Parental vitamin D deficiency during pregnancy is associated with increased blood pressure in offspring via Panx1 hypermethylation

Laura M. G. Meems,1,* Hasan Mahmud,1,* Hendrik Buikema,2 Jörg Tost,3 Sven Michel,4 Janny Takens,1 Rikst N. Verkaik-Schakel,5 Inge Vreeswijk-Baudoinqu, Irene V. Mateo-Leach,1 Pim van der Harst,1 Torsten Plösch,2 and Rudolf A. de Boer1

1Department of Cardiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands; 2Department of Clinical Pharmacy and Pharmacology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands; 3Centre National de Génotypage, CEA-Institute de Génomique, Laboratory for Epigenetics and Environment, Evry, France; 4Department of Pediatric Pneumology and Allergy, University Children’s Hospital Regensburg (KUNO), Regensburg, Germany; and 5Obstetrics and Gynaecology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

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NEW & NOTEWORTHY

Parental vitamin D deficiency is associated with increased blood pressure in the offspring and with epigenetic changes of Panx1, a gene encoding for a hemichannel involved in endothelial-dependent relaxation. Offspring from vitamin D-depleted parents have impaired endothelial relaxation of the large vessels, suggesting an underlying biological mechanism.

Hypertension is a major risk factor in the development of cardiovascular (CV) disease and premature death worldwide. The prevalence of hypertension is on the rise due to the increasing aging population, and it is estimated that in 2025 1.56 billion adults will be affected by this condition (54a).

The underlying mechanisms of hypertension are complex and multifactorial, but among many others, heritable factors play a role (24, 51). This may include epigenetic DNA modifications. Several groups have identified epigenetic markers that could contribute to increased susceptibility to develop hypertension (19a, 37, 44).

Recently, it became apparent that in humans, maternal nutritional status during early pregnancy predictably affects DNA methylation in offspring (9). Previously, it has been demonstrated that in mice changes in maternal diet altered epigenetic development and influenced DNA methylation status during the embryonic phase (8, 52, 53). These data suggest that nutritional deficiencies may induce epigenetic changes that modify gene transcriptional activity and (indirectly) alter disease susceptibility (46). In this context, one of the nutritional components that may be of particular interest is vitamin D. Due to our predominantly indoor lifestyle, vitamin D deficiency has become one of the most common nutritional deficiencies worldwide (18, 19). Interestingly, maternal vitamin D deficiency has been associated with the development of short- and long-term disorders in the offspring, including hypertension (18). However, the mechanism linking parental vitamin D deficiency to increased susceptibility for hypertension in the offspring remains to be elucidated. We used a rat model to study the consequences of parental vitamin D deficiency on blood pressure and epigenetic changes in the offspring.

MATERIALS AND METHODS

Animal care and experimental design. All animal studies were approved by the Animal Ethical Committee of the University of Groningen and conducted in accordance with existing guidelines for the care and use of laboratory animals.

* L. M. Meems and H. Mahmud contributed equally to this work.

Address for reprint requests and other correspondence: R. A. de Boer, Department of Cardiology, University of Groningen, University Medical Center Groningen, P.O. Box 30 001, Hanzeplein 1, 9713 GZ, Groningen, The Netherlands (e-mail: r.a.de.boer@umcg.nl).
We studied 12-wk-old single-caged Sprague-Dawley male and female rats (Harlan) and supplied them with normal chow (st-diet) \((n = 7 \text{ males}, 7 \text{ females})\) or vitamin D-depleted diet (depl-diet) \((n = 8 \text{ males}, 8 \text{ females})\) [Harlan (Teklad), diet code TD.87095, containing 20% lactose, 2% Ca, and 1.25% P] with 12:12-h day-night cycles and ad libitum access to tap water. After 10 wk of dietary intervention, blood pressure in parent (F0) rats was measured with a noninvasive method (tail-cuff method, rats were trained for 2 wk beforehand). We collected blood (via venipuncture under anesthesia) from male and female animals to determine vitamin D status \([25\text{-hydroxyvitamin D}\ [25\text{(OH)}\text{D}]]\) and parathyroid hormone (PTH) levels. Enzyme immunoassay of 25(OH)D. Plasma 25(OH)D levels were analyzed in duplo using a commercial enzyme-linked immunoassay (EIA) according to the manufacturer’s protocol (25-Hydroxyvitamin D EIA Kit, AC-57F1; Immunodiagnostic Systems, Frankfurt am Main, Germany). Coefficient of variance (CV) for intra-assay variation was 5.3–6.7% and for interassay variation 3.8–11.3%.

**Experimental procedures.** After 10 wk of dietary intervention, a single female was mated with one nonsibling male from the same dietary group, and dietary treatment was continued. Following delivery, males were removed from the cage and euthanized, while females and offspring were maintained group caged. From this point on, all animals were supplied with a standard diet that included vitamin D for the entire duration of subsequent experiments (Fig. 1). Offspring (F1 pups) were nursed freely and weaned at 3 wk onto a standard chow diet, provided ad libitum. After 2 wk of training, final tail-cuff blood pressure measurement was performed in 10 6-wk-old F1 rats (10 male, 10 female). Ten other age-matched male pups per dietary arm received telemeter implantation (Data Sciences International, St. Paul, MN). Telemeters were used to continuously monitor blood pressure with a sampling frequency (Data Sciences International, St. Paul, MN). Short-axis view and M-mode tracings were used to measure cardiac hypertrophy and heart dimensions.

Before death, hemodynamic function was measured, using an indwelling pressure tip catheter (Millar Instruments, Houston, TX) that was introduced in the right carotid artery and advanced into the left ventricle (LV) as previously described (47). Briefly, rats were anesthetized (2.5% isoflurane) and body temperature was maintained by placing the rat on a heating pad. Short-axis view and M-mode tracings were used to measure cardiac hypertrophy and heart dimensions.

**Vascular studies with isolated aorta rings.** The thoracic descending aorta was excised and placed in a Krebs bicarbonate solution of the following composition (in mmol/l): 120.4 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 11.5 glucose, and 25.0 NaHCO₃, continuously aerated with 95% O₂-5% CO₂ at 37°C. The vessel was cleaned of adhering fat tissue, and rings of 2 mm in width were cut with a sharp razor blade, while ensuring not to touch the luminal surface. Rings were mounted between two stirrups in organ baths filled with 15 ml of Krebs solution. One stirrup was anchored inside the organ bath, while ensuring not to touch the luminal surface. Rings were mounted between two stirrups in organ baths filled with 15 ml of Krebs solution. One stirrup was anchored inside the organ bath, while ensuring not to touch the luminal surface.

Table 1. Overview of primers used for RT-PCR and DNA methylation status quantification (\(5^\prime\rightarrow 3^\prime\))

| Primers used for quantitative RT-PCR | 36B4
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>GTTGCCATCGTTGGCATTCT</td>
</tr>
<tr>
<td>Reverse</td>
<td>GGAATCCTGAAATTTTT</td>
</tr>
</tbody>
</table>

| Primers used for pyrosequencing | Panx1
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Forward</td>
<td>GTGTGTCGTGGGACTGAC</td>
</tr>
<tr>
<td>Reverse</td>
<td>GAGATTGGCGGATGCCAG</td>
</tr>
</tbody>
</table>

**Sequence (CpG-island) to analyze of Panx1**

TTTTGTTTTTGAAGG

**Primers used for pyrosequencing**

GTTCACTAGTGTTTTTTTTT

**Sequence (CpG-island) to analyze of Panx1**

TTGGGATTTT

**Primers used for pyrosequencing**

GTTGATTTGAGTAAGTTGTTT

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**Sequence (CpG-island) to analyze of Panx1**

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**Primers used for pyrosequencing**

GTTCACTAGTGTTTTTTTTT
were preconstricted with 10 mmol/l KCl (10 to 80 mmol/l) or phenylephrine (PE; 1 nmol/l to 60 mmol/l) twice. Following washout and renewed stabilization, they were primed and checked for viability by evoking a contraction then subjected to 14 mN and allowed to stabilize for 60 min before determination isotonic changes, as previously described (3). Rings were bath while the other was connected to a displacement transducer to determine isotonic changes, as previously described (3). Rings were then subjected to 14 mN and allowed to stabilize for 60 min before they were primed and checked for viability by evoking a contraction with 60 mmol/l KCl twice. Following washout and renewed stabilization, parallel rings were studied (in duplicate) either for contractile responses to KCl (10 to 80 mmol/l) or phenylephrine (PE; 1 nmol/l to 10 µmol/l) or endothelium-dependent relaxation. For the latter, rings were preconstricted with 1 µmol/l PE followed by determination of the dilatory response to the endothelium-dependent vasodilator acetylcholine (ACH: 10 mmol/l to 10 µmol/l). Subsequently, a single high concentration of sodium nitroprusside (SNP; 10 µmol/l) was administered to determine maximal endothelium-independent relaxation.

Quantitative real-time PCR. To measure mRNA gene expression levels, total RNA from LV and kidney tissue was extracted using TRizol reagent (Invitrogen, Carlsbad, CA). cDNA synthesis and quantitative real-time PCR (RT-qPCR) were performed as previously described with the use of 0.5 µg total RNA (36). mRNA levels were expressed in relative units based on a standard curve obtained with serial dilutions of cDNA mixture. RNA expression data were normalized using the 36B4 as a reference. We assayed transcript abundance of genes listed in Table 1.

Western blot analysis. We isolated protein using RIPA buffer (50 mM Tris pH 8.0, 1% Nonidet P40, 0.5% deoxycholate, 0.1% SDS, and 150 mM NaCl) that was supplemented with 10 µl/ml phosphatase inhibitor cocktail 1 (Sigma-Aldrich), protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), and 1 mM phenylmethylsulfonyl fluoride (PMSF; Roche Diagnostics). Regions of interest selected for validation were amplified from the scanner were preprocessed by median-centering and quantile normalization as proposed by Pälmke et al. (41) using the Bioconductor packages Ringo (48) and limma (43). To define differentially methylated regions, a combination of three separate algorithms was applied, including two publicly available methods BATMAN (10) and the dmrFinder from the CHARM package (20) as well as a self-programmed R-script.

Assessment of DNA methylation status by pyrosequencing. According to the DNA methylation quantification method of Tost and Gut (49), we analyzed the quantitative DNA methylation status of the Panx1 gene by pyrosequencing of bisulfite-treated DNA. Briefly, 1 µg of DNA from heart or kidney tissue, respectively, was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research). Regions of interest selected for validation were amplified using 2 µl of bisulfite-treated genomic DNA and 10 µM of forward and reverse primers, covering a total of 4.87 kb of promoter per gene. The probe lengths were between 50 and 75 mer. Arrays were scanned using a MS200 scanner (Nimblegen). The log2 ratios of fluorescence intensity data obtained from the scanner were preprocessed by median-centering and quantile normalization as proposed by Pälmke et al. (41) using the Bioconductor packages Ringo (48) and limma (43). To define differentially methylated regions, a combination of three separate algorithms was applied, including two publicly available methods BATMAN (10) and the dmrFinder from the CHARM package (20) as well as a self-programmed R-script.

Table 2. Baseline characteristics and echocardiographic data of male F1 rats

<table>
<thead>
<tr>
<th>Data</th>
<th>F1-st (n = 10)</th>
<th>F1-depl (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>336 ± 8</td>
<td>325 ± 6</td>
</tr>
<tr>
<td>HW, mg</td>
<td>1,092 ± 17</td>
<td>1,060 ± 17</td>
</tr>
<tr>
<td>LVW, mg</td>
<td>825 ± 13</td>
<td>798 ± 13</td>
</tr>
<tr>
<td>HW/BW, g/m</td>
<td>3.3 ± 0.2</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>LVW/BW, mg/mm</td>
<td>25 ± 0.2</td>
<td>25 ± 0.1</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>398 ± 7</td>
<td>397 ± 8</td>
</tr>
<tr>
<td>MV A, m/s</td>
<td>0.68 ± 0.05</td>
<td>0.63 ± 0.05</td>
</tr>
<tr>
<td>MV E, m/s</td>
<td>1.04 ± 0.02</td>
<td>0.89 ± 0.05*</td>
</tr>
<tr>
<td>MV E/A ratio</td>
<td>1.60 ± 0.11</td>
<td>1.50 ± 0.06</td>
</tr>
<tr>
<td>IVSs, mm</td>
<td>2.78 ± 0.05</td>
<td>2.64 ± 0.05</td>
</tr>
<tr>
<td>LVIDs, mm</td>
<td>2.69 ± 0.18</td>
<td>3.34 ± 0.10*</td>
</tr>
<tr>
<td>LVPWs, mm</td>
<td>2.82 ± 0.07</td>
<td>2.79 ± 0.05</td>
</tr>
<tr>
<td>IVSd, mm</td>
<td>1.62 ± 0.07</td>
<td>1.56 ± 0.03</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>5.86 ± 0.22</td>
<td>6.34 ± 0.09*</td>
</tr>
<tr>
<td>LVPWd, mm</td>
<td>1.63 ± 0.05</td>
<td>1.56 ± 0.04</td>
</tr>
<tr>
<td>LVOT diameter, mm</td>
<td>2.97 ± 0.04</td>
<td>2.92 ± 0.06</td>
</tr>
<tr>
<td>EF, %</td>
<td>88 ± 1.6</td>
<td>84 ± 0.7*</td>
</tr>
<tr>
<td>%FS</td>
<td>54 ± 2.2</td>
<td>48 ± 0.8*</td>
</tr>
<tr>
<td>LV CO, ml/min</td>
<td>147 ± 8</td>
<td>135 ± 8</td>
</tr>
</tbody>
</table>

Data are means ± SE. Baseline characteristics and echocardiographic data at death of the used rats (n = 10 per group, all males). st, Standard chow; depl, vitamin D-deficient chow; BW, body weight; HW, heart weight; LVW, left ventricular weight; HR, heart rate; MV, mitral valve; A, late ventricular filling velocities; E, early ventricular filling velocities; E/A ratio, diastolic relaxation; IVSs, thickness of the interventricular septum in systole; LVIDs, left ventricular internal diameter in systole; LVPWs, thickness of the left ventricular posterior wall in systole; IVSd, thickness of the interventricular septum in diastole; LVIDd, left ventricular internal diameter in diastole; LVPWd, thickness of the left ventricular posterior wall in diastole; LVOT, left ventricular outflow tract; EF, ejection fraction; FS, fractional shortening; LV CO, left ventricular cardiac output. *P < 0.05 F1-st vs. F1-depl, †P < 0.01 F1-st vs. F1-depl.
and reverse primer, one of them being biotinylated (49). Information about PCR and sequencing primers and the sequence-to-analyze is summarized in Table 1 (11). Quantification of the DNA methylation status of the Panx1 was performed on a PyromarkQ24 system (Qiagen) as described by Freitag et al. (15). Results were analyzed the results using the PyroMark CpG software (V.10.0.11.14; Qiagen).

Statistical analyses. Results are reported as means ± SE, unless reported otherwise. Statistical analysis between groups was performed by using the Student’s-t-test if data were normally distributed or the Mann-Whitney U-test if data were not normally distributed. Changes in vitamin D, PTH, and blood pressure over time were analyzed using repeated-measures ANOVA analysis. All P values were two-tailed, and P < 0.05 was considered statistically significant.

Aorta contractile responses to KCl and PE are given in micromoles. Vasodilator responses to ACh are expressed as a percentage of PE-induced precontraction before contraction-tension response curves were generated (Graphpad Prism 5; Graphpad Software, San Diego, CA). The n values represent the number of investigated rats. Full contraction-tension response curves were compared using repeated-measures ANOVA. Analyses were performed using Stata version 11.0 software (StataCorp).

The database required for BATMAN and all following analysis steps were set up according to the developers’ instructions (10). Every region was divided in 100-bp windows representing a probe that was designed on the array. For each window a methylation percentage was assigned. We assumed a region to be differentially methylated if at least five 100-bp windows in a row showed >20% points difference between the two investigated phenotypes. The proposed normalization steps from the CHARM package (20) could not be directly applied as instead of the recommended 2,000 only ~1,000 CpG free probes were placed on the NimbleGen promoter array. Therefore, no methylation percentages could be calculated and the dmrFinder function (21) was used to detect differentially methylated regions without providing distinct methylation values. The dmrFinder function was applied using the standard settings.

A third independent method to define differentially methylated regions was additionally programmed in R (42a). As different biological entities like promoter regions, transcriptions start sites, or exons may be covered by a single region designed on the microarray, varying methylation patterns within such regions may exist. Therefore, we did not compare all probes of one region together at the same time but applied a sliding window approach to analyze each region stepwise. Similar as for BATMAN we combined five probes in one window. This window was then moved by one probe per step until the end of a region. The difference between sets of windows of two different groups was assessed by a Wilcoxon-Mann-Whitney test for every step. As for the other approaches, the combined use of several arrays per phenotype led to an increased possibility to detect differences while reducing the impact of potential artifacts or phenotype-independent variation in DNA methylation patterns. While searching for differentially methylated regions we selected only those regions that displayed significant differences for at least three consecutive

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**Fig. 3.** A: single blood pressure measurement (tail-cuff method) in F1 rats. B: registration of continuous blood pressure measurement (telemetry; Data Sciences International) during 3 consecutive days. *P < 0.05 vs. F1-st rats; n = 10/group. C: dose-dependent thoracic aorta contractile response to phenylephrine. SBP, systolic blood pressure; DBP, diastolic blood pressure. D: dose-dependent thoracic aorta relaxation response to acetylcholine. ***P < 0.001 vs. F1-st rats; n = 6/group.
sliding windows (size 500 bp, step 100 bp) at a significance level of $P < 0.001$.

As in previous projects (S. Michel, J. Tost, unpublished data), we observed the best reproducibility as confirmed by pyrosequencing of differentially methylated regions if the region was identified by all three methods. Therefore, only regions fulfilling the above-mentioned selection criteria in all three methods were further investigated. By combining three distinct and independent approaches to define differentially methylated regions, the number of potential false positives was largely reduced, and therefore, besides the more stringent significance level of 0.001 for the sliding window approach, no further multiple test correction applied.

RESULTS

Vitamin D-deficient diet resulted in a pronounced and prolonged vitamin D deficiency in F0 rats. F0 rats (parents) were fed with standard or vitamin D-depleted diet for 10 wk. The dietary effect on plasma 25(OH)D and PTH levels was verified before rats were mated (Fig. 2). Baseline characteristics and echocardiography data of F1 rats are presented in Table 2. Offspring from parents fed a standard diet (F1-st) were compared with offspring from parents fed on a vitamin D-depleted diet (F1-depl). In these pups, we observed no differences in body weight (BW), total heart weight (HW), or left ventricular weight (LVW) (Table 2). Furthermore, we calculated HW/BW ratio and LVW/BW ratio as a relative measure of cardiac hypertrophy. Again, outcomes were not different between groups (Table 2). Echocardiography was performed to assess the effects of a vitamin D-depleted diet during pregnancy on cardiac performance in the offspring. Although global cardiac function was similar in all rats, we observed a minimally impaired cardiac function in F1-depl rats, as reflected by the increased LV internal diameter and decreased E-velocity, fractional shortening, and ejection fraction (Table 2).

F1-depl rats have elevated systolic and diastolic blood pressure and impaired endothelial relaxation. The vitamin D-deficient diet had no effect on blood pressure levels in F0 rats (systolic blood pressure/diastolic blood pressure: F0-st: $138 \pm 1/119 \pm 4$ vs. F0-depl: $140 \pm 2/120 \pm 2$ mmHg, $P =$ NS). Single blood pressure measurement (by tail cuff) revealed that F1-depl rats had a significant higher systolic and diastolic blood pressure than F1-st rats (Fig. 3A). Continuous blood pressure measurements for 3 days in F1 rats confirmed elevated systolic and diastolic blood pressure in F1-depl rats (Fig. 3B). PE-induced vasoconstrictor activity in isolated aorta rings was similar in all F1 rats (Fig. 3C). In the aortas of F1 rats, we observed a significant difference in acetylcholine (ACh)-induced (endothelium dependent) relaxation of F1-depl rats as compared with F1-st rats (Fig. 3C). Endothelial-independent relaxation to SNP did not differ between both groups (i.e., 3% vs. 7% of preconstriction for F1-st and F1-depl rats).

Fig. 4. mRNA expression levels in F1 rats of atrial natriuretic peptide (Anp; A), vitamin D receptor (Vdr; B), renin (C), and aldosterone-1 receptor (At1r; D) in heart and kidney. mRNA expression levels are presented as fold change. *$P < 0.05$ vs. F1-st rats; $n = 10$/group.
levels of renin and angiotensin-1-receptor (At1r) in F1-depl rats as compared with F1-st rats (Fig. 4B). We observed no changes in mRNA expression levels of renin and angiotensin-1-receptor (At1r).

These results suggest that the observed increase in blood pressure in F1-depl rats is not likely to be associated with altered expression levels of the renin-angiotensin-aldosterone system (RAAS) in heart and kidney (Fig. 4, C and D).

Parental vitamin D depletion induces epigenetic changes in the offspring. We determined the DNA methylation profile (Nimblegene Chip Array) in kidney samples from offspring (3 male animals per dietary arm). Although all F1 rats had normal vitamin D levels and were fed and raised under the same conditions, F1-depl rats showed significant changes in methylation status. As illustrated in Fig. 5, we only selected those genes with an obvious change in methylation levels (n = 10; \( P \leq 0.001 \); Fig. 5).

Subsequently, we checked the National Center for Biotechnology Information (NCBI) Gene database to obtain more information on these 10 genes. First, we found that all genes were conserved in humans. For two genes, RefSeq status was predicted and the rest was provisional. Functional description of six genes (Dqxl, Gyltl1b, LOC499742, Otop2, RGD1562608, and Zbtb7b) was not provided. The Capn2 and Bcl2 genes are associated with regulation of apoptosis, while the Adh7 gene is an alcohol dehydrogenase. Finally, the Pannexin-1 gene (Panx1) encodes for the plasma membrane protein called Pannexin-1, which is involved in various physiological and pathophysiological processes (42). The hemichannel Pannexin-1 is abundantly present in different organs and tissues in mammals, including the heart, skeletal muscle, and vasculature. Within the vasculature, Pannexin-1 is predominantly expressed in the endothelium of the larger vasculature and is associated with regulation of endothelial function (17). Therefore, we nominated Panx1 as the most plausible candidate gene to take forward for further analyses of epigenetic changes.

Validation of Panx methylation status by pyrosequence. To verify if altered DNA methylation status of Panx1 could potentially be associated with the observed impaired endothelial relaxation in F1-depl rats, we used pyrosequencing to perform a quantitative DNA methylation analysis on methylation status of Panx1 in kidney, LV, and aorta tissue. In this analysis we validated the microarray data using an extended set of samples (tissue of 10 kidneys, LV, and aortas per group) that included additional tissue from male sibling and nonsibling rats. We observed a significant hypermethylation of the Panx1 gene in F1-depl rats in kidney tissue and smaller, nonsignificant increases in LV and aorta tissue (vs. F1-st rats, Fig. 6). In general, hypermethylation in the promoter region causes gene silencing of genes. However, this is not an unequivocal response, and different cell types may respond in various ways. In our study we observed differential mRNA expression levels of Panx1: mRNA expression levels of Panx1 in kidney tended to be lower in F1-depl rats (vs. F1-st, \( P = 0.10 \)), while we observed no differences in mRNA expression levels in hearts from F1-depl rats at all. Nevertheless, mRNA expression levels of Panx1 were significantly decreased in aortas of F1-depl rats (vs. F1-st \( P < 0.05 \)) and so were Panx1 protein expression levels (Fig. 7).

DISCUSSION

In this study, we show for the first time that a rat model of parental vitamin D deficiency is associated with elevated blood pressure levels in the offspring, which is accompanied by impaired endothelial relaxation in the large vessels. We propose that hypermethylation of the Panx1 promoter region, resulting in lower gene expression, is an underlying potential mechanism. The observed upregulation of Anp in heart suggests increased cardiac stress, while the reduced expression levels of the Vdr in heart and kidney suggest reduced VDR activity. Our findings add to the mounting literature suggesting that nutritional deficiencies of parents may translate into enhanced susceptibility to several common multifactorial diseases in their children.
A recent large multiethnic genome-wide association and replication study provided new insights regarding genetic predisposition of blood pressure regulation and suggested DNA methylation to be an important player (26). Previously, it has been shown that vitamin D is related to blood pressure and risk of hypertension (4, 27, 31), and this has been confirmed recently (50). Interestingly, maternal vitamin D deficiency has been associated with changes in methylation status of the offspring as well (20). This suggests that vitamin D biology may not only directly affect blood pressure regulation but could also intervene with blood pressure levels in an indirect manner.

DNA methylation is part of the process of epigenetic modifications that control how genes are expressed in an individual without altering the DNA. Especially during gametogenesis and early embryogenesis, this process has a major impact on interindividual variation in gene expression in various organs and tissues. Even relatively small observed changes in methylation status can lead to large phenotypic differences that contribute to altered susceptibility to certain diseases later in life (22, 35). Epigenetic modifications can be provoked by numerous factors, including environmental chemicals, drugs, but also nutrition (23, 30). Nutritional factors, in particular maternal deficiencies, are therefore regarded as well-known modifiers of long-term disease outcomes in offspring. It has repetitively been reported that extreme maternal food deprivation during the first trimester of pregnancy induced increased cardiovascular disease prevalence, risk of metabolic disorders, and breast cancer in the children (6, 7, 40). Interestingly, maternal nutritional deficiencies are also directly associated with blood pressure regulation in offspring: the offspring from rats and mice fed on a low protein diet tend to develop increased blood pressure levels (28, 54), and it has been shown that children from malnourished mothers had an increased susceptibility to develop hypertension later in their lives (6). However, no clear cause- and effect relationship explaining the development of hypertension has been established yet (39).
In our study, parental vitamin D deficiency is associated with increased blood pressure in F1 animals, which was not paralleled by and thus independent of the parental blood pressure. Since the increase in blood pressure, in this study, was not likely to be associated with changes in expression of genes of the RAAS, we hypothesize that this would be due to another in utero programming effect, such as altered DNA methylation status. In this study we did not observe a consistent direct (inverse) relationship between DNA methylation and mRNA expression levels in the aorta, kidney, and heart. Epigenetic heterogeneity, however, is complex and not fully understood yet (14). Previous studies have shown that, via diverse kinetic mechanisms, epigenetic changes on epialleles (CpG islands) are linked to changes in methylation status and that they are likely to affect disease susceptibility and disease outcome (29, 32). However, a relationship between alterations in methylation status and mRNA/protein expression levels may not be as self-evident as generally assumed. In general, hypermethylation in the promoter region causes gene silencing by attracting inhibitory proteins and blocking of transcriptional machinery binding. Demethylation is the opposing process and enhances binding of transcription factors and, hence, initiates transcription. This general idea, however, assumes that all cells use the same set of transcription factors, which is clearly not the case. It is therefore likely that methylation changes have different consequences on gene expression in different cell types, as they may affect different transcription factor binding sites and affinities. In our data set, this can be seen in the variety of mRNA expression levels in kidney, heart, and aorta. Future studies are thus warranted to elucidate as to how the underlying mechanism of the complex, but highly interesting, phenomenon of epigenetic heterogeneity alters disease susceptibility and disease outcomes.

The pannexin (Panx) family exists out of three isoforms (Panx1, Panx2, and Panx3) that are present throughout the entire body including the endothelial cells of the large vasculature. In the vessel wall, Panx1 is most abundantly expressed (33). Panx1 is thought to form ATP-permeable hexameric channels that release ATP. Binding of ACh to its receptor on the endothelial surface activates Panx1 hemichannels, which induce endothelium-derived hyperpolarization (EDH) (22) (42). Interestingly, mice that lack the Panx1 encounter endothelial dysfunction due to impaired ACh-induced relaxation (17), and functional Panx1 hemichannels seem to be essential in EDH-like relaxation (16). We hypothesize that the reduction in Panx1 expression may explain, at least in part, the observed differences in blood pressure levels in F1 rats. From our aggregate data, we propose that parental vitamin D deficiency causes hypermethylation in the promoter region of Panx1. This results in less Panx1 transcription and gene expression and inhibition of EDH. As a consequence, ACh-induced relaxation is impaired: relaxation of the large vessels is hampered and blood pressure levels increase (Fig. 8).

Vitamin D levels and VDR are thought to play an essential role in endothelial function as well. Previously, it has been demonstrated that VDR activation of uremic rats significantly improved endothelial function without alteration of blood pressure (2, 25, 38, 55). As we observed an increased blood pressure in F1-depl rats, we suggest that the effect of reduced Vdr is unlikely to be a major factor in explaining our results. This study has several limitations. 1) Global methylation status is measured on kidney tissue and not on vascular segments or endothelial cells. 2) The Western blot analyses should be regarded semiquantitative as we lacked sufficient material for densitometry. 3) Because of the descriptive nature of this study, we are unable to prove a direct cause and effect relation of parental vitamin D deficiency and the development of increased blood pressure in the offspring. We highly recommend future studies to perform crossover experiments that for example compare offspring from vitamin D-depleted fathers/control mothers with offspring from the reverse group. 4) In this study we show that the increase in blood pressure levels is associated with a decrease of mRNA expression levels of Panx1 in the thoracic aorta. Besides larger arteries, smaller
resistance vessels (arterioles) also regulate blood pressure levels. Panx1 is present in both arteries and arterioles, and it has been hypothesized that Panx1 activity regulates vascular tone, peripheral resistance, and blood pressure levels in larger and smaller vessels (1). Future studies should address if altered Panx1 activity in smaller resistance vessels influences blood pressure levels as well. 5) The analysis of RAAS activity is limited. Vitamin D is known to be a negative regulator of the RAAS, and vitamin D deficiency results in enhanced plasma renin activity (PRA) (45). In addition, increased RAAS activity, on the other hand, is associated with higher blood pressure (34). We measured mRNA expression levels of genes related to RAAS. We were unable to measure PRA in F0 rats and F1 rats due to a limited amount of plasma sample. Nevertheless, the combination of plasma vitamin D levels and mRNA expression data in F1 pups suggests, at very most, a limited role for enhanced RAAS activity in the blood pressure increase in F1-depr rats. 6) We were unable to apply multiple comparison correction. Although we attempted to increase robustness in our analysis by using three different methods to detect differences in methylation status, this strategy does not preclude a higher false discovery rate than conventional multiple comparison methods would have done. Future studies should therefore validate our findings before following up on the methylation changes reported in this study.

Conclusions. In conclusion, this study demonstrates that parental vitamin D deficiency is associated with increased blood pressure in offspring. As a potential and plausible biological mechanism, we suggest Panx1 hypermethylation and a concomitant reduction in gene expression to be contributing to the observed disturbed endothelial relaxation. This phenomenon may not only directly result in higher blood pressure but may eventually also contribute to adverse cardiovascular outcomes later in life.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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