

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

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**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 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, 0.4 g sodium/kg feed versus 6.8 g 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* ( $P<0.05$ ) compared with high dietary sodium. Strong immunostaining of the KCNJ5 (G protein-activated inward rectifier potassium channel 4), which is frequently mutated in primary aldosteronism, was demonstrated in the zG. mRNA sequencing transcriptome analysis identified significantly altered expression of genes modulated by the renin-angiotensin-aldosterone system in the zG ( $n=1172$ ) and zona fasciculata ( $n=280$ ). 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. (*Hypertension*. 2020;76:1769-1777. DOI: 10.1161/HYPERTENSIONAHA.120.15998.)

• **Data Supplement**

**Key Words:** adrenal cortex ■ aldosterone ■ hydrocortisone ■ hyperaldosteronism ■ hypertension ■ models, animal ■ sodium

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.<sup>1-3</sup> Sodium depletion activates the RAAS and causes expansion of the zG layer and an increase in

aldosterone secretion.<sup>4,5</sup> 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.<sup>5-7</sup>

Humans and commonly used experimental surrogates display distinct differences in adrenal physiology. For example, the *Cyp17a1* gene (encoding 17 $\alpha$ -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

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glucocorticoid, instead of cortisol as in humans. Another major difference involves the absence of the zona reticularis and adrenal androgen synthesis in these rodents.<sup>8</sup> Furthermore, potassium channels that function in the maintenance of zG cell membrane potential show different gene expression profiles and patterns of immunostaining in rat and human adrenals.<sup>9,10</sup> Notably, the rat adrenal does not express *KCNJ5* (G protein-activated inward rectifier potassium channel 4), which is frequently mutated in primary aldosteronism.<sup>9</sup> Regulation of *KCNJ5* gene expression and channel activity modulate the zG membrane depolarization that normally initiates aldosterone production in humans.<sup>11</sup> *KCNJ5* mutations result in uncontrolled zG membrane depolarization and cause aldosterone excess.

The adrenal cortex is divided into 3 morphologically distinct layers (zG, zona fasciculata [zF], and zona reticularis). In the human adrenal, restricted expression of *CYP11B2* (encoding aldosterone synthase) in the zG and *CYP11B1* (encoding 11 $\beta$ -hydroxylase) in the zF and zona reticularis sustains the functional zonation of aldosterone biosynthesis in the zG and cortisol in the zF. These two CYP11B (cytochrome P450 11B1) enzymes, with their specific zonal distributions, are present in multiple species including mice, rats, hamsters, and guinea pigs.<sup>8</sup> 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<sup>12</sup> and, by way of an unknown mechanism, their biosynthetic zonal specificity is maintained.<sup>13,14</sup>

Large animals such as pigs are useful to model complex human diseases due to their comparable anatomy and physiology to humans.<sup>15–17</sup> Such models provide an opportunity to screen for disease biomarkers and test novel therapeutic strategies.<sup>18,19</sup> 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 Methods section is available in the [Data Supplement](#).

The authors declare that all supporting data are available within the article and in the [Data Supplement](#). mRNA sequencing (mRNA-seq) data are publicly available and can be accessed at <https://github.com/MedIVLMUMunich/PigAdrenalRNAseq>.

## Animal Handling

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

## Immunohistochemistry and Immunofluorescence

Primary antibodies for immunohistochemistry and immunofluorescence are shown in Table S2. A pig polyclonal CYP11B antibody was generated using a synthetic peptide (acetyl-<sup>95</sup>EDVERLQKVEGLHPQR<sup>110</sup>C; Figure S1), which was validated for use in Western blotting and immunohistochemistry and immunofluorescence (Figure S2). CYP17A and *KCNJ5* monoclonal antibodies were produced as described previously.<sup>20,21</sup>

## Steroid Measurements and Renin Assays

Liquid chromatography tandem mass spectrometry for adrenal steroid measurements was performed according to Peitzsch et al.<sup>22</sup>

Renin activity was determined by liquid chromatography tandem mass spectrometry quantification (Attoquant Diagnostics, Vienna, Austria) as reported elsewhere.<sup>23</sup>

## Matrix-Assisted Laser Desorption/Ionization–Mass Spectrometry Imaging

In situ metabolic imaging analysis of fresh-frozen adrenal sections was performed with on-tissue derivatization using Girard T reagent according to Suguira et al.<sup>24</sup> and matrix-assisted laser desorption/ionization–mass spectrometry imaging analysis was performed as reported by Sun et al.<sup>25</sup>

## Transcriptome Profiling by mRNA-Seq

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

## Statistical Analysis and Bioinformatics

IBM SPSS Statistics 26 (IBM Corp, Armonk, NY) and GraphPad PRISM 8.0a (La Jolla, CA) 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 *U* test for independent measures. *P*<0.05 was considered statistically significant. Biological pathway enrichment was analyzed using Reactome (<https://reactome.org/PathwayBrowser/#/>) and Gene Ontology (<http://geneontology.org/>) databases.<sup>26–28</sup>

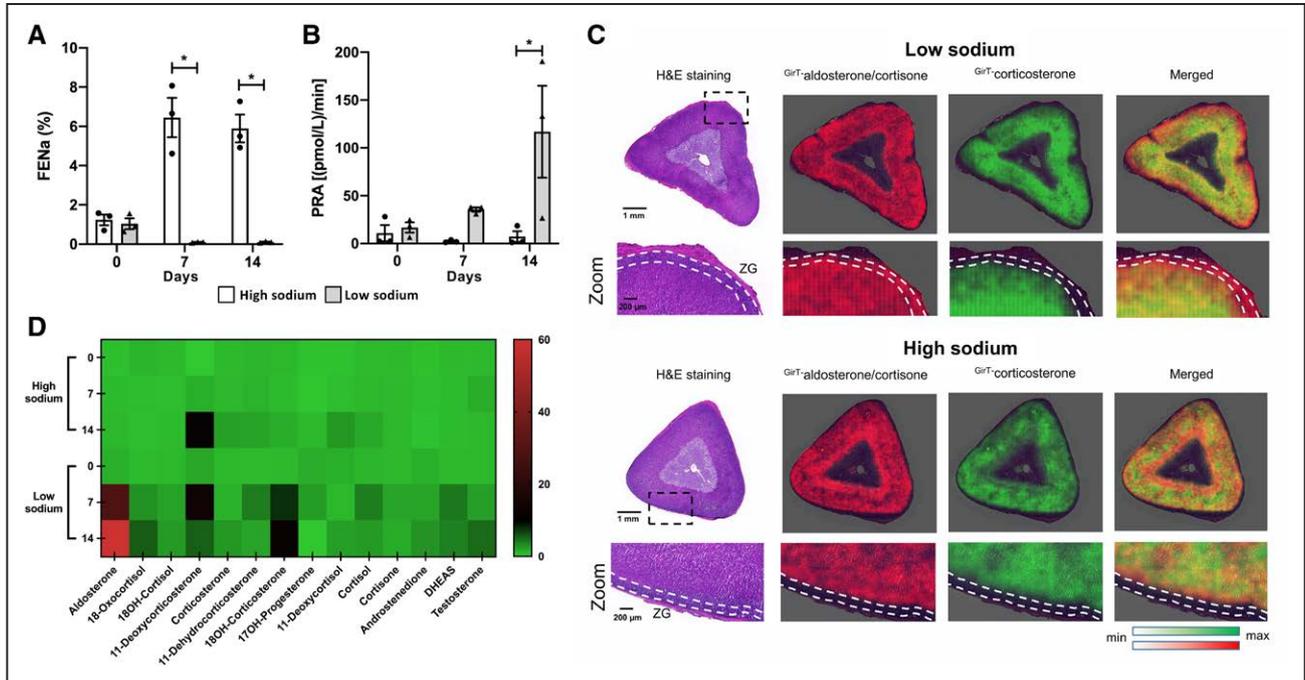
## Results

### 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 two groups (Figure 1A). At day 14, plasma renin activity increased 7-fold compared with day 0 reaching 116.91 pmol/L per minute (9.13 ng/mL per hour  $\pm$ 3.7) under the low-sodium diet (Figure 1B).

Fourier-transform ion cyclotron resonance mass spectrometry imaging of adrenal sections combined with on-tissue derivatization of steroids with Girard T 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 coexpression of CYP17A (steroid 17- $\alpha$ -hydroxylase/17,20 lyase)-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 liquid chromatography tandem mass spectrometry 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 $\pm$ 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 $\pm$ 11.185 ng/mL) at day 14 compared with 3.92 nmol/L (1.421 $\pm$ 0.082 ng/mL) at day 0, but the difference was not significant. The hybrid



**Figure 1.** Activation of the pig renin-angiotensin-aldosterone system by dietary sodium restriction. Fractional excretion of sodium (FENa) measurements confirmed the higher sodium intake at 7 and 14 d of pigs on the high- compared with the low-sodium diet (A). Plasma renin activity showed a 7-fold increase at day 14 compared with day 0 (B). Matrix-assisted laser desorption/ionization–mass spectrometry imaging demonstrated the increased aldosterone or cortisone production in the zona glomerulosa (zG) of the low-sodium group. The absence of CYP17A (steroid 17- $\alpha$ -hydroxylase/17,20 lyase, 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 (C). Multiple adrenal steroids were measured by liquid chromatography tandem mass spectrometry showing the progressive increase of aldosterone production up to day 14 in pigs under sodium restriction. Data for individual pigs are 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 (D). Bars represent mean $\pm$ SEM (n=3 for each group); a Friedman nonparametric test for repeated measures was used to detect paired differences. Circles, high sodium; triangles, low-sodium diet. C, Scale bar=1 mm and 200  $\mu$ m in zoomed images as shown. DHEAS indicates dehydroepiandrosterone sulfate, 17OH-progesterone, 17-hydroxyprogesterone; 18OH-corticosterone, 18-hydroxycorticosterone; 18OH-cortisol, 18-hydroxycortisol; GirT, Girard reagent T; H&E, hematoxylin and eosin; and PRA, plasma renin activity. \* $P$ <0.05.

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 $\pm$ 1.178 ng/mL]; 18-oxocortisol, 0.27 nmol/L [0.102 $\pm$ 0.046 ng/mL]).

### Morphological Changes in Adrenal Cortex in Response to Dietary Sodium Restriction

Pig adrenals showed strong immunostaining of VSNL1 (visinin-like 1) and KCNJ5 in the zG layer (Figure 2A and 2B). VSNL1 immunostaining highlighted an increased thickness of the zG layer from 6.5 $\pm$ 0.21% of the total adrenal cortex on the high-sodium diet to 13.8 $\pm$ 0.17% ( $P$ <0.001) on the low-sodium diet. Similar results were observed with measurements from KCNJ5 immunostaining (Figure 2B).

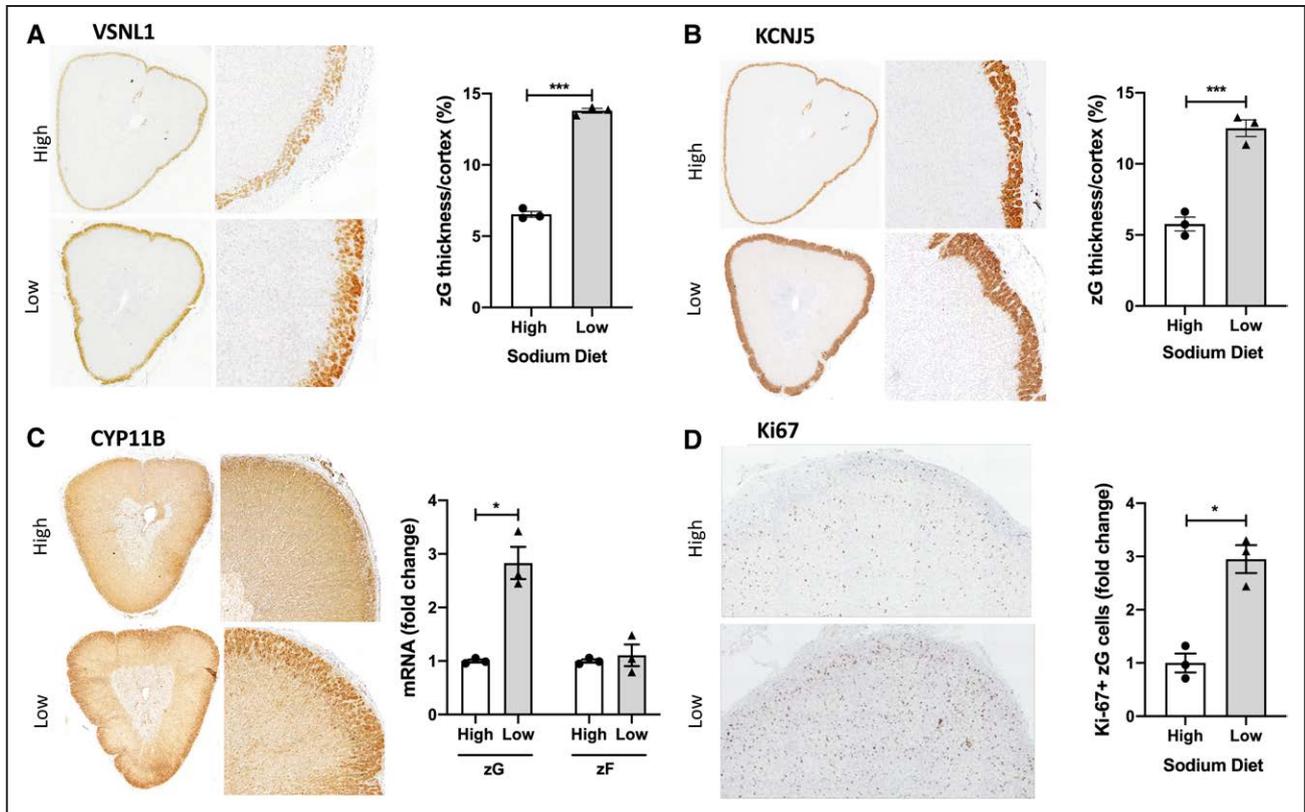
CYP11B immunohistochemistry showed positive staining throughout the adrenal cortex with more intense immunostaining throughout the thickened zG layer. *CYP11B* gene expression displayed a 2.8 $\pm$ 0.3-fold increase ( $P$ =0.045) in the zG under sodium restriction with no change observed in the zF (Figure 2C). Increased zG cell proliferation was demonstrated under sodium restriction with a 2.9 $\pm$ 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 zona reticularis 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 through 4C). These included 1172 significantly altered annotated genes in the zG of the low- versus high-sodium groups comprising 768 upregulated and 404 downregulated genes. In the zF, 280 significantly altered annotated genes were identified with 172 upregulated and 108 downregulated. The full list of annotated DEGs in the zG and the zF can be downloaded using the links in the Table legend.

Significantly altered transcripts that 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).



**Figure 2.** Morphological and functional expansion of the pig adrenal zona glomerulosa (zG) by dietary sodium restriction. Immunostaining of VSNL1 (visinin-like 1) and KCNJ5 (G protein-activated inward rectifier potassium channel 4) highlighted the increase in the zG in the pigs fed a 14-d 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 (A and B). CYP11B (cytochrome P450 11B1) 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 (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- vs high-sodium diet (D). Bars represent mean $\pm$ SEM (n=3 per group); a Mann-Whitney *U* test was used to detect differences. Circles, high sodium; triangles, low-sodium diet. zF indicates zona fasciculata. \**P*<0.05, \*\*\**P*<0.001.

A heat map representation of differentially expressed genes (DEGs; defined as a log<sub>2</sub>-fold change  $\geq 2$ ) under low versus high sodium intake in the zG (59 genes) and the zF (21 genes) is shown in Figure 4D and 4E. The top 20 upregulated and down-regulated 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 (protein fosB), FOS (proto-oncogene c-Fos), VDR (vitamin D receptor) and SF-1 (steroidogenic factor 1 encoded by *NR5A1*), RGS4 (regulator of G-protein signaling 4), the calcium-binding proteins VSNL1 and SMOC (secreted modular calcium-binding protein 1), STARD4 (StAR [steroidogenic acute regulatory protein]-related lipid transfer protein 4), the cytochrome P450 enzymes CYP27A1, CYP21A2, and CYPB1, and VAV2 (Vav guanine nucleotide exchange factor 2; Table).<sup>29–43</sup>

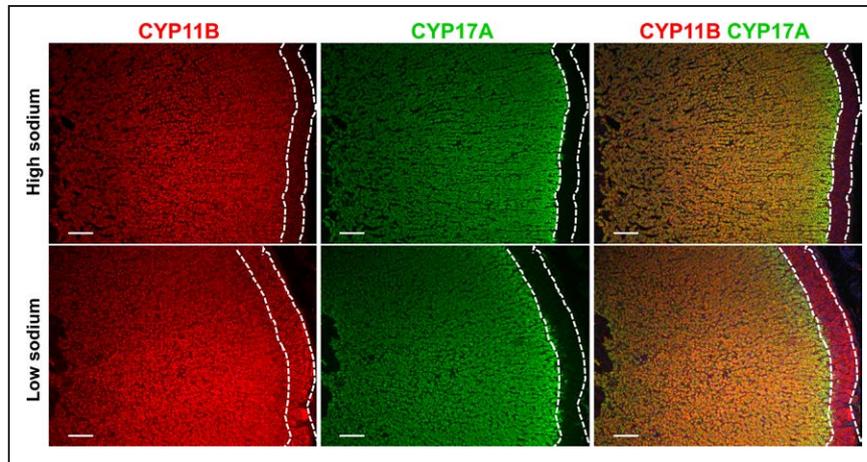
### Enriched Biological Pathways in the zG in Response to Dietary Sodium Restriction

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*=5.17 $\times 10^{-2}$ ; DEGs, n=31), cholesterol biosynthesis (*P*=2.51 $\times 10^{-4}$ ; DEGs, n=12), and regulation of cholesterol biosynthesis by SREBP

(sterol regulatory-element binding protein; *P*=9.79 $\times 10^{-6}$ ; DEGs, n=12), the cell cycle (*P*=9.82 $\times 10^{-1}$ ; DEGs, n=40), and potassium channels (*P*=5.18 $\times 10^{-1}$ ; DEGs, n=9; Table S6). The DEGs in the steroid metabolism pathway included *VDR*—the top DEG related to this pathway—and *STARD4*. 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* 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,<sup>4,5</sup> 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 versus 2.38 nmol/L, respectively)—a



**Figure 3.** CYP11B (cytochrome P450 11B1)-CYP17A (steroid 17- $\alpha$ -hydroxylase/17,20 lyase) double immunofluorescence staining. In the adrenal, CYP17A is exclusively expressed in the zona fasciculata (zF) and the zona reticularis, and CYP11B-CYP17A double immunostaining allows the clear visualization of the zona glomerulosa (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  $\mu$ m.

population noted for their low salt consumption<sup>44</sup>—and 20-fold higher than in a group of 525 adult humans from Europe.<sup>45</sup>

We show strong immunostaining of *KCNJ5* in the zG of the pig adrenal as in humans,<sup>21</sup> 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.<sup>11</sup> 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.<sup>46</sup> 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 angiotensin II.<sup>11</sup> *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.<sup>9</sup>

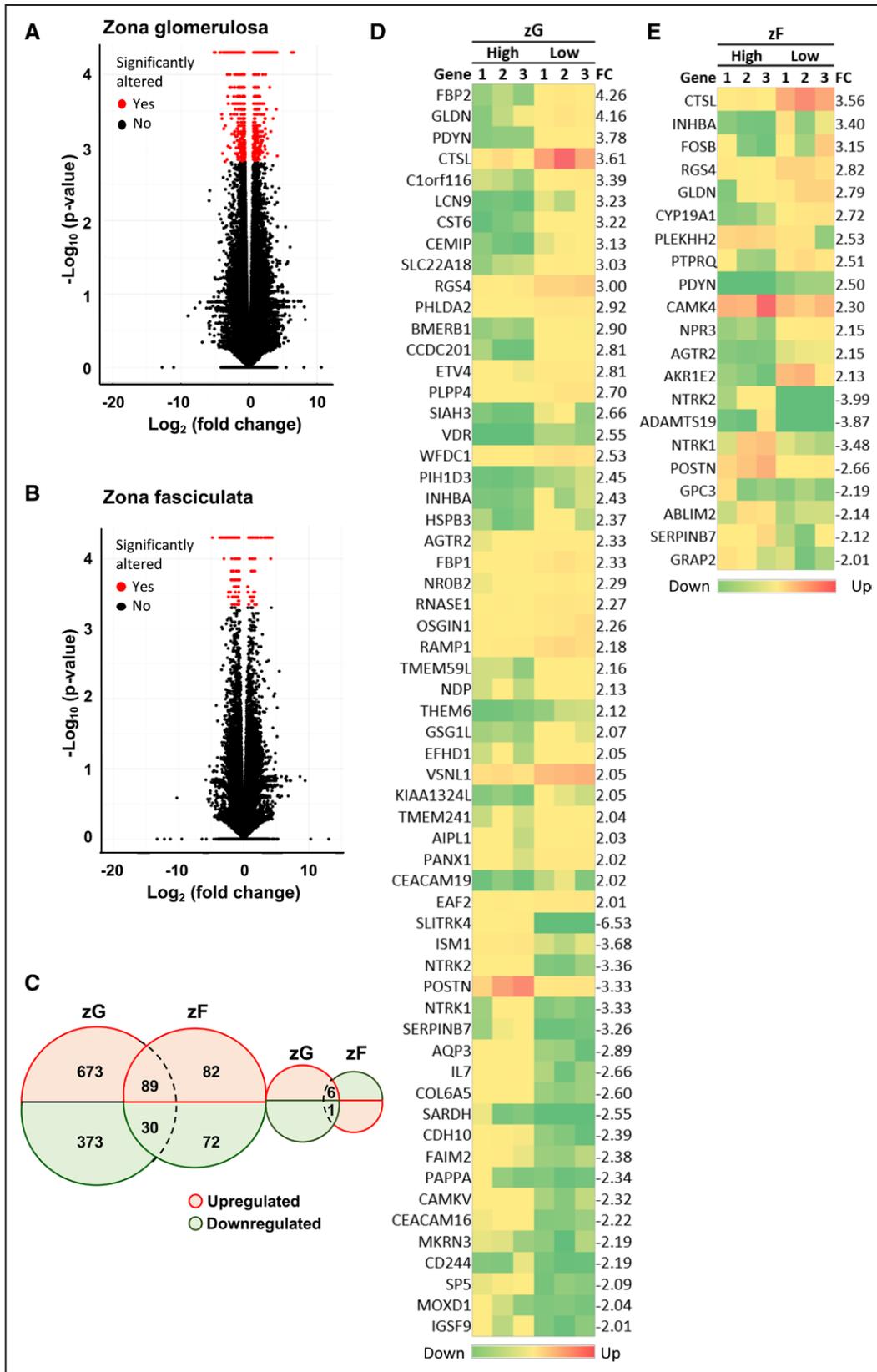
We used mRNA-seq analysis to gain further insight into transcriptome changes of zG cells associated with increased aldosterone synthesis in the pig as a model for the regulation of human aldosterone production. RNA-seq analysis offers the possibility to accurately quantify expression of genes from very low to high levels and allowed the identification of a large number of genes in the zG transcriptome (768 and 404 up- and downregulated annotated genes), which are regulated by chronic RAAS activation compared with the rat using microarray analysis (201 and 68 up- and downregulated genes, low-versus high-sodium diet).<sup>5</sup> 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* 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.<sup>29</sup> The *VDR* gene is upregulated in aldosterone-producing adenomas—a major cause of primary aldosteronism—compared with normal adrenals,<sup>47</sup> and *VDR* gene expression is positively correlated with *CYP11B2* mRNA levels consistent with a role in pathophysiological aldosterone production.<sup>48</sup> Other upregulated genes in the pig zG with a previously reported role in aldosterone secretion include *RGS4*, which is upregulated in the rat adrenal by a low-sodium diet and angiotensin II infusion<sup>6,30</sup> and *VSNL1* encoding the calcium sensor protein VSNL1, which regulates basal and angiotensin II-stimulated *CYP11B2* gene expression in human adrenocortical cells in vitro.<sup>34</sup>

Transcriptome alterations were also identified in the zF transcriptome by stimulation of the RAAS. This is consistent with the report of a >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.<sup>49</sup> Furthermore, an analysis of transcription regulatory genes modulated by angiotensin II in cultured human adrenocortical cells identified several genes that significantly activate an 11 $\beta$ -hydroxylase reporter gene, with a relatively much smaller effect on the expression of an aldosterone synthase reporter plasmid.<sup>50</sup> 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



**Figure 4.** Transcriptome alterations in the zona glomerulosa (zG) and the zona fasciculata (zF) under low- vs high-sodium diets. Volcano plots indicate transcripts with significantly altered levels (red dots; online) by dietary sodium manipulation in the zG (A) and zF (B). The numbers of significantly altered transcripts in the zG and zF (low vs high dietary sodium) are indicated in the Venn diagram (C). There were 6 transcripts upregulated in response to sodium restriction in the zG that were downregulated in the zF and one transcript that was downregulated in the zG but upregulated in the zF (low vs high sodium). The heat maps represent the up- and downregulated genes (defined as  $\log_2$ -fold change >2) in the zG (D) and zF (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  $\log_2$ -fold change on the right. FC indicates  $\log_2$ -fold change.

**Table. Top Differentially Expressed Genes With a Described Functional Role in the Adrenal**

Gene	zG_FPKM		Log <sub>2</sub> _fc LS vs HS	P value	zF_FPKM		Log <sub>2</sub> _fc LS vs HS	P value	Reference
	LS	HS			LS	HS			
<i>FOSB</i>	ND	ND	...	...	8.25	0.93	3.15	0.00005	29
<i>RGS4</i>	174.01	21.70	3.00	0.00005	21.96	3.10	2.82	0.00005	6,30
<i>VDR</i>	1.63	0.28	2.55	0.00005	ND	ND	...	...	29
<i>AGTR2</i>	19.85	3.93	2.33	0.00005	1.94	0.44	2.15	0.00005	31–33
<i>VSNL1</i>	346.14	83.42	2.05	0.00005	34.53	8.78	1.98	0.00005	34
<i>SMOC2</i>	4.31	14.45	-1.75	0.00005	3.11	6.91	-1.15	0.00005	30
<i>STARD4</i>	16.91	8.72	0.95	0.0001	12.66	3.76	1.75	0.00005	35
<i>FOS</i>	55.93	28.42	0.98	0.00005	164.14	55.98	1.55	0.00005	36
<i>CYP27A1</i>	14.10	32.12	-1.19	0.00005	ND	ND	...	...	37
<i>NR5A1</i>	229.67	118.83	0.95	0.00005	ND	ND	...	...	38
<i>VAV2</i>	27.20	14.46	0.91	0.00005	ND	ND	...	...	39
<i>KCNJ5</i>	221.11	118.62	0.90	0.00005	ND	ND	...	...	40
<i>CYP21A2</i>	5786.23	3275.67	0.82	0.00025	ND	ND	...	...	41
<i>CEBPB</i>	74.41	42.09	0.82	0.00005	ND	ND	...	...	42
<i>CYP11B1</i>	23.04	38.24	-0.73	0.00025	ND	ND	...	...	43

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 log<sub>2</sub>-fold changes under a low- vs 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](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](https://github.com/MedIVLMUMunich/PigAdrenalRNAseq/raw/master/2)zF_All%20annotated%20DEGs.xlsx). DEG indicates differentially expressed gene; fc, fold change; FPKM, fragments per kilobase of transcript, per million mapped reads; HS, high sodium; LS, low sodium; ND, not detected; zF, zona fasciculata; and zG, zona glomerulosa.

analysis in rats.<sup>5</sup> *AGTR2* 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,<sup>31</sup> strikingly higher than in the adult,<sup>32</sup> where it may mediate apoptosis during adrenal gland development.<sup>33</sup> 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% to 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.<sup>51,52</sup> 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 sex-related effects on steroid production,<sup>45</sup> and thus we could not address sex-related effects of dietary sodium on aldosterone production.<sup>53</sup> 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.

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## Disclosures

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 (cytochrome P450 11B1).
- Matrix-assisted laser desorption/ionization–mass spectrometry imaging 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 d.
- 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.