology. Values are given as mean ± SEM. No significant differences were observed.

5.5.2 Kidney histology

Increased interstitial extracellular matrix deposition in the kidney is a hallmark feature of different chronic kidney diseases, and fibrosis of functional tissues contributes to loss of function in the organ. In this study, perivascular fibrosis and interstitial fibrosis of the kidney were not significantly different in HS fed animals compared with NS fed animals, either in males or in females. Hypertension may cause vascular remodeling of kidney vessels, but the media-lumen-ratio as a readout of vascular remodeling was not changed by the HS diet, either in males or in females. Morphologic changes of the glomerulus may be indicative of glomerular diseases and are therefore pathophysiologically relevant. However, glomerular size and glomerulosclerosis were not significantly different in the HS groups compared with the NS groups.

Parameters	Normal-salt males	High-salt Males	Normal-salt females	High-salt females
kidney media-lumen ratio	2.42±0.12	2.79±0.25	2.58±0.17	3.11±0.11
Kidney interstitial fibrosis (%)	2.08±0.89	3.12±0.91	2.14±0.21	4.74±0.39
Kidney perivascular fibrosis (%)	1.47±0.13	1.52±0.13	1.52±0.0.4	1.44±0.02
Kidney glomerulosclerosis index	1.08±0.03	1.23±0.13	1.12±0.03	1.2±0.06

Glomerular size (x 10^3 square pixels)	443.50±22.11	444.86±29.54	350.86±10.59	390.98±17.80

Table 8. Parameters of kidney histology. Values are given as mean ± SEM. No significant differences were observed.

5.6 Gene expression

One aim of this study was to investigate sex-specific changes of the genome in response to a HS diet. Numerous genes demonstrated significant differences when compared between treatment groups and between sexes. Here, only genes with sex-specific differences are presented. Data from all other genes analyzed during this study are displayed in the supplementary table.

5.6.1 Effects of a high-salt diet on gene expression in the heart

The feeding of a HS diet led to a significant up-regulation of the transforming growth factor and beta 1(*Tgfβ1*) in the heart in males compared to females. Matrix metalloproteinase-2(*Mmp-2*) was significantly up regulated in HS males compared with NS males and HS females; on the other hand, this gene was down-regulated in NS females versus NS males. HS males had a significant up-regulation of the cardiac gene matrix metalloproteinase-9 (*Mmp-9*) compared to HS females. Lipocalin-2(*Lcn-2*) was up-regulated in in HS females and NS males compared to NS females. The gene for osteopontin (*Opn*) was up-regulated in HS males compared to HS females. Remarkably, monocyte chemoattractant protein-1(*Mcp-1*) was down-regulated in HS females compared to HS males.

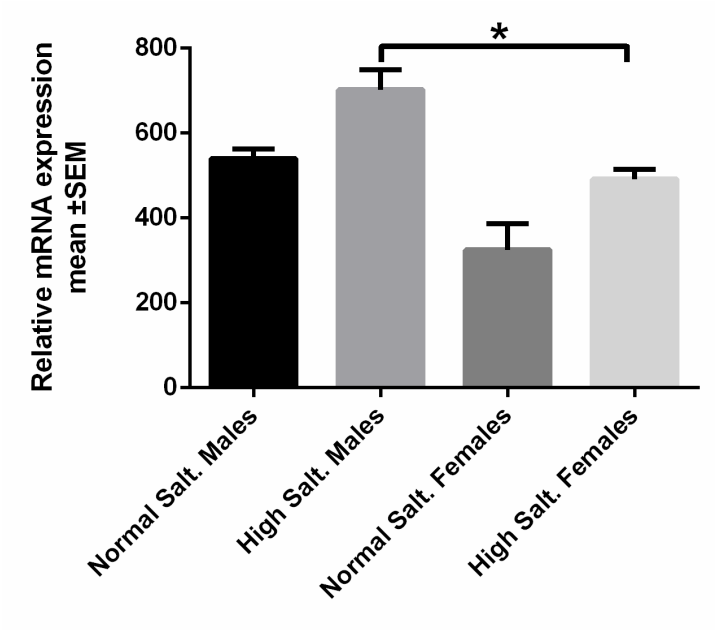

Figure 10. Relative gene expression levels of transforming growth factor and beta 1 in the heart. Values are given as mean ± SEM. *P < 0.05. Data were found to be significantly different using anova.

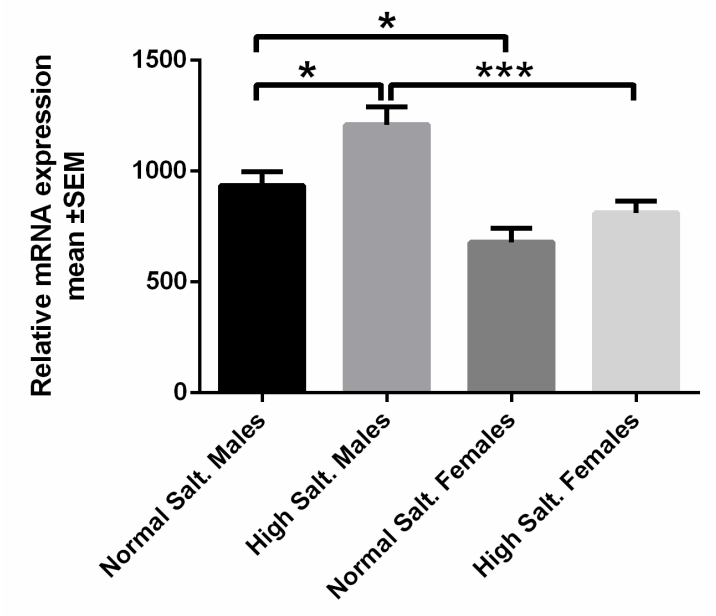

Figure 11. Relative gene expression levels of matrix metalloproteinase-2 in the heart. Values are given as mean ± SEM. ***P <0.001, *P < 0.05. Data were found to be significantly different using anova.

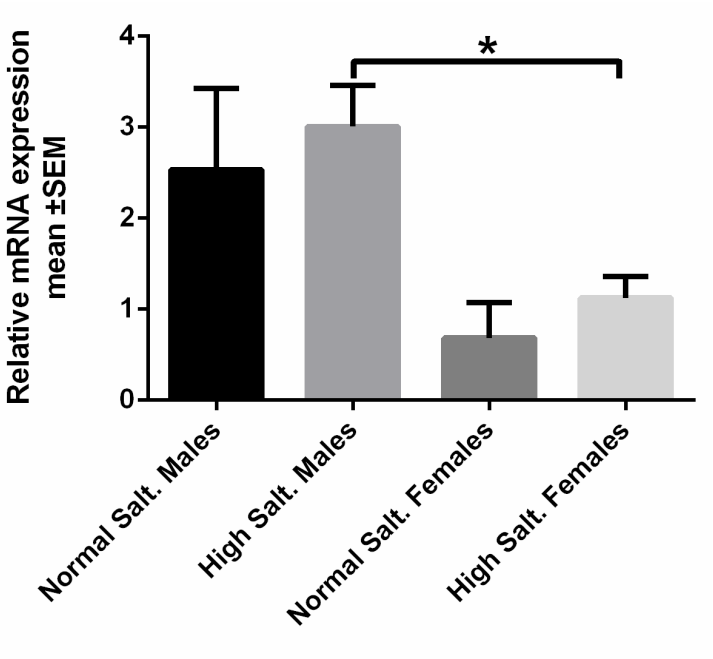

Figure 12. Relative gene expression levels of matrix metalloproteinase-9 in the heart. Values are given as mean ± SEM. *P < 0.05. Data were found to be significantly different using anova.

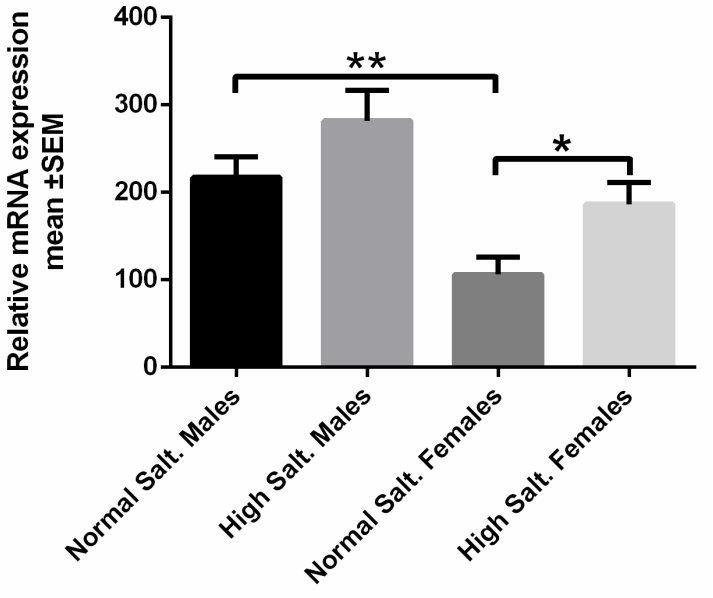

Figure 13. Relative gene expression levels of lipocalin-2 in the heart. Values are given as mean ± SEM. *P < 0.05, **P < 0.01. Data were found to be significantly different using anova.

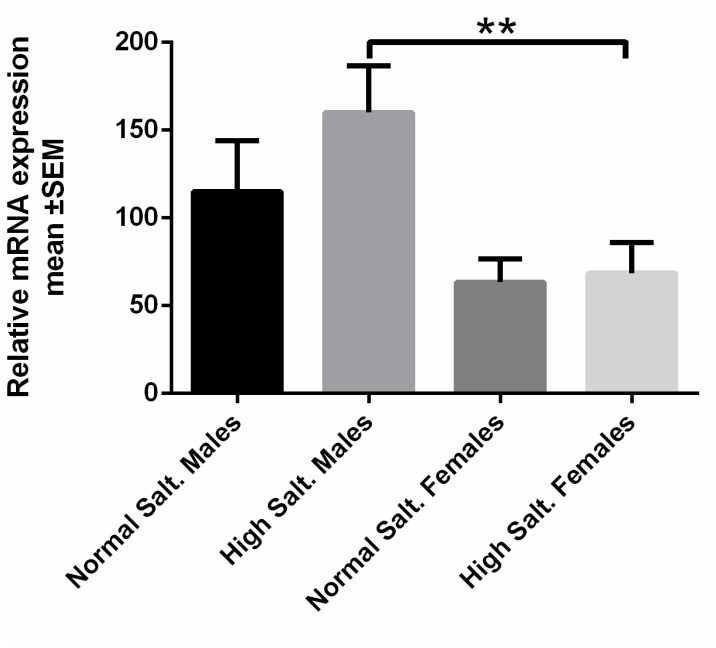

Figure 14. Relative gene expression levels of osteopontin in the heart. Values are given as mean ± SEM. **P < 0.01. Data were found to be significantly different using anova.

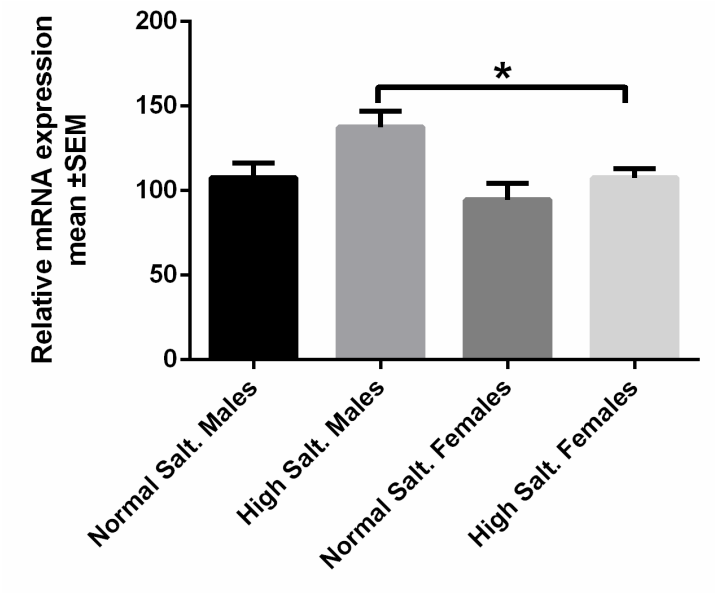

Figure 15. Relative gene expression levels of monocyte chemoattractant protein-1 in the heart. Values are given as mean ± SEM. *P < 0.05. Data were found to be significantly different using anova.

5.6.2 Effects of a high-salt diet on gene expression in the kidney

The feeding of a HS diet led to a significant up-regulation of the renal *Lcn-2* gene in males compared to NS males and HS females. Zinc finger and BTB domain-containing 16(*Zbtb16*), cytochrome P450 family 27 subfamily B member 1(*Cyp27b1*) and *Tgfβ1* were

up-regulated in HS males compared to NS males and HS females. Feeding of a HS diet led to a significant down-regulation of the renal genes cytochrome P450 family 24 subfamily A member 1(*Cyp24a1*) expression in males and females compared to the NS diet groups. Finally, the gene period circadian clock 1(*Per-1*) was significantly up-regulated in HS females compared to HS males.

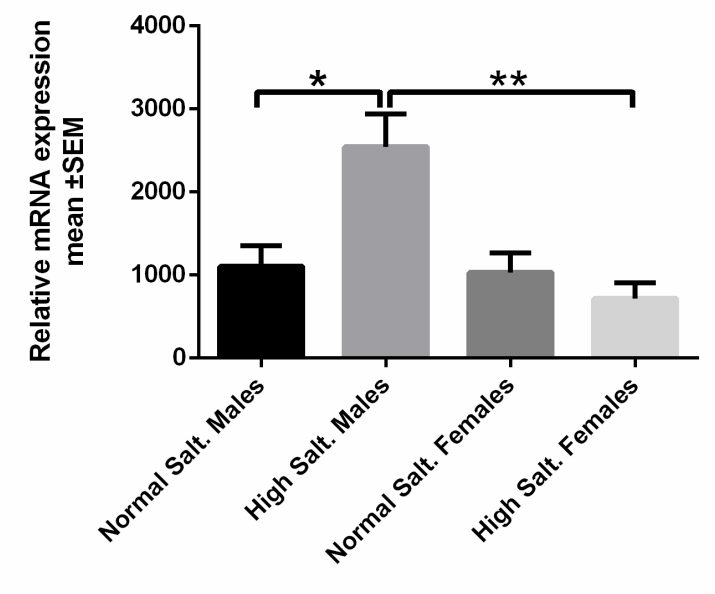

Figure 16. Relative gene expression levels of lipocalin-2 in the kidney. Values are given as mean ± SEM. *P < 0.05, **P < 0.01. Data were found to be significantly different using anova.

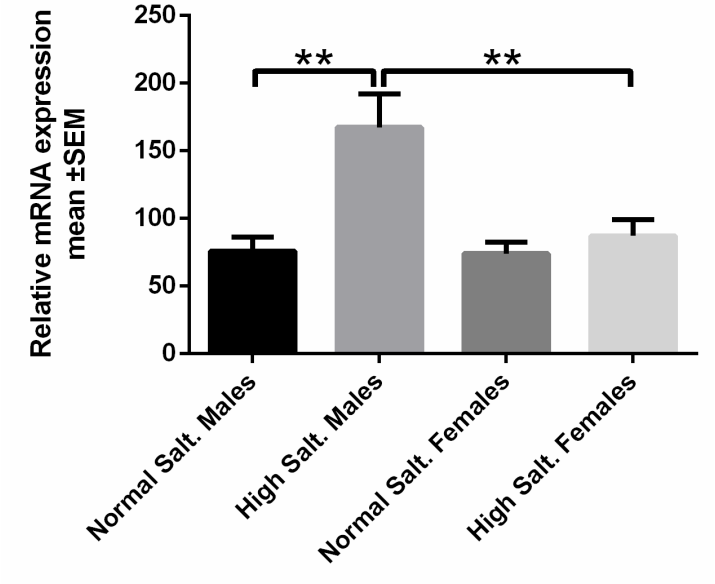

Figure 17. Relative gene expression levels of zinc finger and BTB domain containing 16 in the kidney. Values are given as mean ± SEM. **P < 0.01. Data were found to be significantly different using anova.

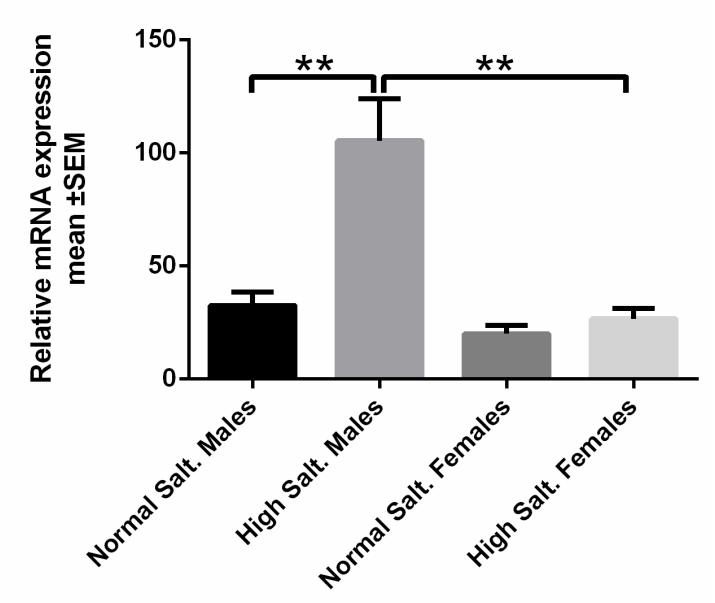

Figure 18. Relative gene expression levels of cytochrome P450 family 27 subfamily B member 1 in the kidney. Values are given as mean ± SEM. **P < 0.01. Data were found to be significantly different using anova.

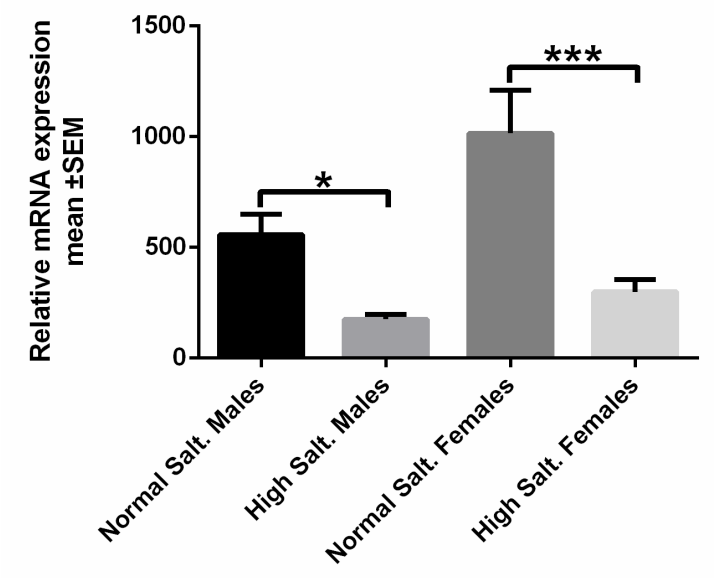

Figure 19. Relative gene expression levels of cytochrome P450 family 24 subfamily A member 1 in the kidney. Values are given as mean ± SEM. *P < 0.05, ***P < 0. 001. Data were found to be significantly different using anova.

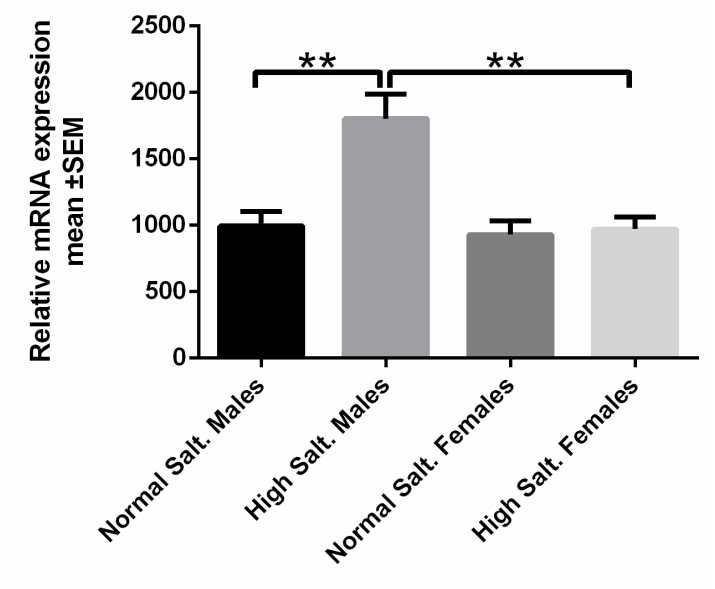

Figure 20. Relative gene expression levels of transforming growth factor and beta 1 in the kidney. Values are given as mean ± SEM. **P < 0.01. Data were found to be significantly different using anova.

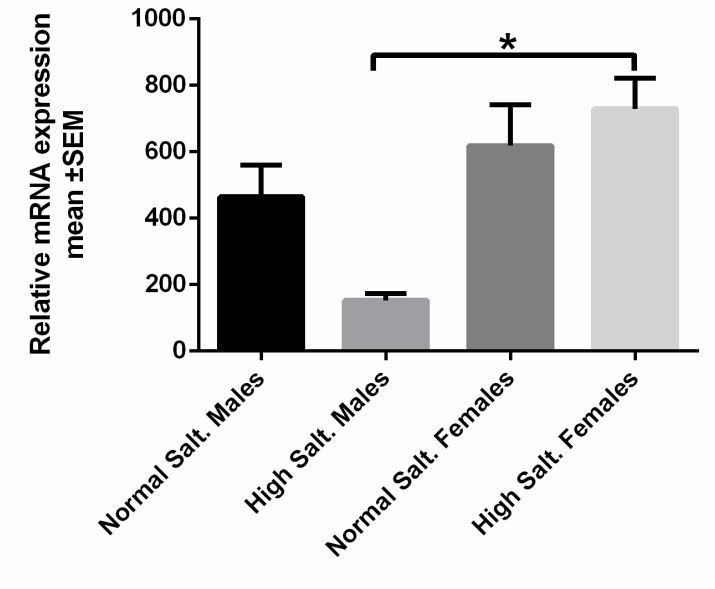

Figure 21. Relative gene expression levels of period circadian clock 1 in the kidney. Values are given as mean ± SEM. *P < 0.05. Data were found to be significantly different using anova.

6. DISCUSSION

The animal experiment investigated the effects of a HS diet on the expression of various genes in different organs of Wistar rats. Physiologic parameters such as blood pressure and kidney function as well as morphology in the heart and the kidney were analyzed. The HS diet was given to rats after weaning and they were compared to rats on a NS diet. A special focus of the study was put on revealing possible sex specific effects of the HS diet on a genetic level. Therefore data were calculated in a sex-specific manner.

Animals on the HS diet developed higher blood pressure and greater organ weights of the heart and the kidneys compared to rats on a NS diet. Urinary total protein-to-creatinine ratio, sodium and chlorine were increased in the HS group, whereas urinary potassium was decreased. Expression of several genes in the heart and the kidney were differently regulated in animals on a HS diet compared to the animals on a NS diet. Interestingly, in some of these genes the effects of the HS diet were different in female versus male rats. Moreover, the effect of the HS diet on blood pressure was also sex-dependent, as female animals had a later onset of high blood pressure than males. Reflecting this finding, the urinary total protein-to-creatinine ratio was significantly higher in male than in female animals.

6.1 Blood pressure and kidney function

Animals on the HS diet demonstrated higher blood pressure and greater weights of the heart and the kidney compared to rats on a NS diet. Moreover, urinary total protein-to-creatinine ratio, sodium and chlorine were higher in the HS groups, whereas urinary potassium was lower in comparison with animals receiving the NS diet. The effect of the HS diet on blood pressure seemed to be sex-dependent, as female animals on HS diet had a later onset of hypertension (defined as BP being significantly higher compared to the control group) than males. In line with this finding, the urinary total protein-to-creatinine ratio at the study end was significantly higher in males on the HS diet than in females. HS intake as an environmental risk factor for the development of hypertension and associated end organ damage has been shown in studies all over the world (6, 7, 77, 78). Studies using experimental animal models as well as studies in humans have led to

two main theories about the primary mechanisms causing salt-dependent hypertension: i) sodium and water retention in the extracellular fluids, which according to the Guyton's theory of impaired renal pressure-natriuresis may explain the associated alterations in blood volume and peripheral resistance; ii) the neurogenic mechanisms that lead to sympatho-excitatory responses, and consequently to an increase in blood pressure (79). Likewise, a sex-specific effect of a HS diet on blood pressure is well known for humans (66) and for several salt-sensitive rat strains (68, 80). Explanatory models for the known sex differences in the development of salt-induced hypertension usually include an important role of sex hormones (80). Whereas female hormones are believed to increase renal sodium excretion and thus elicit protective effects on blood pressure regulation (67, 81), male hormones may contribute to a characteristic resistance to sodium excretion, thus contributing to increased blood pressure in response to a HS intake (69, 82). Other mechanistic explanations for sex hormone dependent actions of blood pressure regulation include endothelium-dependent relaxation as an effect of estrogens, lipoprotein level regulation by testosterone, and others (83). In our study, we were able to show a sex-specific regulation of several genes in the heart and the kidney possibly involved in blood pressure regulation and kidney function.

An important mechanism for the control of the salt and water balance and blood pressure regulation is the renin-angiotensin system (RAAS), and the essential genes of the RAAS pathway were indeed differentially expressed in the analyzed organs. In the kidney, renin expression (supplementary table) was significantly down-regulated by the HS intake in males and in females, which can be interpreted as an overall suppression of the RAAS as a physiologic response to the increased salt load (84). In contrast, other animal experiments showed that a HS diet induced an up-regulation of different renal RAAS components (85, 86). However, the contribution of an activated intrarenal RAAS for the development of salt-induced hypertension is not well defined so far. An explanation for this discrepancy could be that a 4% NaCl HS diet was used in the present study, whereas the studies reporting a HS-diet-induced up-regulation of renal RAAS used an 8% NaCl HS diet (85, 86). As also observed by other researchers, (87, 88) there were no sex-specific differences in the renal renin expression, whereas renal angiotensinogen and

heart angiotensin converting enzyme (*Ace*) expression were significantly higher in male than in female rats in the NS groups. A higher expression of the renal angiotensinogen gene has been described for male Wistar Kyoto rats and spontaneously hypertensive rats in comparison to the female rats of the same strains. Castration decreased the expression, whereas testosterone treatment increased it (87, 88). Male mice and ovariectomized female mice showed a higher expression of renal *Ace2* than intact females. Interestingly, estradiol treatment led to a decreased expression in both sexes (89). These experiments showed that sex-specific differences in the expression of renal RAAS components are probably androgen/estrogen dependent. Another main factor of the RAAS pathway is aldosterone. Binding of aldosterone to the mineralocorticoid receptor regulates the expression of several genes in the pathway of aldosterone-regulated renal Na reabsorption (90). One of these aldosterone-regulated genes, *Zbtb16*, is a transcriptional repressor with pleiotropic effects and is possibly involved in the pathogenesis of hypertension (91). Renal *Zbtb16* was significantly up-regulated in males on a HS diet compared with males on a NS diet. This was not the case in the female groups, and the expression of renal *Zbtb16* was significantly higher in male versus female rats on a HS diet. A possible explanation for these observations could be a compensatory up-regulation of *Zbtb16* because of the higher blood pressure in the male HS group. Since *Zbtb16* has a negative regulatory effect on aldosterone-stimulated sodium reabsorption in the kidney (90), an increased expression could be interpreted as an adaptive mechanism to decrease sodium reabsorption in order to control blood pressure.

In the heart, we saw an increased expression (supplementary table) of atrial natriuretic peptide (*Anp*) and brain natriuretic peptide (*Bnp*) in male and female HS rats, which can be interpreted as a compensatory response to the elevated salt intake and increased blood pressure. Anp and Bnp are released from cardiomyocytes in the atria and ventricles in response to cardiac wall stretch. As physiologic regulators of blood pressure and salt and water excretion, natriuretic peptides (Nps) reduce intravascular fluid and cardiac filling, and decrease blood pressure (92). Due to their vasodilatative action and inhibitory effects on renin and aldosterone secretion, Anp and Bnp are important protective players in cardiovascular diseases and high blood pressure (93). We

did not measure plasma levels of these hormones, but a good correlation between mRNA levels and peptide synthesis of Nps has been reported (94). The increase observed in cardiac gene expression of *Anp* and *Bnp* in animals on a HS diet in comparison to the control animals on a NS diet may represent an increased production of these cardiac hormones in order to induce vasodilatation, diuresis, natriuresis and a reduction of the increased blood pressure. When comparing groups on a HS diet according to sex, *Anp* and *Bnp* expression were significantly higher in male than in female rats. This is rather contrary to sex differences for *Np* often describe in the literature. Female spontaneously hypertensive rats (95), mice (96), and women (97-100) showed higher plasma levels and cardiac expression than their male counterparts. As estrogens (101-104) and apparently also androgens (105, 106) seem to have profound effects on the production and secretion of Nps, the sex specific differences might be partially attributed to the influence of sex hormones (94). Sex specific differences in the cardiac expression of *Nps* in response to an oral HS challenge have not been well defined. In context with the earlier onset of high blood pressure observed in males in our experiment, the increased *Anp* and *Bnp* expression in male rats could reflect an increased compensatory up-regulation of *Nps* as a response to the developing hypertension.

6.2 Histology of the heart, and the kidney

Histomorphological analyses of the heart and the kidney did not show abnormalities in rats on a HS diet. This is to some extent surprising as end organ damage both due to salt-induced hypertension as well as blood pressure independent detrimental effects of HS intake, have been described extensively (107-110). However, there are well known differences in susceptibility to HS for different rat strains (111, 112) and experimental approaches investigating the effects of a high dietary salt intake use specifically sensitive rat strains like the DAHL or spontaneously hypertensive rats (SHR) and/or a rather high amount of 8% salt in the diet (112, 113). Lower doses of dietary salt are usually combined with a chronic infusion of angiotensin for the angiotensin II-induced hypertension model (114). The rats used in this project were conventional normotensive Wistar rats fed with a 4% HS diet. Wistar rats are known to be more resistant to morphologic consequences

of experimental renal mass reduction than other strains and apparently the structural lesions are less pronounced in males than in females (115). It is possible that this rather resistant rat strain and the 4% instead of 8% salt might explain in part the lack of structural changes in the heart and the kidney despite clinically overt hypertension and proteinuria. Similar to the findings from our study, *Gomes et al.* did not see any morphological changes in the heart and kidney after 12 weeks of feeding a HS diet to Wistar rats despite a significant elevation of blood pressure (116). In their study, though, the salt content of the diet was only half of the amount used in our experiment. In addition, *Berger et al.* found that feeding a HS diet of 3% for 6 months to SHR, although increasing SBP, did not lead to morphological changes like hypertrophy or fibrosis of the heart (117). We also had a study duration of 6 months, so maybe this period was not long enough to detect of structural organ changes like fibrosis and hypertrophy on a histological level.

The investigation of molecular pathways from different perspectives can increase the chances of identifying developing pathomorphological processes. Therefore, we supported the histological analyses by investigating of gene expression in the heart and the kidney. Changes on the genetic level may precede visible morphological alterations and can be considered as early markers of disease development and progression (118). Interestingly, we found an up-regulation of several genes indicative of structural adaptations in the kidney and in the heart.

6.3 Expression of extracellular matrix (ECM)/fibrosis-related and inflammatory genes/pathways

Investigation of tissue-specific gene expression in the heart and the kidney revealed a significant up-regulation of several genes in male HS rats but not in female HS rats compared to the respective control group on a NS diet. There were also differences in the expression between male and female rats on a NS diet, but except for *Ace*, these sex specific differences in expression were more evident in the groups on a HS diet, especially for genes known to be involved in cardiac and renal remodeling processes such as inflammation, hypertrophy and fibrosis. Comparable to our findings, a recently published meta-analysis aimed at identifying changes in cardiac gene expression during

the development of heart failure in the Dahl salt-sensitive rat fed a high-sodium diet found changes in pathways and genes of cardiac remodeling, inflammation and fibrosis. However, in this meta-analysis there was no information on sex differences (119).

Inflammatory, hypertrophic and fibrotic changes of the heart are known responses to pressure overload and are closely interconnected processes (120). Moreover, myocardial and renal fibrosis are suggested to be blood pressure independent consequences of a HS intake (107). Tissue fibrosis is characterized by an increased biosynthesis and a decreased degradation of collagen and other extracellular matrix (ECM) components. By inducing the proliferation of fibroblasts and stimulating ECM deposition, Tgfβ1 plays an essential role in the formation of fibrosis (121). Other factors of tissue fibrosis are matrix metalloproteinases (Mmps), which represent the crucial enzymes for the ECM turnover, and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (Timps) and the plasminogen activator inhibitor type1 (Pal1) (122). If the tight balance between Mmps, Timps and Pal1 is disturbed, this may result in an excessive deposition of fibrotic ECM material, including collagen, resulting in a pathologic remodeling of the organ (123). In our experiment, we saw an up-regulation of renal collagen III and *Tgf-β1* as well as an up-regulation of cardiac *Timp1* and *Mmp2* in male rats on a HS diet in comparison to male rats on the NS diet. The up-regulation of profibrotic markers probably indicated an abnormal ECM turnover as a consequence of the HS intake and developing hypertension. We did not see the same effects of the HS diet in female rats, which is in line with their later onset of hypertension.

In general, a sexual dimorphism for hypertension and cardiovascular health and disease has been the subject of sex based research for a long time (124). An important part of that field is investigations of molecular sex-based differences on a transcriptional level (125-127). It is known that younger women are generally less susceptible to the deleterious effects of volume overload and the manifestation of certain other cardiomyopathies, and that there are tissue-specific differences in gene transcription between the sexes (124-128). In our study, we saw sex differences in cardiac and renal gene expression for several genes (supplementary table) between male and female rats in the groups with a NS diet, and as such, animals representing healthy control cohorts:

neutrophil gelatinase associated lipocalin 2 *(Ngal),* endothelial nitric oxide synthase 3 (*Nos3*), Interleukin 1 Receptor Like 1(*Il1rl1*), angiotensin I converting enzyme (*Ace*), *Timp4, Mmp2,* and myosin heavy chain-7 (*Mhc7*). Of these genes, the expression of *Mmp2* and *Mhc7* was significantly higher in males on a HS diet compared to males on the NS diet, but it was not significantly different between female rats with a HS diet versus a NS diet. This allows the conclusion that, despite preexisting baseline differences, the HS diet still led to a more pronounced up-regulation of *Mmp2* and *Mhc7* in the male heart.

Moreover, a sexually dimorphic response to HS intake and blood pressure was shown by significant differences between male and female rats on a HS diet for several other genes (supplementary table) involved in remodeling processes such as hypertrophy and fibrosis. Male HS rats had a significant higher expression of renal *Tgf-β1* and cardiac *Mhc7,* growth differentiation factor 15 *(Gdf-15), Galectin-3, Ace, Opn, Timp1, Mmp9, Tgf-β1* and *Serpine 1* (the gene for Pal 1). These findings suggest that male rats were more affected by the detrimental effects of the HS consumption.

Sex-specific differences in adverse myocardial remodeling have been described for rodent models of myocardial infarction (129), volume overload (65), and ischemia-reperfusion injury (130) as well as for human cohorts of different cardiomyopathies (128). So far, the detailed mechanisms and molecular basis for a sex-specific remodeling are not completely clear, but besides genetic and/or epigenetic factors (131), a modifying role of sex hormones and their respective receptors is being assumed (132). Though some studies have looked at the effects of testosterone (133-135), the role of male sex hormones has not been as well investigated as the impact of estrogens. In ovariectomized rats with left ventricular hypertrophy, estrogen replacement improved ventricular TIMP-MMP balance, blocked MMP-9 activation, and attenuated perivascular fibrosis (136). Interestingly, gonadoectomized rats also showed an increased activity of MMP-9 and MMP-2, probably an effect of testosterone insufficiency, as testosterone replacement normalized the activity of these enzymes (135).

Pre-menopausal women have a lower prevalence of cardiovascular diseases than post-menopausal women, and men have an earlier onset of cardiovascular disease than women, and this has been attributed to the influence of estrogens (131). However, data

from large interventional trials with hormones have come up with conflicting results (132, 137) and there are still many unanswered questions about the role of sex hormones in the pathogenesis and treatment of cardiovascular diseases. In addition, it is known that estrogens modulate cardiac fibroblast growth, which may account for protective effects on remodeling (138). The cardioprotective mechanism in females level may involve an interaction on a molecular between estrogen and expression of proteins of the extracellular matrix, like cardiac MMPs, TIMPs, TGF-β1 and collagens (132, 136, 138-141). Oestrogenic effects on the heart tissue could include inhibition of the expression of estrogen-responsive genes such as collagens and MMPs (142, 143). Fittingly, we observed a significantly lower expression of such markers in the female rats on a HS diet compared to the male rats on a HS diet.

Other potentially protective mechanisms of estrogens include effects on immune cell function and inflammation (144, 145). In general, there are known sex discrepancies in immune responses which may be modulated by an estrogen/estrogen receptor (ER) signaling (145). Interestingly, ER-knockout mice with pressure overload-induced hypertrophy showed a significant increased activation of cardiac inflammatory signaling compared to wild-type mice (146). Moreover, in heart biopsy samples of patients with aortic stenosis, inflammation-related genes were repressed to a higher degree in the female than in the male biopsy samples (140). In our experiment, a possible estrogen-related protective effect against HS -induced pro-inflammatory pathomechanisms could be reflected by a decreased expression of pro-inflammatory markers in female cardiac tissue. We saw a significant lower expression of monocyte chemoattractant protein-1 (*Mcp-1*), *Galectin-3* and *Opn* in female rats on a HS diet compared to male rats on a HS diet. MCP-1 is a known pro-inflammatory cytokine, and animals and patients with heart failure show elevated plasma levels of MCP-1 (147, 148). Transgenic mice over-expressing murine MCP-1 in the heart display an increased recruitment of monocytes to the myocardium and subsequent myocarditis and heart failure (149, 150). Increased myocardial levels of *Mcp-1* might play an important role for fibroblast proliferation and collagen production, and therefore for cardiac remodeling (151). Blocking of *Mcp-1* signaling in a mouse model of myocardial infarction attenuated interstitial fibrosis and

macrophage infiltration, possibly due to a decreased *Mmp-9* activation and myocardial *TNF-α* and *Tgf-β* gene expression (151). Furthermore, *Mcp-1* knockout mice are protected against the inflammatory and fibrotic response of a continuous infusion of angiotensin-II (152). Like MCP-1, the two matricellular proteins Galectin-3 and OPN have been suggested to be crucial regulators of cardiac inflammation and fibrosis (123, 153). Both osteopontin and galectin 3 can act as cytokines and could therefore play a role in collagen disposition and degradation, and as such in cardiac fibrosis and remodeling (119, 153). This may be partly due to their chemoattractant properties for monocytes and macrophages (154) and their impact on fibroblast adhesion and proliferation (123, 155).

A higher expression of the proinflammatory markers *Mcp-1*, *Galectin-3* and *Opn* in male rats on a HS diet compared to female rats on a HS diet could suggest a blunted activation of pro-inflammatory pathophysiological pathways, because of a protective influence of estrogens.

The overall picture we saw in our study was a higher expression of pro-inflammatory and pro-fibrotic markers in male rats on a HS diet, probably reflecting a higher activation of pro-inflammatory and pro-fibrotic pathophysiological pathways which are closely interlinked.

6.4 Expression of Clock and Vitamin D metabolism-associated genes

The circadian clock is responsible for synchronizing our daily physiological activities with time, even on a molecular level. Many physiological functions, including blood pressure, show circadian rhythms (156, 157). *Solocinski et al* reported the involvement of the clock protein Per1 in the regulation of blood pressure (157). They showed that the administration of a 4% HS diet plus mineralocorticoid led to an increased blood pressure in *Per1*-knockout mice but not in wild-type mice. This indicated that genetic loss of *Per1* could increase the sensitivity to HS, leading to a higher blood pressure. In line with these results, we found that the renal expression of *Per1* was significantly up-regulated in female rats on a HS diet (the group with a later onset of high blood pressure) compared to male rats receiving a HS diet.

Kidney dysfunction, impaired vitamin D metabolism and cardiovascular diseases are interrelated. Patients with end-stage renal disease are at a higher risk of mortality due to cardiovascular disease. Chronic kidney disease usually leads to uraemia, the formation of uremic toxins like indoxyl sulphate, elevated levels of circulating fibroblast growth factor 23 *(Fgf23)* and disturbances in parathyroid hormone secretion. These events are associated with impaired vitamin D production and a disturbed mineral homeostasis, leading to an increased risk for uremic vascular calcification. Current literature suggests that uremic vascular calcification might be the major culprit for poor cardiovascular outcomes in patients with chronic kidney disease (158). In the present study, the renal expression of the vitamin D activating enzyme 1-α hydroxylase (*Cyp27b1*) was significantly up-regulated in male rats on a HS diet compared to female rats receiving a HS diet, whereas the renal expression of the vitamin D deactivating enzyme 24-α hydroxylase (*Cyp24a1*) was significantly down-regulated in male and female rats receiving a HS diet in comparison to control groups. These results indicate that a HS diet led to an impaired vitamin D metabolism and probably disturbed mineral homeostasis. Moreover, these effects seem to be more pronounced in male rats on a HS diet compared to female rats receiving a HS diet.

6.5 Limitations

In the present study, the systolic blood pressure measurement was performed using the non-invasive tail-cuff method. However, the invasive measurement method is still considered the gold standard in this regard. We decided to use the non-invasive tail-cuff method due to the technical complexity of the surgical procedure of the invasive method as well as the associated complications, including death.

In order to investigate the effects of the high salt diet on the transcriptome level, we performed a candidate gene-approach analysis on the mRNA level using PCR. Thus, we cannot exclude the possibility that important genes that might be related to the effects of the high salt diet were not analyzed.

Moreover, we analyzed the gene expression only on the mRNA level and for technical reasons we did not confirm the findings on the proteome level due.

The rats used in this project were conventional normotensive Wistar rats fed with a 4% HS diet. Wistar rats are known to be more resistant to the morphologic consequences of experimental renal mass reduction than other strains. It is possible that this rather resistant rat strain and the 4% instead of 8% of salt might in part explain the lack of structural changes in the heart and the kidney despite clinically overt hypertension and proteinuria. Moreover, we had a study duration of 6 months, so maybe this period was still not long enough to detect of structural organ changes such as fibrosis and hypertrophy on a histological level.

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8. AFFIDAVIT

Eidesstattliche Versicherung

„Ich, Jingli Guo, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Sex-specific alterations in blood pressure and gene expression in Wistar rats on a high-salt diet“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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9. CURRICULUM VITAE

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

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11. SUPPLEMENTARY TABLE

Heart genes

Gene code	Gene full name	Normal salt males	High-salt males	Normal salt females	High-salt females
Fibrosis-associated genes					
Timp4	Tissue Inhibitor Of Metalloproteinases 4	421.9±26	398.1±26.82	297.1±23.29**	339.8±17.20
Timp1	Tissue Inhibitor Of Metalloproteinases 1	180.50±9.57	233.7±15.61**	152.8±12.56	169.9±7.79^{bbb}
Mmp2	Matrix Metallopeptidase 2	935.1±61.39	1210±81*	679.5±63.98*	812.5±53.54^{bbbb}
Mmp9	Matrix Metallopeptidase 9	2.535±0.89	3.01±0.45	0.68±0.39	1.124±0.24^{b}
Serpine1	Serpin Family E Member 1	105.4±8	165.3±17.1	83.47±12.36	94.28±7.95^{bb}
Myh7	Myosin Heavy Chain 7	40342±3608	62873±5091****	13785±2220****	22267±2119^{bbbb}
Galec3	Galectin-3	635.4±35.41	733.8±46.06	536.9±42.77	589.1±17.47^{b}
Col1a1	Collagen Type I Alpha 1	765±52.85	942.1±59.58	416.8±104.6	872.3±64.02^{a}
Col3a1	collagen, Type III, alpha 1	1814±449.4	3986±1021	1578±376.5	2113±667.3
Fn1	Fibronectin 1	632.6±34.26	918.1±84.46	443.8±121.5	760.9±83.06
Spock2	SPARC (Osteonectin), Cwcv And Kazal Like Domains Proteoglycan 2	116.2±23	206.9±43	60.6±13.6	87.85±18.82
Tgfb1	Transforming Growth Factor, Beta 1	540.5±21.73	702.4±47.56	325.3±61.81	491.7±22.5^{b}

Renin–angiotensin–aldosterone system					
Nppb	Natriuretic Peptide B	6686±575.1	11066±768.1****	3839±559.5**	6171±573.6^{a,bbbb}
Ace	Angiotensin I Converting Enzyme	252.2±8.6	242.9±23.73	161.6±9.14***	143.2±7.28^{bbb}
Agt	Angiotensinogen	7.9±1	9±1	5±0.8	5.8±0.7
Apn	Atrial Natriuretic Peptide	7460±893.8	37074±7219****	5252±1283	8910±1221^{a,bbbb}
Agtr1a	Angiotensin II Receptor, Type 1	0.47±0.039	0.57±0.05	0.59±0.03	0.49±0.03
Inflammation					
Mcp-1	Monocyte Chemoattractant Protein-1	107.7±8.53	137.6±9.54	94.58±9.85	107.6±5.36^{b}
Gdf15	Growth Differentiation factor	6.11±1.41	22.51±5.39*	2.99±1.02	5.46±1.41^{bbbb}
Il1rl1	Interleukin 1 Receptor Like 1	17.61±0.81	16.99±1.53	12.52±0.73**	14.78±0.93
Ptgs2	Prostaglandin-Endoperoxide Synthase 2	5.94±0.97	8.36±1.59	5.42±0.63	5.11±0.53
Vascular-associated gene					
Nos3	endothelial nitric oxide synthase	941.7±57.34	958.6±46.54	749.5±30.51**	657.5±17.59^{bbbb}
Metabolism-associated genes					
Atp2a2	ATPase Sarcoplasmic/Endoplasmic	123750±5924	138064±5626	137335±4623	132873±3818

	Reticulum Ca2+ Transporting 2				
Lcn2	Lipocalin 2	217.2±23.77	281.7±34.73	106±19.99**	186.4±25.11[a]
Cell cycle regulator					
Lyve1	Lymphatic Vessel Endothelial Hyaluronan Receptor 1	207.9±18.13	199.7±17.93	177.6±8.59	176.6±10.83
Others					
Opn	Osteopontin	115.10±28.79	160.10±26.46	63.48±13.25	68.62±17.31[bb]

Kidney genes

Gene code	Gene full name	Normal salt males	High-salt males	Normal salt females	High-salt females
Fibrosis-associated genes					
Timp1	Tissue Inhibitor Of Metalloproteinases 1	192.6±9.3	222.3±35.78	195.9±10.76	188.6±19.22
Timp4	Tissue Inhibitor Of Metalloproteinases 4	144.3±5.79	122.8±19.39	159.8±11.05	117.7±14.39
Mmp2	Matrix Metallopeptidase 2	153.7±27.14	182±41.11	193.8±25.6	162.2±33
Mmp9	Matrix Metallopeptidase 9	33.84±12.02	24.43±10.19	59.66±13.25	28.65±12.31
Serpine1	Serpin Family E Member 1	16.77±2	19.24±3.74	16.46±1.92	14.11±2.72
Serpine3	Serpin Family E Member 3	5.9±1.28	6.33±0.71	2.78±0.52*	2.43±0.37^{b}
Alpl	Alkaline Phosphatase, Liver/Bone/Kidney	35084±5514	34244±7750	56124±9252	32734±5819
Spock2	Sparc/Osteonectin, Cwcv And Kazal-Like Domains Proteoglycan (Testican) 2	108.8±23.28	108.2±24.36	259.1±43.37*	177±45.96
Col1a1	Collagen Type I Alpha 1	214±43.07	353±29.67	204.2±33.99	244.5±44.84
Col3a1	Collagen Type III Alpha 1	70.37±21.07	187.7±16.71*	82.58±23.07	98.85±28.02
Fn1	Fibronectin 1	177.7±35.31	353.3±28.84	183.7±28.85	253±46.35
Tgfb1	Transforming Growth Factor Beta 1	993.9±109.1	1804±183.9**	930.2±102.7	969.8±93.12^{bb}

Renin–angiotensin–aldosterone system					
Zbtb16	Zinc Finger And BTB Domain Containing 16	75.94±10.24	167.3±24.8**	74.04±8.32	87.21±12.40bb
Ren	Renin	692.5±92.1	304.9±71.97**	919.9±89.72	202.7±21.91aaaa
Agt	Angiotensinogen	4604±801.3	1683±497.1	592.8±141.1***	2130±516hs
Ace	Angiotensin I Converting Enzyme	751.6±70.52	716.5±92.79	471.2±51.62**	473.3±47.12b
Ace2	Angiotensin I Converting Enzyme 2	487.8±37.26	497.9±65.67	329.1±27.85*	377.3±22.79
Inflammation					
Mcp1	Monocyte Chemoattractant Protein-1	165.6±13.56	184.6±38.30	201.4±25.99	154.7±26.94
Gdf15	Growth Differentiation Factor 15	9.71±1.60	43.93±22.91	6.17±0.79	6.78±3.80
Lgals3	Lectin, Galactoside Binding Soluble 3	10967±760.4	12100±1315	9872±820.2	7652±597.5bb
Il1rl1	Interleukin 1 Receptor Like 1	41.9±8.44	50.2±12.51	51.7±9.29	29.11±6.16
Tsc22d3	TSC22 Domain Family Member 3	213.4±25.14	231±28.98	285.8±31.08	295.4±29.18
Ddit4	DNA Damage Inducible Transcript 4	183.9±30.04	125.9±10.56	393±57.53**	289.9±76.87
Vascular-associated gene					
Angpt2	Angiopoietin 2	625.7±96.28	532.2±105.1	8060±1494****	4334±854.3

Tnfrs11b	Tumor Necrosis Factor Receptor Superfamily Member 11b	345.6±114.6	851.2±284.5	574.8±165.6	226.5±80.48
Adm	Adrenomedullin	41.47±4.77	81.69±12.06*	62.43±7.764	70.78±7.4
Circadian rhythm regulator					
Per1	Period Circadian Clock 1	464.8±95.93	152.7±19.98	618.7±122.8	728.4±98.92b
Vitamin-associated genes					
Cyp27b1	Cytochrome p450 Family 27 Subfamily B Member 1	32.61±5.84	105.4±18.65**	20±3.79	26.88±4.61bb
Cyp24a1	Cytochrome P450 Family 24 Subfamily A Member 1	558.1±91.66	176.2±20.68*	1016±195.3	299.9±57.97aaaa
Neurotransmitters synthesis					
Vamp1	Vesicle-associated Membrane Protein 1	5.08±0.52	5.42±0.42	4.54±0.47	4.9±0.34
Th	Tyrosine Hydroxylase	1.65±0.3	1.91±0.36	1.38±0.19	1.78±0.18
Ddc	Dopa Decarboxylase	2229±282.6	2999±672.7	2206±247.8	2414±297.7
Cell cycle regulators					
Cdkn1a	Cyclin-Dependent Kinase Inhibitor 1A	86.89±17.05	174.4±27.26*	103.6±21.73	116.1±25.85
Nupr1	Nuclear Protein 1, Transcriptional Regulator	1248±209.3	2015±385	1376±228.4	1507±156.6
Epigenetic regulator					

Dnmt3a	DNA (Cytosine-5-)-Methyltransferase 3 Alpha	276.7±51.79	533.3±41.55**	294.2±38.94	445.3±42.18
Metabolism-associated genes					
Retn	Resistin	24.1±6.72	5.67±1.87	15.9±6.29	6.44±2.8
Lcn2	Lipocalin 2	1106±248.4	2544±396.5*	1031±236.9	707.3±197.9bb
Atp2a2	ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 2	4465±396.8	4681±1013	4769±587.1	2726±563.5
Cps1	Carbamoyl-Phosphate Synthase 1	1.167±0.301	3.875±2.01	6.29±1.41*	5.63±1.84
Slc51a	Solute Carrier Family 51 Alpha Subunit	1580±192.7	1378±196.6	1505±394	1097±187
Csad	Cysteine Sulfinic Acid Decarboxylase	8175±681.0	10006±807.3	4179±450.3***	4711±508.6bbbb
Dhcr24	24-Dehydrocholesterol Reductase	83.53±13.6	161.6±14.15**	91.9±16.5	111.9±15.55
Others					
Opn	Osteopontin	4306±395.3	5421±1247	7201±841.7	7620±1637
Havcr1	Hepatitis A virus cellular receptor 1	164.9±37.93	247±103.8	101.2±17.67	102.5±15.15

Supplementary table. **Heart and kidney genes. Values are mean ±SEM. *p<0.05; **p<0.01; ***p<0.001; ****P<0.0001 versus normal salt males. ap<0.05; aap<0.01; aaap<0.001; aaaaP<0.0001 versus normal salt females. bp<0.05; bbp<0.01; bbbp<0.001; bbbbP<0.0001 versus high-salt males.**