Effect of Dietary Sodium Modulation on Pig Adrenal Steroidogenesis and Transcriptome Profiles

MS ID#: HYPE/2020/15998_R1

Effect of dietary sodium modulation on pig adrenal steroidogenesis and transcriptome profiles

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Total words**:** 2700 excluding references, 1 table, 4 figures;
online-only supplement with 6 tables, 3 figures
Running title**:** Pig adrenal response to sodium restriction

Abstract

Primary aldosteronism is a frequent form of endocrine hypertension caused by aldosterone overproduction from the adrenal cortex. Regulation of aldosterone biosynthesis has been studied in rodents despite differences in adrenal physiology with humans. We therefore investigated pig adrenal steroidogenesis, morphology, and transcriptome profiles of the zona glomerulosa (zG) and zona fasciculata (zF) in response to activation of the renin-angiotensin-aldosterone system by dietary sodium restriction. Six-week-old pigs were fed a low or high sodium diet for 14 days (3 pigs per group, 0.4g sodium/kg feed *versus* 6.8g sodium/kg). Plasma aldosterone concentrations displayed a 43-fold increase ($p=0.011$) after 14-days of sodium restriction (day 14 *versus* day 0). Low dietary sodium caused a 2-fold increase in thickness of the zG ($p<0.001$) and an almost 3-fold upregulation of *CYP11B* (cytochrome P450 11B1) ($p<0.05$) compared with high dietary sodium. Strong immunostaining of the KCNJ5 potassium channel, which is frequently mutated in primary aldosteronism, was demonstrated in the zG. mRNA-seq transcriptome analysis identified significantly altered expression of genes modulated by the renin-angiotensin-aldosterone system in the zG (n= 1,172) and zF (n= 280). These genes included many with a known role in the regulation of aldosterone synthesis and adrenal function. The most highly enriched biological pathways in the zG were related to cholesterol biosynthesis, steroid metabolism, cell cycle and potassium channels. This study provides mechanistic insights into the physiology and pathophysiology of aldosterone production in a species closely related to humans and shows the suitability of pigs as a translational animal model for human adrenal steroidogenesis.

Key words: aldosterone; cortisol; hyperaldosteronism; adrenal cortex; sodium restriction; steroidogenesis; hypertension

Introduction

The mineralocorticoid hormone aldosterone is synthesized in zona glomerulosa (zG) cells of the adrenal cortex and stimulates sodium reabsorption in epithelial cells of the kidney distal tubule and colon for the maintenance of blood volume and blood pressure. The main physiological regulators of aldosterone production are the renin-angiotensin-aldosterone system (RAAS) and circulating potassium; although other factors are likely also involved.[1-3] Sodium depletion activates the RAAS and causes expansion of the zG layer and an increase in aldosterone secretion.[4,5] Manipulation of dietary sodium in rats has been used as an approach to study the effects of RAAS activation on zG gene expression to identify genes that function in the regulation of aldosterone production.[5-7]

Humans and commonly used experimental surrogates display distinct differences in adrenal physiology. For example, the *Cyp17a1* gene (encoding 17α-hydroxylase and 17,20-lyase) is not expressed in the adrenal glands of laboratory rats and mice resulting in the production of corticosterone as the major glucocorticoid, instead of cortisol as in humans. Another major difference involves the absence of the zona reticularis (zR) and adrenal androgen synthesis in these rodents.[8] Furthermore, potassium channels which function in the maintenance of zG cell membrane potential show different gene expression profiles and patterns of immunostaining in rat and human adrenals.[9,10] Notably, the rat adrenal does not express the inwardly rectifying potassium channel KCNJ5 which is frequently mutated in primary aldosteronism.[9] Regulation of *KCNJ5* gene expression and channel activity modulate the zG membrane depolarization that normally initiates aldosterone production in humans.[11] KCNJ5 mutations allow uncontrolled zG membrane depolarization and cause aldosterone excess.

The adrenal cortex is divided into three morphologically distinct layers (zG, zF, and zR). In the human adrenal, restricted expression of *CYP11B2* (encoding aldosterone synthase) in the zG and *CYP11B1* (encoding 11β-hydroxylase) in the zF and zR sustains the functional zonation of aldosterone biosynthesis in the zG and cortisol in the zF. These two CYP11B enzymes, with their specific zonal distributions, are present in multiple species including mice, rats, hamsters, and guinea pigs.[8] In contrast, others, such as pigs, cattle, sheep and dogs, express a single CYP11B enzyme that performs the final steps of both aldosterone and cortisol biosynthesis[12] and, by way of an unknown mechanism, their biosynthetic zonal specificity is maintained.[13,14]

Large animals such as pigs are useful to model complex human diseases due to their comparable anatomy and physiology to humans.[15-17] Such models provide an opportunity to screen for disease biomarkers and test novel therapeutic strategies.[18,19] In this study, we evaluated the role of dietary sodium manipulation on adrenal morphology, steroidogenesis and transcriptome profiles in 6-week-old male pigs. Our objective was to identify the transcriptional response of the adrenal to RAAS activation and determine the suitability of the pig as a translational animal model for human adrenal steroidogenesis.

Methods

An expanded online methods section is available in the online supplementary file.

The authors declare that all supporting data are available within the article and its online supplementary files. mRNA-seq data are publicly available and can be accessed at https://github.com/MedIVLMUMunich/PigAdrenalRNAseq

69 Animal handling

70 The study used 6-week-old male German Landrace DanBred pigs on a controlled 14-day diet of
71 0.04% sodium (n= 3; low sodium group) or 0.7% sodium (n= 3; high sodium group, around 5-times
72 higher than in standard pig feed) of equivalent metabolic energy with free access to water (Table
73 S1).

74 Immunohistochemistry and immunofluorescence

75 Primary antibodies for immunohistochemistry and immunofluorescence are shown in Table S2. A
76 pig polyclonal CYP11B antibody was generated using a synthetic peptide (acetyl-
77 ^{95}EDVERLQKVEGLHPQR^{110}C) (Figure S1) which was validated for use in Western blotting and
78 immunohistochemistry and immunofluorescence (Figure S2). CYP17A and KCNJ5 monoclonal
79 antibodies were produced as described previously.[20,21]

80 Steroid measurements and renin assays

81 Liquid chromatography tandem mass spectrometry (LC-MS/MS) for adrenal steroid measurements
82 was performed according to Peitzsch et al.[22] Renin activity was determined by LC-MS/MS
83 quantification (Attoquant Diagnostics, Vienna, Austria) as reported elsewhere.[23]

84 Matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI)

85 *In situ* metabolic imaging analysis of fresh frozen adrenal sections was performed with on-tissue
86 derivatization using Girard´s T reagent according to Suguira et al.[24] and MALDI-MSI analysis was
87 performed as reported by Sun et al.[25]

88 Transcriptome profiling by mRNA sequencing

89 mRNA-seq transcriptome profiling was done by Eurofins Genomics (Ebersberg, Germany).

90 Statistical analysis and bioinformatics

91 IBM SPSS Statistics 26 (IBM Corp., Armonk, New York, USA) and GraphPad PRISM 8.0a (La Jolla,
92 California, USA) were used for statistical analyses. Significant differences were analyzed using a

Friedman test for repeated measures, with correction for multiple comparisons, where appropriate, and a Mann-Whitney test for independent measures. P-values less than 0.05 were considered statistically significant. Biological pathway enrichment was analyzed using Reactome (https://reactome.org/PathwayBrowser/#/) and Gene Ontology (http://geneontology.org/) databases.[26-28]

Results

Low dietary sodium intake and activation of the RAAS

Pigs consumed comparable amounts and ate all daily allocated food. Measurements of urinary sodium excretion levels confirmed the difference in sodium intake between the 2 groups (Figure 1A). At day 14, plasma renin activity increased 7-fold compared with day 0 reaching 116.91 pmol/L/min (9.13 ng/mL/hr ± 3.7) under the low sodium diet. (Figure 1B).

Fourier-transform ion cyclotron resonance MSI of adrenal sections combined with on-tissue derivatization of steroids with Girard T (GirT) reagent facilitated the clear visualization of aldosterone or cortisone (which have an identical *m/z* ratio) in the zG layer of pigs on the sodium-restricted diet and low detection in the zG of pigs on the high sodium diet (Figure 1C). Because co-expression of CYP17A (which is not expressed in the zG) with CYP11B is required for cortisol and cortisone synthesis, the signal in the zG layer indicates increased synthesis of aldosterone in the zG under dietary sodium restriction.

Blood plasma steroid concentrations measured by LC-MS/MS are shown in Table S3 and are also represented in a heat map (Figure 1D) to illustrate the progressive time-related increase of plasma aldosterone levels in pigs on the sodium-restricted diet only. This corresponded to a 43.2-fold

increase in plasma aldosterone concentrations (day 14 *versus* day 0, p= 0.011) to reach 3.20 nmol/L (1.153 ± 0.583 ng/mL) at day 14 (Table S3). No significant differences in concentrations of steroids other than aldosterone were observed. The plasma 18-hydroxycorticosterone concentration progressively increased on the low sodium diet to 48.66 nmol/L (17.638 ± 11.185 ng/mL) at day 14 compared with 3.92 nmol/L (1.421 ± 0.082 ng/mL) at day 0, but the difference was not significant. The hybrid steroids 18-hydroxycortisol and 18-oxocortisol were identified in all pig blood plasma samples. In the plasma samples measured, both 18-hydroxycortisol and 18-oxocortisol displayed maximum levels in the sodium restricted pigs at day 14 (18-hydroxycortisol, 5.79 nmol/L {2.190 ± 1.178 ng/mL}; 18-oxocortisol, 0.27 nmol/L {0.102 ± 0.046 ng/mL}).

Morphological changes in adrenal cortex in response to dietary sodium restriction

Pig adrenals showed strong immunostaining of VSNL1 and KCNJ5 in the zG layer (Figure 2A and B). VSNL1 immunostaining highlighted an increased thickness of the zG layer from 6.5% ± 0.21 of the total adrenal cortex on the high sodium diet, to 13.8% ± 0.17 ($p<0.001$) on the low sodium diet. Similar results were observed with measurements from KCNJ5 immunostaining (Figure 2B).

CYP11B immunohistochemistry showed positive staining throughout the adrenal cortex with more intense immunostaining throughout the thickened zG layer. *CYP11B* gene expression displayed a 2.8 ± 0.3-fold increase (p=0.045) in the zG under sodium restriction with no change observed in the zF (Figure 2C). Increased zG cell proliferation was demonstrated under sodium restriction with a 2.9 ± 0.26-fold increase (p=0.028) in Ki-67 immunoreactive cells relative to pigs on a high sodium diet (Figure 2D). CYP11B-CYP17A double immunofluorescence delineated the zG layer more clearly, as CYP17A expression and thus immunostaining is restricted to the zF and zR, and

confirmed increased intensity of CYP11B immunostaining in the zG but not in the zF under the low *versus* high sodium diets (Figure 3).

mRNA-seq transcriptome analysis of the zG under a low and high sodium diet

An overview of the mRNA-seq analysis is shown in Figure S3. Comparison of the transcriptomes of the zG and zF demonstrated a higher number of transcripts with significantly altered expression levels in the zG than the zF (Figure S3, Figure 4A-C). These included 1,172 significantly altered annotated genes in the zG of the low *versus* high sodium groups comprising 768 upregulated and 404 downregulated genes: https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/1)zG_All%20annotated%20DEGs.xlsx. In the zF, 280 significantly altered annotated genes were identified with 172 upregulated and 108 downregulated: https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/2)zF_All%20annotated%20DEGs.xlsx.

Significantly altered transcripts which were common to the zG and the zF under low *versus* high dietary sodium comprised 89 upregulated and 30 downregulated genes. In addition, 6 transcripts were upregulated in the zG but downregulated in the zF (*FHIT*, *ssc-mir-202*, *WWOX*, *GTDC1*, *ZNF592* and *SLC2A9*) and 1 transcript was downregulated in the zG and upregulated in the zF (*HEATR3*) in response to salt restriction (Figure 4C).

A heat map representation of differentially expressed genes (DEGs) (defined as a log2-fold change ≥2) under low *versus* high sodium intake in the zG (59 genes) and the zF (21 genes) is shown in Figure 4D-E. The top 20 upregulated and downregulated genes in the zG (low *versus* high sodium

diet) with gene loci, expression levels and respective functions are shown in Table S4 and in the zF in Table S5. Several DEGs were identified with a described role in aldosterone production or adrenal function. These included genes encoding the transcription factors FOSB, FOS, VDR and NR5A1 (also called SF-1), RGS4 (regulator of G protein signaling 4), the calcium-binding proteins VSNL1 (visinin-like 1) and SMOC (secreted modular calcium-binding protein 1), STARD4 (StAR-related lipid transfer protein 4), the cytochrome P450 enzymes CYP27A1, CYP21A2 and CYPB1, and VAV2 (Vav Guanine Nucleotide Exchange Factor 2) (Table).[29-43]

Enriched biological pathways in the zG in response to dietary sodium restriction

Biological pathway analysis of significantly DEGs in the pig adrenal zG transcriptomes (low *versus* high sodium diet) identified enriched pathways related to steroid metabolism (p= 5.17e-2; number of DEGs= 31), cholesterol biosynthesis (p= 2.51e-4; DEGs= 12) and regulation of cholesterol biosynthesis by SREBP (sterol regulatory-element binding protein; p= 9.79e-6; DEGs= 12), the cell cycle (p= 9.82e-1; DEGs= 40) and potassium channels (p= 5.18e-1; DEGs= 9) (Table S6). The DEGs in the steroid metabolism pathway included *VDR* (vitamin D receptor), the top DEG related to this pathway, and *STARD4* (StAR related lipid transfer domain containing 4). Seven DEGs were common to the regulation of cholesterol biosynthesis by SREBP and the cholesterol biosynthesis pathways (*HMGCS1*, *HMGCR*, *FDFT1*, *PMVK*, *MVK*, *SQLE* and *MVD*). DEGs associated with the cell cycle included *AGTR2* (angiotensin II receptor type 2) and *PRKAR2B* (cAMP-dependent protein kinase type II-beta regulatory subunit). *KCNJ5* was the DEG in the potassium channel pathway with the highest expression level in the zG (Table S6).

Discussion

We demonstrate the effect of RAAS activation by dietary sodium restriction on adrenal morphology, steroidogenesis and transcriptome profiles in pigs as a translational animal model for humans. In pigs, as described previously in rats,[4,5] RAAS activation induced by dietary sodium restriction caused zG expansion, increased zG expression of aldosterone synthase (called CYP11B in pigs and CYP11B2 in rats and humans) at both transcript and protein levels and an increased production of aldosterone from the zG. After 14 days, pig plasma aldosterone concentrations were comparable to those reported in Yanomami Indians (3.20 nmol/L *versus* 2.38 nmol/L, respectively), a population noted for their low salt consumption[44], and 20-fold higher than in a group of 525 adult humans from Europe.[45]

We show strong immunostaining of KCNJ5 in the zG of the pig adrenal as in humans[21] and *KCNJ5* transcripts were detected in the zG but not the zF. KCNJ5 is a potassium inwardly rectifying channel that contributes to normal membrane polarization and may contribute to the mechanism of aldosterone synthesis in response to angiotensin II stimulation.[11] Germline and somatic *KCNJ5* mutations that cause membrane depolarization of the zG cell have been identified as the main known molecular variants causing primary aldosteronism in humans.[46] However, there are redundant mechanisms for the maintenance of membrane potential and knocking down the expression of KCNJ5 in human adrenal cortical carcinoma cells did not alter basal aldosterone synthesis nor abrogate its stimulation by angiotensisn II.[11] *KCNJ5* transcripts were undetectable in laser-captured samples of rat adrenal zG and specific KCNJ5 immunostaining was undetectable indicating a difference between rats and humans in the regulation of zG membrane potential and aldosterone production.[9]

We used mRNA-seq analysis to gain further insight into transcriptome changes of zG cells associated with increased aldosterone synthesis in the pig as a model for the regulation of human aldosterone production. RNA-seq analysis offers the possibility to accurately quantify expression of genes from very low to high levels and allowed the identification of a large number of genes in the zG transcriptome (768 and 404 up- and down-regulated annotated genes) which are regulated by chronic RAAS activation compared with the rat using microarray analysis (201 and 68 up- and down-regulated genes, low *versus* high sodium diet).[5] In the latter case, the rats were fed a sodium-controlled diet for 3 days, compared with the 14-days in the current study, and the high dietary sodium levels used in the rat study were almost 10-times greater than in the present study, with relatively mild high sodium conditions, which may also account for this difference.

We report several upregulated genes in the zG in response to sodium restriction with a previously reported role in aldosterone production. *VDR* (encoding the vitamin D receptor) was the top DEG in the zG with an annotated function related to steroid metabolism which was expressed at a low level in the zG and undetected in the zF. *VDR* gene expression was upregulated by angiotensin II stimulation of a human adrenocortical cell line and *VDR* overexpression caused an increase in aldosterone secretion under basal and angiotensin II stimulated conditions.[29] The *VDR* gene is upregulated in aldosterone-producing adenomas, a major cause of primary aldosteronism, compared with normal adrenals[47] and *VDR* gene expression is positively correlated with *CYP11B2* mRNA levels consistent with a role in pathophysiological aldosterone production.[48] Other upregulated genes in the pig zG with a previously reported role in aldosterone secretion include *RGS4* (regulator of G protein signaling 4) which is upregulated in the rat adrenal by a low sodium diet and angiotensin II infusion[6,30] and *VSNL1* encoding the calcium sensor protein VSNL1 which

regulates basal and angiotensin II-stimulated CYP11B2 gene expression in human adrenocortical cells *in vitro*.[34]

Transcriptome alterations were also identified in the zF transcriptome by stimulation of the RAAS. This is consistent with the report of a more than 2-fold significant increase in cortisol secretion, in addition to an increase in aldosterone secretion, by angiotensin II stimulation of human adrenocortical cells *in vitro*.[49] Furthermore, an analysis of transcription regulatory genes modulated by angiotensin II in cultured human adrenocortical cells identified several genes which significantly activate an 11β-hydroxylase reporter gene, with a relatively much smaller effect on the expression of an aldosterone synthase reporter plasmid.[50] These genes included members of the *FOS* (fos proto-oncogene) gene family, *FOS* and *FOSB*, which encode leucine zipper proteins which dimerize with proteins of the *JUN* family (Jun proto-oncogene), to form the transcription factor complex AP-1 (activator protein-1). *FOS* and *FOSB* were highly upregulated in the zF by sodium restriction in the present study. *FOSB* transcripts were not detected in the zG whereas *FOS* was expressed in both zones with a greater transcriptional response in the zF.

The cell cycle was one of the most enriched biological pathways in the zG in sodium restricted pigs reflecting the expansion of the zG layer, in agreement with a zG microarray analysis in rats.[5] *AGTR2* (angiotensin II type 2 receptor) transcription showed the highest response of the DEGs annotated to the cell cycle in the zG. The *AGTR2* is highly expressed in the human fetal adrenal,[31] strikingly higher than in the adult,[32] where it may mediate apoptosis during adrenal gland development.[33] The pronounced altered expression of *AGTR2* in the zG transcriptome in response to sodium restriction suggests a potential role in the control of adrenal morphology after birth.

The strengths of our study include the use of an animal species closely related to humans. We used a relatively mild high sodium diet to represent a level of salt intake relevant to that of many humans which is in contrast to the highly abnormal experimental conditions of 4-8% NaCl in high salt diets given to rats. Moreover, unlike in rats and mice, pigs display clear, high-level KCNJ5 expression in the zG, and produce the hybrid steroids 18-hydroxycortisol and 18-oxocortisol which display elevated levels in patients with primary aldosteronism carrying KCNJ5 mutations.[51,52] This highlights the superiority of the pig over rodents for the study of human adrenal biology and indicates the potential utility of the pig to model aspects of primary aldosteronism.

A limitation of our study is the low number of animals used in each diet group, which may explain why differences in aldosterone precursor steroids were not detected despite the identification of differences in aldosterone production. We also used only male animals to circumvent gender-related effects on steroid production[45] and thus we could not address sex-related effects of dietary sodium on aldosterone production.[53] An additional limitation of the pig is the expression of a single CYP11B enzyme in the adrenal cortex. Despite this, shared mechanisms of aldosterone physiology with animals that express both CYP11B2 and CYP11B1 in their adrenals are likely because many genes involved in the transcriptional response to sodium restriction were identified common to pigs and rats.

Perspectives

This study presents a proof of concept for the suitability of the pig as a model of human steroidogenesis and provides a rich source of transcriptome data for studies on aldosterone physiology and pathophysiology and for the identification of potential novel pharmacological targets to treat aldosterone excess.

Acknowledgements

The technical assistance of Christina Blechinger, Diana Jaquin, Petra Rank and Youssra Ahmed-Mohammed is gratefully acknowledged.

Sources of funding

This work was supported by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement No [694913] to M Reincke) and by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) project number: 314061271-TRR 205 to G Eisenhofer, M Peitzsch, M Reincke, A Walch, and TA Williams and Deutsche Forschungsgemeinschaft SFB 824 C04 to A Walch. CE Gomez-Sanchez is supported by National Heart, Lung and Blood Institute grant R01 HL144847, and the National Institute of General Medical Sciences grant U54 GM115428 and BX004681 from the Department of Veterans Affairs, USA.

Conflicts of interest/Disclosure

None

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Novelty and Significance

What is new?

- The level of sodium intake significantly alters pig adrenal morphology, steroidogenesis and transcriptome profiles
- Under a low sodium diet, the pig adrenal displays zona glomerulosa expansion with increased expression of CYP11B
- MALDI-MSI demonstrated that aldosterone production in the zona glomerulosa increased in response to sodium restriction
- Plasma aldosterone concentrations and plasma renin activity were increased in pigs fed a low sodium diet for 14-days
- The KCNJ5 potassium channel is highly expressed in the pig zona glomerulosa and absent in the zona fasciculata
- Use of the pig adrenal as a surrogate to study adrenal steroidogenesis offers advantages over that of the rat or mouse

What is relevant?

- Pig adrenal transcriptome profiling by mRNA seq analysis identifies genes responsive to activation of the renin-angiotensin aldosterone system
- An abundance of genes in the zona glomerulosa and the zona fasciculata respond to dietary sodium restriction
- In some respects, pig adrenal steroidogenesis is more like that of the human than the adrenals of rats and mice.

Summary

The pig is an appropriate model to study the regulation of aldosterone secretion and will be useful to discover and assess novel pharmacological targets of aldosterone excess.

Figure Legends

Figure 1. Activation of the pig renin-angiotensin-aldosterone system by dietary sodium restriction

Fractional excretion of sodium measurements confirmed the higher sodium intake at 7 and 14 days of pigs on the high compared with the low sodium diet (**Panel A**). Plasma renin activity showed a 7-fold increase at day 14 compared with day 0 (**Panel B**). MALDI-MSI demonstrated the increased aldosterone or cortisone production in the zG of the low sodium group. The absence of CYP17A (required for cortisol synthesis) in the zG indicates that the increased intensity of the aldosterone or cortisone signal in the zG is aldosterone. The zG is delineated with white broken lines (**Panel C**). Multiple adrenal steroids were measured by LC-MS/MS showing the progressive increase of aldosterone production up to day 14 in pigs under sodium restriction. Data for individual pigs is normalized to the average plasma steroid concentration at day 0 for high and low sodium groups and the intensity scale indicates fold change over baseline (**Panel D**).

FENa, fractional excretion of sodium; GirT, Girard's Reagent T; H&E, hematoxylin and eosin; 18OH-cortisol, 18-hydroxycortisol; 18OH-corticosterone, 18-hydroxycorticosterone; 17OH-progesterone, 17-hydroxyprogesterone; PRA, plasma renin activity. Bars represent mean ± SEM (n=3 for each group), a Friedman non-parametric test for repeated measures was used to detect paired differences. Circles, high sodium; triangles, low sodium diet. *$p<0.05$. Panel C, scale bar = 1mm and 200 μm in zoomed images as shown.

Figure 2. Morphological and functional expansion of the pig adrenal zona glomerulosa by dietary sodium restriction

Immunostaining of VSNL1 and KCNJ5 highlighted the increase in the zG in the pigs fed a 14-day low sodium diet. The thickness of the zG layer is shown as a percentage of the total thickness of the adrenal cortex. The average of 6 measurements for each adrenal is shown (**Panel A and B**). CYP11B immunostaining shows the expansion of the zG layer is complemented by an increase in aldosterone synthase-positive cells in pigs on a low sodium diet (**Panel C**) and immunostaining for Ki67, a marker of cell proliferation, shows a significant increase in Ki67-positive cells in the zG layer on a low *versus* high sodium diet (**Panel D**). Bars represent mean ± SEM (n=3 per group), a Mann-Whitney test was used to detect differences. Circles, high sodium; triangles, low sodium diet. *p<0.05, ***p<0.001.

Figure 3. CYP11B-CYP17A double immunofluorescence staining

In the adrenal, CYP17A is exclusively expressed in the zF and the zR and CYP11B-CYP17A double immunostaining allows the clear visualization of the zG layer showing the increased thickness and increased CYP11B immunostaining in the zG compared with the zF in pigs on the low compared with the high sodium diet. The zG is delineated with white broken lines. Scale bar= 50 µm

Figure 4. Transcriptome alterations in the zona glomerulosa and the zona fasciculata under low *versus* high sodium diets.

Volcano plots indicate transcripts with significantly altered levels (red dots) by dietary sodium manipulation in the zG (**Panel A**) and zF (**Panel B**). The numbers of significantly altered transcripts in the zG and zF (low *versus* high dietary sodium) are indicated in the Venn diagram (**Panel C**). There were 6 transcripts upregulated in response to sodium restriction in the zG which were

downregulated in the zF and one transcript which was downregulated in the zG but upregulated in the zF (low *versus* high sodium). The heat maps represent the up- and down-regulated genes (defined as log2-fold change>2) in the zG (**Panel D**) and zF (**Panel E**) of each pig (n= 3 in each of the high and low sodium diet groups). The gene names are indicated on the left of each heat map and log2-fold change on the right. FC, log2-fold change; zG, zona glomerulosa; zF, zona fasciculata

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Figure 1.

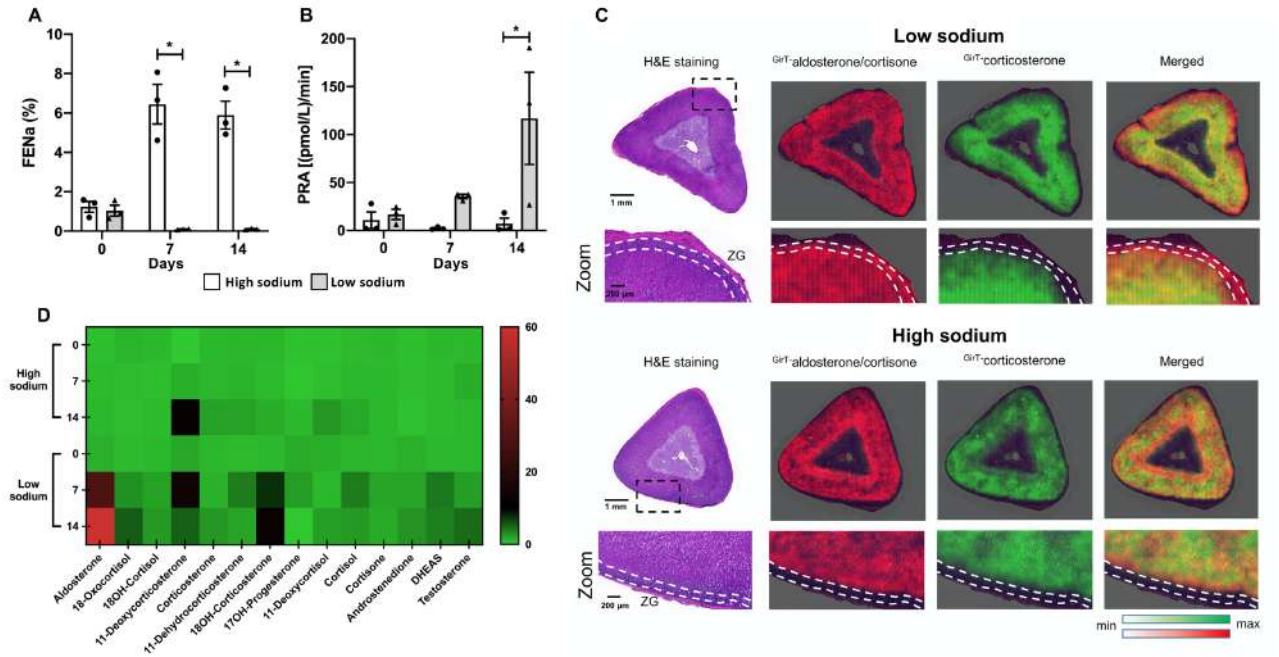

Figure 2.

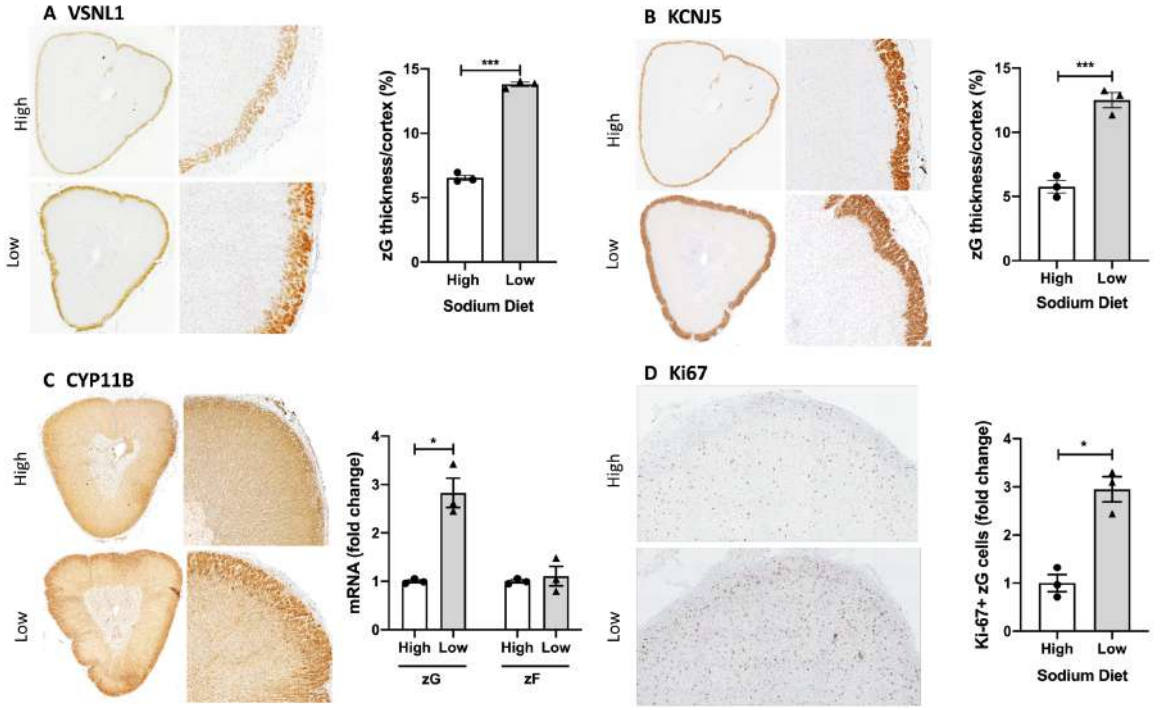

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Figure 3.

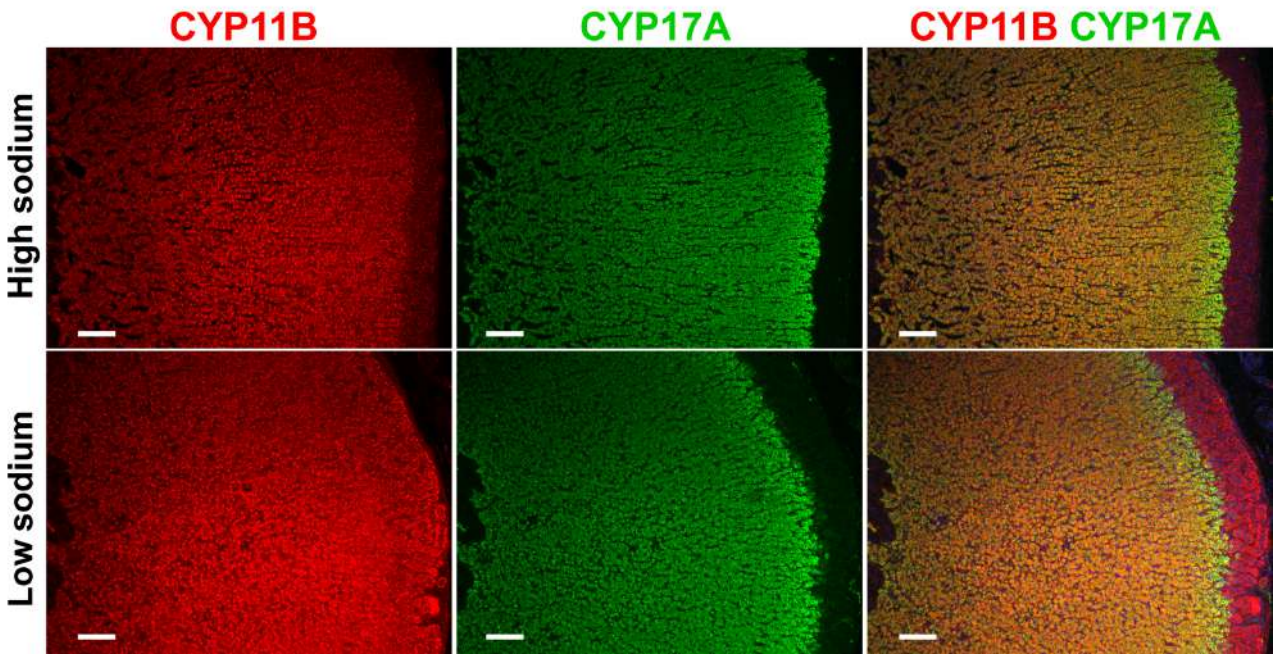

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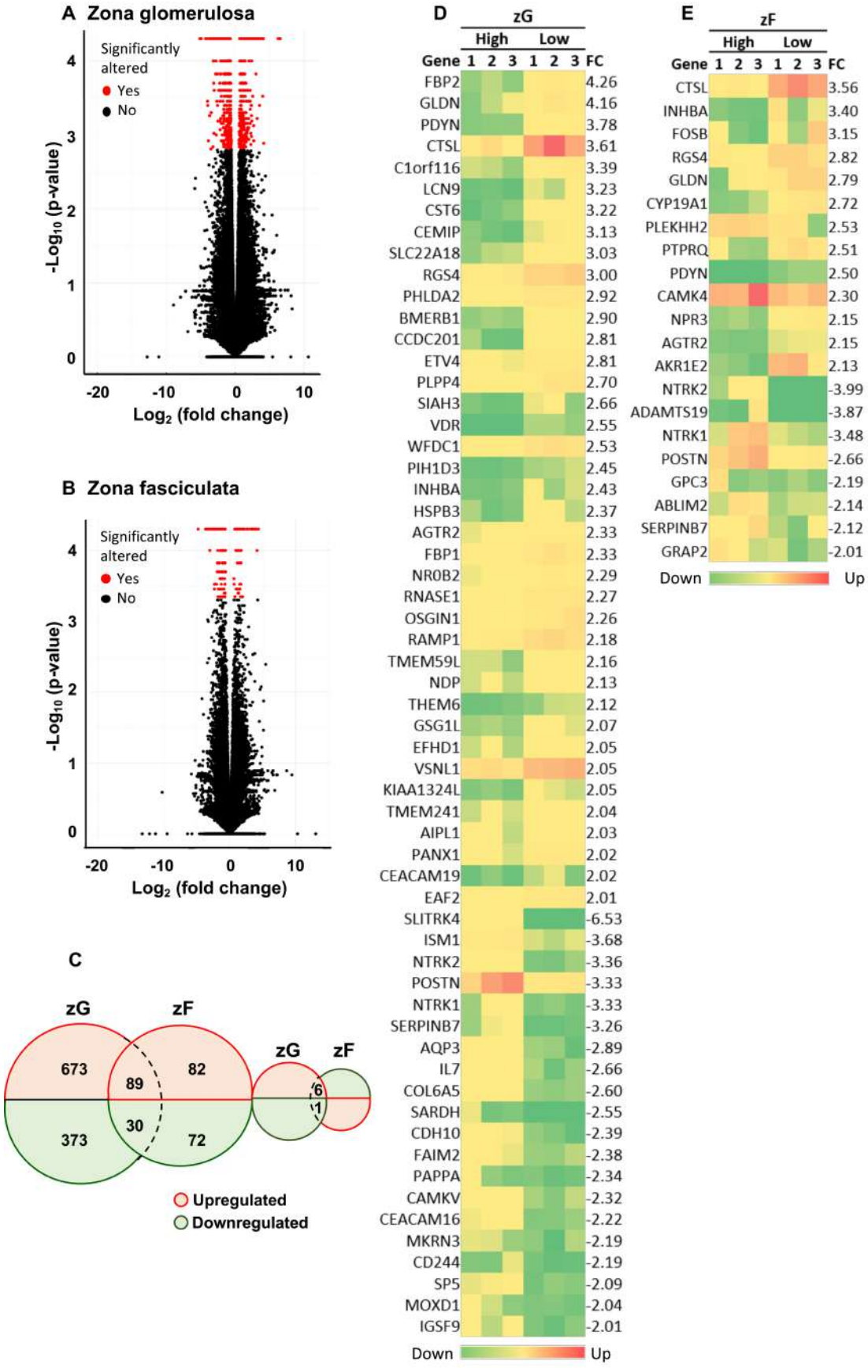
Figure 4.


A Zona glomerulosa
Significantly altered
Yes
No
-Log10 (p-value)
Log2 (fold change)
B Zona fasciculata
Significantly altered
Yes
No
-Log10 (p-value)
Log2 (fold change)
C
zG
zF
673
89
82
373
30
72
zG
zF
6
1
Upregulated
Downregulated
D
zG
High
Low
Gene 1 2 3 1 2 3 FC
FBP2 4.26
GLDN 4.16
PDYN 3.78
CTSL 3.61
C1orf116 3.39
LCN9 3.23
CST6 3.22
CEMIP 3.13
SLC22A18 3.03
RGS4 3.00
PHLDA2 2.92
BMERB1 2.90
CCDC201 2.81
ETV4 2.81
PLPP4 2.70
SIAH3 2.66
VDR 2.55
WFDC1 2.53
PIH1D3 2.45
INHBA 2.43
HSPB3 2.37
AGTR2 2.33
FBP1 2.33
NR0B2 2.29
RNASE1 2.27
OSGIN1 2.26
RAMP1 2.18
TMEM59L 2.16
NDP 2.13
THEM6 2.12
GSG1L 2.07
EFHD1 2.05
VSNL1 2.05
KIAA1324L 2.05
TMEM241 2.04
AIPL1 2.03
PANX1 2.02
CEACAM19 2.02
EAF2 2.01
SLITRK4 -6.53
ISM1 -3.68
NTRK2 -3.36
POSTN -3.33
NTRK1 -3.33
SERPINB7 -3.26
AQP3 -2.89
IL7 -2.66
COL6A5 -2.60
SARDH -2.55
CDH10 -2.39
FAIM2 -2.38
PAPPA -2.34
CAMKV -2.32
CEACAM16 -2.22
MKRN3 -2.19
CD244 -2.19
SP5 -2.09
MOXD1 -2.04
IGSF9 -2.01
Down Up
E
zF
High
Low
Gene 1 2 3 1 2 3 FC
CTSL 3.56
INHBA 3.40
FOSB 3.15
RGS4 2.82
GLDN 2.79
CYP19A1 2.72
PLEKHH2 2.53
PTPRQ 2.51
PDYN 2.50
CAMK4 2.30
NPR3 2.15
AGTR2 2.15
AKR1E2 2.13
NTRK2 -3.99
ADAMTS19 -3.87
NTRK1 -3.48
POSTN -2.66
GPC3 -2.19
ABLIM2 -2.14
SERPINB7 -2.12
GRAP2 -2.01
Down Up

Gene	zG_FPKM		Log_2_fc LS *vs*. HS	P value	zF_FPKM		Log_2_fc LS *vs*. HS	P value	REF
	LS	HS			LS	HS			
FOSB	n.d.	n.d.	-	-	8.25	0.93	3.15	0.00005	29
RGS4	174.01	21.70	3.00	0.00005	21.96	3.10	2.82	0.00005	6, 30
VDR	1.63	0.28	2.55	0.00005	n.d.	n.d.	-	-	29
AGTR2	19.85	3.93	2.33	0.00005	1.94	0.44	2.15	0.00005	31-33
VSNL1	346.14	83.42	2.05	0.00005	34.53	8.78	1.98	0.00005	34
SMOC2	4.31	14.45	-1.75	0.00005	3.11	6.91	-1.15	0.00005	30
STARD4	16.91	8.72	0.95	0.0001	12.66	3.76	1.75	0.00005	35
FOS	55.93	28.42	0.98	0.00005	164.14	55.98	1.55	0.00005	36
CYP27A1	14.10	32.12	-1.19	0.00005	n.d.	n.d.	-	-	37
NR5A1	229.67	118.83	0.95	0.00005	n.d.	n.d.	-	-	38
VAV2	27.20	14.46	0.91	0.00005	n.d.	n.d.	-	-	39
KCNJ5	221.11	118.62	0.90	0.00005	n.d.	n.d.	-	-	40
CYP21A2	5786.23	3275.67	0.82	0.00025	n.d.	n.d.	-	-	41
CEBPB	74.41	42.09	0.82	0.00005	n.d.	n.d.	-	-	42
CYP1B1	23.04	38.24	-0.73	0.00025	n.d.	n.d.	-	-	43

Table. Top differentially expressed genes with a described functional role in the adrenal
The genes with the highest level of differential expression with a described functional role in the adrenal are shown. The table indicates the expression levels and log2-fold changes under a low *versus* high sodium diet in either the zG or zF.

The full list of annotated DEGs in the zG can be downloaded at:
https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/1)zG_All%20annotated%20DEGs.xlsx

The full list of annotated DEGs in the zF can be downloaded at:
https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/2)zF_All%20annotated%20DEGs.xlsx

Fc, fold change; FPKM, fragments per kilobase of transcript, per million mapped reads; n.d., not detected; REF, reference