

High aldosterone-to-renin variants of *CYP11B2* and pregnancy outcome

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Abstract

Background. Increased aldosterone concentrations and volume expansion of normal pregnancies are hallmarks of normal pregnancies and blunted in pre-eclampsia. Accordingly, we hypothesized an active mineralocorticoid system to protect from pre-eclampsia.

Methods. In pregnant women (normotensive $n = 44$; pre-eclamptic $n = 48$), blood pressure, urinary tetrahydroaldosterone excretion and activating polymorphisms (SF-1 site and intron 2) of the aldosterone synthase gene (*CYP11B2*) were determined; 185 non-pregnant normotensive individuals served as control. Amino acid-changing polymorphisms of the DNA- and agonist-binding regions of the mineralocorticoid receptor were evaluated by RT-PCR, SSCP and sequencing.

Results. Urinary tetrahydroaldosterone excretion was reduced in pre-eclampsia as compared to normal pregnancy ($P < 0.05$). It inversely correlated with blood pressure ($r = 0.99$, $P < 0.04$). Homozygosity for activating *CYP11B2* polymorphisms was preferably present in normotensive as compared to pre-eclamptic pregnancies, identified (intron 2, $P = 0.005$; SF-1 site, $P = 0.016$). Two mutant haplotypes decreased the risk of developing pre-eclampsia (RR 0.16; CI 0.05–0.54; $P < 0.001$). In contrast, intron 2 wild type predisposed to pre-eclampsia ($P < 0.0015$). No functional mineralocorticoid receptor mutant has been observed.

Conclusions. High aldosterone availability is associated with lower maternal blood pressure. In line with this observation, gain-of-function variants of the *CYP11B2* reduce the risk of developing pre-eclampsia. Mutants of the mineralocorticoid receptor cannot explain the frequent syndrome of pre-eclampsia.

Keywords: aldosterone synthase; aldosterone; arterial hypertension; pre-eclampsia; pregnancy outcome

Introduction

Pre-eclampsia (PE), as defined by maternal arterial hypertension and proteinuria in pregnancy beyond the 20th week of gestation, causes severe morbidity often in pre-

viously healthy individuals [1]. In addition to the immediate effects of the disease that may include cerebral accidents, liver disease and renal failure, PE is associated with remote cardiovascular and metabolic disease [2,3]. Thus, the ability to predict PE, especially with tests based on pathophysiological mechanisms, would be an important step towards eventually preventing and managing the disease.

Extracellular and plasma volume expansion of normal pregnancy is linked to changes in renal haemodynamics and of circulating levels of several steroid hormones including markedly increased circulating aldosterone concentrations [4–10]. Following production by the enzyme aldosterone synthase (*CYP11B2*), aldosterone acts in target tissues via the mineralocorticoid receptor (MR).

Catalyzed by *CYP11B2*, aldosterone is synthesized from deoxycorticosterone in a three-step reaction with 18-methyl oxidation being rate limiting. In a group of pre-eclamptic women, we recently observed a reduced *CYP11B2* activity characterized by a compromised 18-methyl oxidase step due to a loss-of-function mutation of *CYP11B2* [11].

In non-pregnant subjects, essential hypertension with a high aldosterone-to-renin ratio, suggesting a high activity of *CYP11B2*, has previously been attributed to gain-of-function mutants representing biallelic gene polymorphisms of the *CYP11B2*. This is a -344C/T exchange located in the putative steroidogenic binding factor-1 (SF-1) within the 5' regulatory region and an intron 2 conversion [Int2 (c)] resulting from the replacement of this region with that of the neighbouring gene encoding *CYP11B1* [12].

With respect to our initial finding of an increased prevalence of loss-of-function mutants of *CYP11B2* in PE, such gain-of-function alleles might protect from developing PE. Several functional relevant mutants of the aldosterone effector, the MR, are known to lead either to a loss or to a gain of function, which could as such further affect the aldosterone response [13].

We hypothesized that in pregnancy, gain-of-function mutants of the *CYP11B2* gene in the absence of loss-of-function polymorphisms of the MR sustain a lower blood pressure and reduce the risk of developing PE.

Table 1. Characteristics of the pregnant women studied

Type of pregnancy	Normotensive	<i>P</i> <	Pre-eclampsia
Information from present pregnancy			
<i>N</i>	44		48
Age (years)	33.2 ± 1.3	n.s.	31.2 ± 0.7
BMI			
At delivery (kg/m ²)	27.4 ± 0.9	n.s.	29.0 ± 0.9
Before pregnancy (kg/m ²)	23.0 ± 0.8	0.04	26.3 ± 1.3
Blood pressure			
Systolic (mmHg)	117 ± 3	0.001	159 ± 16
Diastolic (mmHg)	75 ± 2	0.001	97 ± 2
Fetal birth weight (g)	3286 ± 98	0.001	1774 ± 179
Birth weight percentiles corrected for gestation	42.3 ± 4.8	0.01	26.2 ± 4.1
Low birth weight <2.5 kg (%)	7	0.0001	79
SGA <10th birth weight percentile (%)	21	0.04	42
Gestation at time of sampling (days)	221.9 ± 9.1	n.s.	224.7 ± 4.9
Proteinuria (g/day)	n.d.	0.002	2.3 ± 0.5
Time to delivery (day)	44 ± 6	0.001	6 ± 4
Information from previous pregnancies			
Total number of pregnancies (n/woman)	1.8 ± 0.2	n.s.	1.8 ± 0.2
Primiparous (%)	53	n.s.	57
Multiparous (%)	47	n.s.	43
History of pre-eclampsia (%)	0	0.001	22
History of HELLP syndrome (%)	0	n.s.	4
Fetal loss (n/woman)	0	0.02	0.4 ± 0.1
Premature birth (n/woman)	0	0.001	1.1 ± 0.1
Normal birth weight (n/woman)	1.8 ± 0.2	0.001	0.4 ± 0.1

n.d. = not detectable on either dipstick urinalysis or quantitative assessment of proteinuria, n.s. = not significant, SGA = small for gestational age.

Material and methods

Patients

Ninety-two pregnant women, 44 with normal pregnancies and 48 with PE, and 185 normotensive control subjects (115 males and 70 females) were investigated. Approval by the appropriate hospital ethics review committee and informed consent were obtained from all individuals. Basic demographic data, including blood pressure, information on concurrent diseases and the present medication, were obtained. Women with pre-existing arterial hypertension, diabetes mellitus or renal disease were excluded from the study. Patients were included in the PE group if blood pressure was $\geq 140/90$ mmHg and proteinuria > 0.3 g/day in the absence of urinary tract infection beyond the 20th week of gestation (Table 1) [14]. Healthy pregnant women were selected as case controls according to the gestational age at initial presentation of pre-eclamptic women. Both groups of women were followed to delivery.

Collection of urine and blood samples and isolation of genomic DNA

Twenty-four-hour urine samples were collected from all women with PE at initial presentation. These samples were matched with those from women with a normal pregnancy at the same gestational age (± 2 weeks); however, analysis was postponed until delivery in order to establish the presence of an uncomplicated pregnancy. Ten millilitres of blood samples were drawn into tubes containing EDTA, mixed and stored at 4°C. Genomic DNA was extracted from peripheral blood leucocytes following the protocol supplied by the nucleon BACC3 kit (Bioscience; Piscataway, USA). Purified DNA was stored at 4°C in ddH₂O.

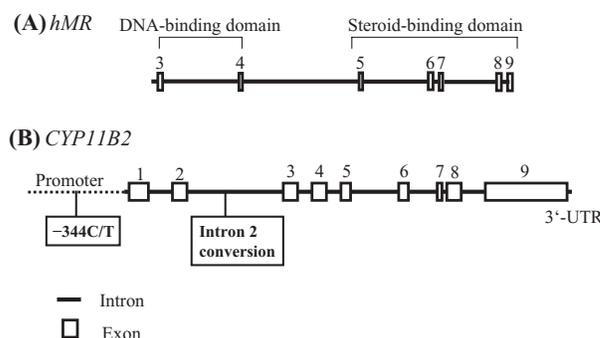


Fig. 1. Schematic representation of the human mineralocorticoid receptor (MR) (A) and aldosterone synthase (CYP11B2) (B) gene. Boxes represent exon, straight lines intron and dashed lines the 5' promoter regions. Mutations are depicted in the frame.

Gas chromatography–mass spectrometry (GC-MS) of tetrahydro-aldosterone

Urinary tetrahydro-aldosterone was analysed by GC-MS according to the method described by Shackleton [15]. Sample preparation consisted of pre-extraction, enzymatic hydrolysis, extraction from the hydrolysis mixture, derivatization and gel filtration. Medroxyprogesterone (2.5 µg) was added as recovery standard to 1.5 ml urine. The sample was extracted on a Sep Pak C18 column, dried, reconstituted in a 0.1 M acetate buffer (pH 4.6) and hydrolyzed with powdered Helix pomatia enzyme (12.5 mg) and 12.5 µl of β -glucuronidase/arylsulfatase liquid enzyme at 55°C for 3 h. The resulting free steroids were extracted on a Sep Pak C18 cartridge, and a standard mixture (5 α -androstane-3 α -17 α -diol, stigmasterol and cholesteryl butyrate, 2.5 µg of each and 0.15 µg of 3 β 5 β -tetrahydro-aldosterone) was added. The sample was derivatized to form the methyloxime-trimethylsilyl ethers and gel filtration on a Lipidex 5000 column added. Samples were analysed on a Hewlett Packard gas chromatograph 6890 equipped with the mass selective detector 5973 and the auto injector 7683 by selective ion monitoring for each measured compound against a known calibration standard (3 β 5 β -tetrahydro-aldosterone against 3 α 5 β -tetrahydro-aldosterone). Ions were chosen with respect to their retention time during a temperature-programmed 35-min run (210–265°C). The urinary concentration of 3 α 5 β -tetrahydro-aldosterone was normalized against urinary creatinine excretion.

Screening of genomic DNA for the gain-of-function mutants of CYP11B2 [SF-1–344C/T and Int2 (w)/(c)] and functionally relevant mutants of the MR (Figure 1)

Polymerase chain reaction (PCR) for SF-1 and Int2 polymorphisms of CYP11B2 was performed using the primers and conditions reported by Davies *et al.* [16]. The region of DNA containing the HaeIII/SF-1 polymorphism was amplified by PCR by use of conditions similar to those previously described. A PCR product of 228 bp was amplified. This was digested with HaeIII and subjected to electrophoresis in 3% MetaPhor agarose. The 228 bp amplicon contains two HaeIII restriction enzyme sites (GG CC). The presence of a C–T transition at position -344 (GG CT) removes one of these sites. After digestion, individuals homozygous for the transition (TT) produce two bands of 175 and 53 bp, individuals homozygous for the wild-type (CC) three bands of 104, 71 and 53 bp, and heterozygous individuals (TC) four bands.

The Int2 conversion was genotyped by using two separate PCR reactions, one that amplifies the normal gene (WT) and one that amplifies the conversion. The size of the amplicon in each reaction is ~418 bp.

The functionally important domains of the MR for DNA and agonist binding, exons 3 to 9, respectively, were amplified by using the following primers: forward 5'-TGT GCA GCA TGT TAA ATG TGC-3', 5'-TTC AAG GCA GGA AAG AAG TAG-3', 5'-TGG CCT TAT CAT TTA TGG TTT C-3', 5'-TTC CTG GAA TGT ACT TAA ATG C-3', 5'-CAG TCT GGG TTT GAT AAT G-3', 5'-CCT GGA AAT TTA CAT GTC TG-3' or 5'-AAA GTC AGA AGG CAG AGG GAT C-3', respectively. As reverse primers served: 5'-AAC ACC CAC ATG AAC ATT TAC C-3', 5'-TGC TGT TGC ATT ACC TAT TAA C-3', 5'-TGC AGC CTG TGA AAG GAG AG-3', 5'-AAA TCA TAA CGC ATA ACT CTG C-3', 5'-TAG AAA CAG

TGC CAG AAT G-3', 5'-TCA CTT AGG AAC CAA GGA G-3' or 5'-CTT AAG GCA AAG TTC TTC TGG G-3', respectively. The known mutation Ser⁸¹⁰ Leu, which was created by site-directed mutagenesis of wild-type exon 6 of the MR, served as positive control [17]. All reactions were carried out with 25 pmol of each primer in a final volume of 50 μ L, containing a 4 mmol/l MgCl₂ buffer, 0.2 mmol/l of each dNTP and 1 unit of *AmpliTaq Gold* polymerase (PE Biosystems, Foster City, CA, USA). The DNA was amplified for 35 cycles with denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min. PCR products were investigated using a combined strategy of single strand conformation polymorphism and direct sequencing to identify polymorphisms. Non-denatured and denatured (100 mM NaOH, 0.25% bromophenol blue/xylene cyanol in formamide heated 6 min at 95°C) PCR products were run on 12% acrylamide/piperazine-diacrylamide (PDA) gels containing 7.25% glycerol in a two-buffer system. Four μ l of the PCR sample were loaded, separated at 12°C, and DNA was visualized by silver staining [18]. Sequence changes were detected by double-band shifts on the gel. Sequence identity was verified by direct sequencing.

Statistical analysis

All data are presented as means \pm SEM. To test for statistically significant differences, Student's *t*-test was used for continuous variables by nonparametric analysis and the Wilcoxon or Kruskal–Wallace test for variables that were not normally distributed. Linear regression analysis was performed for the continuous parameters (birth weight and systolic/diastolic blood pressure as related to urinary tetrahydro-aldosterone/creatinine). Standardized calculations of relative risk (RR) and confidence intervals (CI) were performed with log transformations and a normal approximation used for the CI. For comparison of genotype distributions, the chi-square test and Fisher's exact test were used, taking the appearance of PE as threshold. Comparisons for the number of mutated haplotypes were performed for each allele contributing, for each gene, and by grouping haplotypes as follows: no mutated haplotype [SF-1 C/C, Int2 (w)/(w)], one mutated haplotype of both genes [SF-1-344C/T and Int2 (w)/(c)] and two mutated haplotypes [SF-1 T/T and Int2 (c)/(c)]. Significance was assigned at $P < 0.05$. All statistical analyses were performed using either SYSTAT version 10 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Patients characteristics

Basic demographic, clinical and biochemical data of the 92 pregnant women, dichotomized as to the occurrence of PE, are reported in Table 1. There was no difference in age and body mass index in the two groups of women. The clinical status was confirmed to be uneventful within the current and all former pregnancies in the 44 women included as normally pregnant. PE was diagnosed in 48 women by the presence of proteinuria and hypertension as defined above. The birth weight of children from pre-eclamptic pregnancies was >40% lower than that of children from normal pregnancies at similar gestational weeks (Table 1). Comparable numbers of women with either normal pregnancy or PE were primiparous (53 versus 57%). Twenty percent of multiparous women experienced a first pre-eclamptic episode, and 22% were exposed to recurrent PE. Women with a PE in the current pregnancy had former pregnancies complicated by fetal losses, premature births and low birth weight of their children (Table 1). Antihypertensive treatment in PE consisted of labetalol, α -methyl dopa and nifedipine.

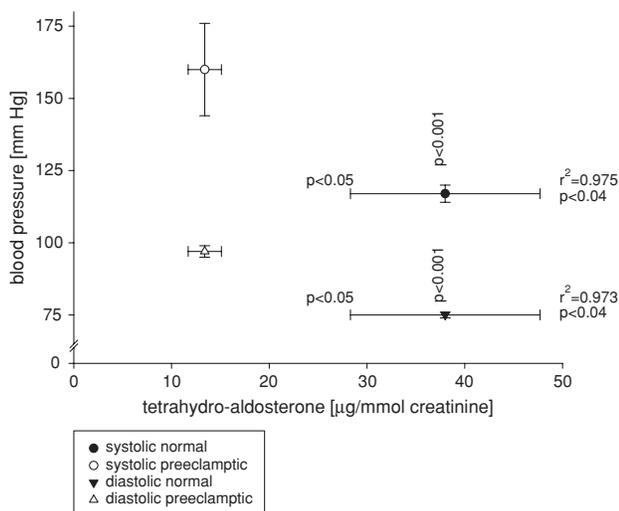


Fig. 2. Correlation between blood pressure and urinary tetrahydro-aldosterone. Tetrahydro-aldosterone was measured by GC-MS in 24-h urine of pregnant women experiencing either a normal ($n = 44$) or a pre-eclamptic ($n = 48$) pregnancy. Systolic and diastolic blood pressure is depicted as circles and triangles, respectively, with values from normotensive and pre-eclamptic pregnant women closed and open, respectively.

Correlation of tetrahydro-aldosterone secretion with clinical patterns

Urinary tetrahydro-aldosterone excretion was significantly reduced in pre-eclamptic women when compared to normal pregnancies ($13.4 \pm 1.8 \mu\text{g/mmol}$ versus $38.0 \pm 9.8 \mu\text{g/mmol}$ creatinine) (Figure 2). Lower blood pressure correlated with a higher urinary tetrahydro-aldosterone excretion (systolic $r = 0.99$, $P < 0.04$; diastolic $r = 0.99$, $P < 0.04$) (Figure 2). High urinary tetrahydro-aldosterone excretion was found in the presence of higher birth weights in normal pregnancies as compared to the lower tetrahydro-aldosterone excretion and birth weights in PE (38.0 ± 9.8 versus $13.4 \pm 1.8 \mu\text{g/mmol}$ creatinine; $P < 0.05$ and 3286 ± 98 g versus 1774 ± 179 g; $P < 0.05$).

Genotype and haplotype analysis of MR and CYP11B2 gene polymorphisms (Figure 1)

A PCR-based screening for mutations in the DNA and steroid-binding domains of the MR was performed, and no amino acid-changing mutation leading to structural or functional changes was identified.

The CYP11B2 genotype distribution within the population of the pregnant women and the controls studied were in Hardy–Weinberg equilibrium. Normotensive pregnancies more likely took place in the presence of homozygosity for Int2 (c)/(c) ($P = 0.0046$), but less frequently with Int2 (w)/(w) ($P = 0.015$). Homozygosity for SF-1 T/T was associated with reduced occurrence of hypertensive pregnancies ($P = 0.016$, Figure 3), yet heterozygosity did not protect from PE ($P = 0.012$). Combining alleles associated with high aldosterone-to-renin ratios indicated an increased frequency of these alleles in normotensive

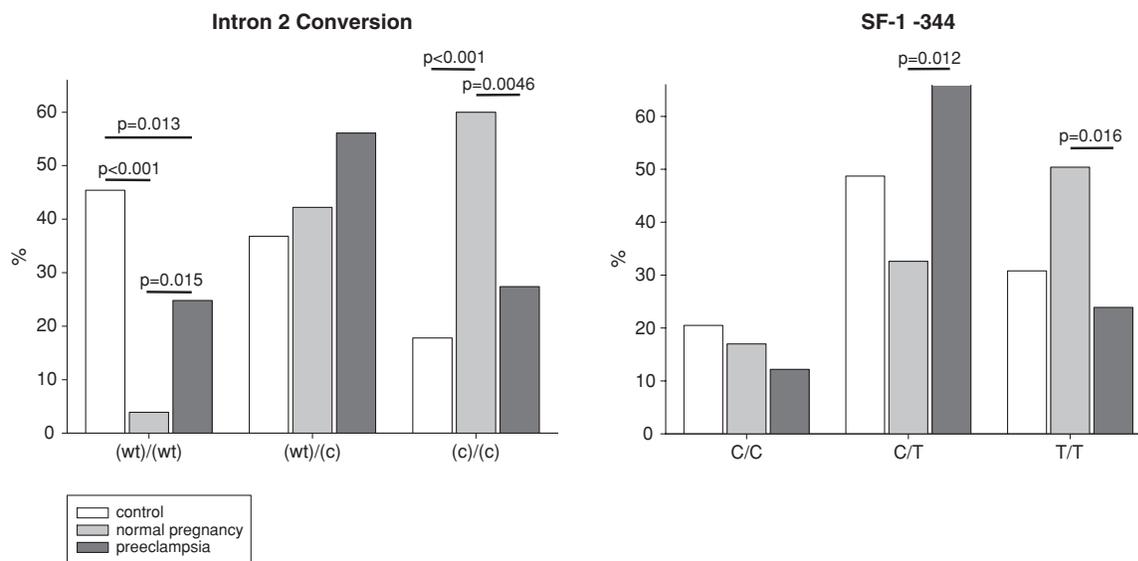


Fig. 3. Allelic distribution of SF-1 T and Int2 (c) haplotypes of *CYP11B2*. Percentage of patients by allelic distribution of SF-1 T and Int2 (c) haplotypes of *CYP11B2* according to pregnancy outcome. Open bars indicate healthy normotensive males, grey bars indicate normal pregnancies and black bars PE.

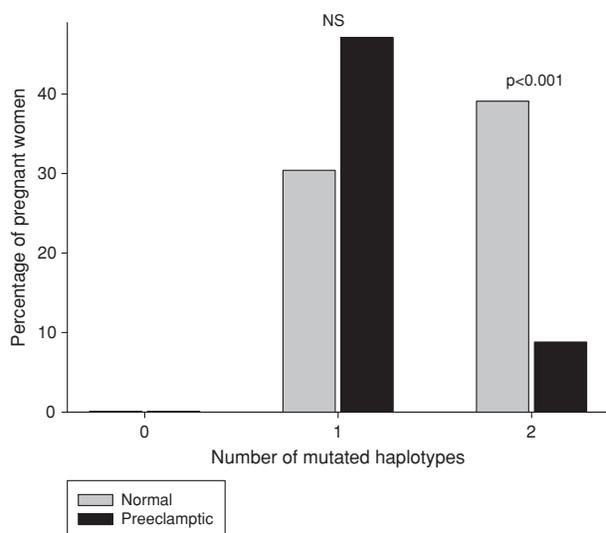


Fig. 4. Percentage of patients by number of SF-1 T and Int2 (c) haplotypes of *CYP11B2* according to pregnancy outcome. Grey bars indicate normal pregnancies and black bars PE.

when compared to pre-eclamptic pregnancies (2.6 ± 0.3 versus 1.9 ± 0.2 alleles; $P = 0.023$). So far, these results point towards a relationship between pregnancy outcome and the number of mutant haplotypes. The presence of two, yet not one, mutant haplotypes for each gene locus was highly significantly linked to uneventful pregnancies ($P < 0.001$, Figure 4). The relative risk of developing PE was not reduced with one mutant haplotype, but with two (compared to no and one mutant haplotype RR 0.16; CI 0.05–0.54).

Discussion

This study shows an association of maternal blood pressure and fetal birth weight with aldosterone availability. As proposed, in normal pregnancies a high aldosterone production, determined via the measurement of urinary tetrahydro-aldosterone excretion, the most sensitive marker of circadian aldosterone production, was related to low maternal blood pressure levels [19]. Confirming our hypothesis, the number of high aldosterone-to-renin ratio alleles was increased in normal pregnancies. The absence of a high aldosterone-to-renin ratio allele of intron 2 was associated with an augmented frequency of PE. Furthermore, the presence of both mutants significantly favoured normal pregnancies.

The clarification of mechanisms of PE and hypertension in pregnancy is of practical relevance for multiple reasons with preventing the disease being the most important one [20–25]. If high aldosterone levels and the increased circulating plasma volume found in normal pregnancies represent a physiological adaptation crucial to support an adequate circulating fluid volume in pregnancy, two observations should be possible: first, a phenotypical adaptation in response to the aldosterone available, and second, mechanisms within the mineralocorticoid system either leading to or mimicking high aldosterone availability should prove beneficial to avoid PE [4–9,26].

If the primary impact of sufficient aldosterone availability would be to benefit pregnancy by avoiding PE, a genetic predisposition to produce high quantities of aldosterone or to appropriately respond to a given aldosterone concentration via the MR would be reasonable. Such a predisposition has been recognized by two gene loci of Mendelian forms of the *CYP11B2* gene, the promoter SF-1 single nucleotide polymorphism -344C/T and a conversion in intron 2, which

are variably linked to arterial mineralocorticoid hypertension with increased aldosterone-to-renin ratios in the non-pregnant population [12]. With these findings we stay in line with our initial finding of a compromised aldosterone synthesis being associated with PE [11].

In our study, the most pronounced differences between normal pregnancies and PE could be observed, if either an increased number of high ARR alleles or homozygosity for the mutant form were present. The RR to develop PE was reduced by >80%, if women were homozygous for both mutant alleles. Thus, combinations of activating polymorphisms seem to add to an uneventful pregnancy outcome, most likely due to an amplified aldosterone production as has been demonstrated during postural changes, when the maximal aldosterone response was found in subjects with two mutant haplotypes [12]. This observation is in line with our results and supports the view that variations in *CYP11B2* may well lead to an intermediate phenotype of aldosterone excess supporting arterial hypertension in non-pregnant subjects and normotension during pregnancy due to an improved utero-placental perfusion.

One of these polymorphisms, the SF-1-344C/T conversion, had been studied in a pre-eclamptic population from Turkey with a lack of association [27]. A direct comparison of these data with our current findings is limited by the missing information on primiparity, which was high in our study group, and the ethnic divergence between the study groups. Also, we investigated a group of pregnant women as controls without any abnormalities in their former pregnancy history, which may have resulted in a bias towards genotypes of the *CYP11B2* with a high aldosterone production. Furthermore, PE appears to be a multifactorial disease with each factor potentially contributing to a different extent, even more in a genetically divergent context. Interestingly, the low expression of high aldosterone-to-renin ratio variants in PE resembled the pattern seen in the healthy, normotensive, non-pregnant subjects in our and earlier studies [12]. This observation does support the notion that *CYP11B2* variants, which predispose women to PE, are rather frequent and any additional contributing factor could easily expose women to the risk of developing PE.

This study supports the assumption that an uncompromised aldosterone production is importantly contributing to an uneventful pregnancy outcome. If aldosterone production is diminished due to a polymorphism within the *CYP11B2* gene, the reduced aldosterone availability in the presence of increasing concentrations of the partial aldosterone antagonist progesterone throughout gestation might reduce the compensatory potential for shifts in circulating volume warranting the genotypes discussed here with a high capacity to produce aldosterone [11]. Our current observations well support the concept of a genetic susceptibility of PE as has been repetitively demonstrated in family and twin studies, though stable environmental factors must also be considered in recurrent PE and have not been addressed here [28–30].

The exact mechanisms determining the magnitude of aldosterone secretion in the presence of the *CYP11B2* variants have not yet been elucidated [31]. Adrenal release of

aldosterone is reduced in hypoxia as shown by Raff and Kohandarvish [32] *in vitro* and by Lawrence *et al.* [32,33]. Thus, one might speculate that during established PE factors such as adrenal hypoxemia further contribute to a diminished aldosterone release.

Earlier, we ruled out aldosterone levels to be decreased due to an excess of alternate mineralocorticoid-like steroid hormones [11]. Yet, aldosterone concentrations might be affected by an altered activity of the MR by either gain- or loss-of-function mutations within functionally relevant regions of the MR gene [13,17,34]. Our present data do support the assumption that structural or functional mutations of the MR are extremely rare and will not have to be judged as a contributing major factor to explain the frequently occurring PE, a finding supporting observations in very small subsets of pre-eclamptic patients by other groups [35,36].

The present investigation revealed a close relationship between aldosterone availability, gestational maternal blood pressure and fetal birth weight. High aldosterone-to-renin ratio variants of the *CYP11B2*, which provide for an increased aldosterone availability, were associated with a lower frequency of pre-eclamptic pregnancies. Most strikingly, double homozygosity for both high aldosterone-to-renin ratio variants, predicted a significantly reduced risk of developing PE. Though the exact regulatory events for *CYP11B2* in pregnancy still have to be elucidated more in detail, this observation is of practical importance since it might help to define subgroups of patients with an altered chance to develop pregnancies complicated by PE. Thus, women could be given a tailored follow-up throughout their pregnancies according to their *CYP11B2* genotype in addition to further risk predictors. These results should be verified in a larger cohort also including women with an intermediate phenotype.

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Conflict of interest statement. None to be declared.

(See related article by M. D. Lindheimer and P. August. Aldosterone, maternal volume status and healthy pregnancies: a cycle of differing views. *Nephrol Dial Transplant* 2009; 24: 1712–1714.)

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