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Angiotensin converting enzyme intron 16 insertion/deletion genotype is associated with plasma C-reactive protein concentration in uteroplacental dysfunction

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Abstract

Introduction: Disturbance of the uteroplacental circulation (UPC) and the renin-angiotensin system are involved in the pathogenesis of preeclampsia. In women with history of preeclampsia persistently elevated C-reactive protein (CRP) levels have been described. The angiotensin-converting enzyme (ACE) intron 16 insertion/deletion (I/D) genotype is associated with ACE activity and assumed to be a risk factor for preeclampsia. As ACE generates proinflammatory angiotensin II, we analysed, whether ACE intron 16 I/D genotype is associated with CRP and whether this association exhibited a relation to uteroplacental dysfunction.

Materials and methods: A total of 639 women have been followed during pregnancy with repeated measurements of CRP levels (observations: n=2333). ACE intron 16 I/D genotype was determined, and its association with CRP was assessed with adjustment for non-independent observations.

Results: CRP levels of ACE D allele carriers were significantly higher than those of the ACE II (wild-type) genotype (p=0.0003, p_{adj}=0.04). This relation was allele-dose dependent ($p<10^{-4}$, $p_{adj}<0.02$). Association between ACE I/D and CRP was significantly restricted to patients presenting with impaired UPC in univariate ($p<0.04$) and multivariate analyses (p=0.01).

Conclusions: The ACE I/D genotype is significantly associated with CRP elevations during pregnancies complicated by disturbed UPC. Whether this effect on CRP is involved in pathogenesis of preeclampsia has to be elucidated.

Keywords

Angiotensin-converting enzyme, C-reactive protein, uteroplacental circulation, inflammation, genotype

Introduction

Preeclampsia is a complication of pregnancy characterised by maternal hypertension and proteinuria that is associated with significantly increased maternal and neonatal morbidity and mortality. There is evidence that women with a history of preeclampsia or eclampsia exhibit an increased cardiovascular comorbidity.[1] Vascular endothelial dysfunction plays an important role in the pathogenesis of this disease,[2] and impaired uteroplacental circulation indicated by increased impedance to flow, notching or foetal growth restriction is a putative precursor to preeclampsia.[3] An involvement of insulin resistance, dyslipidaemia, and inflammation in the pathogenesis of preeclampsia is well documented.[4] Recently, prolonged elevations of plasma C-reactive protein (CRP) levels up to 30 years after a preeclamptic pregnancy have been described.[5] Other groups reported that elevated CRP levels are a risk factor for preeclampsia.[6,7] Furthermore, there is some evidence that CRP levels are associated with

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severity of preeclampsia[8] and that variants in the CRP gene are associated with risk of preeclampsia.[9] However, a relationship between CRP levels and preeclampsia could not be corroborated in all studies on this topic (e.g.[10]) and there are some data that indicate that the relation between CRP and preeclampsia is modified by confounders like weight status.[7] Finally, it is assumed that the renin-angiotensin-aldosterone system (RAAS) is part of a pathway implicated in the pathogenesis of preeclampsia.[11,12] In this context, the intron 16 insertion/deletion (I/D) genotype of the angiotensin converting enzyme (ACE) is discussed as a potential genetic risk factor for preeclampsia with some studies supporting such an association as well as others that indicate a null association.[13–16] Carriers of the ACE intron 16 D allele are assumed to exhibit higher plasma ACE activities as compared to individuals with the ACE intron 16 II (wild-type) genotype.[17] As angiotensin II, which is generated from angiotensin I by an ACE dependent reaction, is known as a mediator of inflammation,[18] we hypothesised that the ACE intron 16 I/D genotype could be associated with plasma CRP levels, which in part could explain the relation between elevated CRP levels and history of preeclampsia. Based on these results, we assessed, whether this association would exhibit a relation to uteroplacental insufficiency.

Materials and methods

Study population

We studied the association of the ACE intron 16 I/D genotype with plasma CRP levels in 639 consecutive pregnant women presenting in our haemostaseologic outpatient unit for medical attendance during pregnancy due to thrombophilia, history of venous thromboembolism, and/or history of foetal loss. Information on uteroplacental circulation was available in 476 patients. Of these, 76 presented with signs of uteroplacental insufficiency in uteroplacental Doppler ultrasound examination indicated by high impedance to flow, notching, and/or foetal growth restriction. Patients were followed up to 12 weeks after delivery. The median interval between consecutive CRP measurements was 36 days (interquartile range (IQR): 31–43 days). As CRP levels are critically influenced by a multitude of environmental factors, which are nearly uncontrollable, we used a time-averaged approach including all CRP measurements and adjusted for non-independent observations.[19] Analyses were performed on 639 patients with a total of 2333 CRP measurements (number of presentations: one (n=72), two (n=147), three (n=103), four (n=98), more than four (n=219)). Five hundred and sixty-five patients (88%) were of German origin and the other women were of Middle East and Asian origin. The study was approved by the local ethics committee, and the patients were enrolled after giving informed consent.

Genotyping and CRP measurements

The ACE intron 16 I/D genotypes were characterised by amplification fragment length polymorphisms, as described previously.[20] Briefly, after extraction of genomic DNA from whole blood using GenoPrep Cartridges B and the GenoM-6 system (GenoVision, Vienna, Austria), a primer pair consisting of 5'GAC CTG CTG CCT ATA CAG T 3' and 5'GGG TAA AAC TGG AGG ATG GCT C 3' was used for amplification, generating a 234 bp product for the ACE intron 16 deletion allele, and a 521 bp amplification product for the ACE intron 16 insertion allele. Additionally, the primer pair 5'GAT TAC AGG CGT GAT ACA GT 3' and 5'GGG TAA AAC TGG AGG ATG GCT C 3', which is specific for the ACE intron 16 insertion allele, was used. All primers were synthesised by TIB Molbiol (Berlin, Germany). Plasma CRP levels were quantified by an immunoturbidimetric method standardised according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) with a lower detection limit of 3 mg/l (Roche Diagnostics, Germany).[21]

Statistical analyses

The ACE intron 16 I/D genotype was assessed by the Pearson χ^2 test to determine if the observed genotype distribution was in accordance to expected Hardy-Weinberg proportions.

Nonparametric comparisons of CRP levels between the different ACE intron 16 I/D genotype combinations were done by Mann-Whitney U test or in the case of allele-dose dependency by the Cuzick nonparametric test for trend. For univariate analyses on the association between ACE intron 16 I/D genotype and CRP elevations above 10 mg/l, odds ratios (ORs) and exact 95% confidence intervals (95% CIs) were calculated. For calculations on allele-dose dependency, the ACE intron 16 II genotype was used as reference group, and a test for linear trend of the log odds (trend test) was performed. For multivariate analyses, ORs and 95% CIs were calculated by a logistic regression model adjusting for age (quartiles), weeks of gestation (≤12 weeks, 13–24 weeks, and >24 weeks), ethnicity, and body mass index (BMI) (>25 kg/m^2).

As CRP levels are influenced by a multitude of uncontrollable environmental factors, we decided to use a time-averaged approach. Several CRP measurements per patient have been included in the analyses. Even though, the usage of clustered data not necessarily biases statistical analyses, intracluster correlation has to be considered and statistically assessed. Consequently, adjustments for non-independent observations within clusters have been performed (p_{adj}) for all analyses, as described previously.[19] These adjustments relax the independence assumption so that independence is only required across clusters.[22]

Table 1. Characteristics of the study population.

Characteristics	Total	Normal UPC	Disturbed UPC
Age (years), median (IQR)	32 (28–36)	32 (28–36)	33 (28–35)
BMI, median (IQR) kg/m^2	23.6 (21.5–26.9)	22.7 (21.0–25.3)[a]	26.0 (23.7–28.7)[a]
Fbg, mean (95% CI) g/l	4.53 (4.47–4.59)	4.49 (4.42–4.55)[b]	4.80 (4.63–4.98)[b]
DD, mean (95% CI) mg/l	0.24 (0.22–0.25)	0.23 (0.22–0.24)	0.27 (0.23–0.31)

BMI: body mass index; CI: confidence interval; CRP: C-reactive protein; DD: D-dimer concentration; Fbg: fibrinogen concentration; IQR: interquartile range; *p*: *p*-value for nonparametric comparison; p_{adj}: *p*-value after adjustment for non-independent observations; UPC: uteroplacental circulation. [a]$p<10^{-4}$; [b]$p<10^{-3}/p_{adj}$=0.007.

Table 2. Plasma levels of C-reactive protein (CRP) in dependence of the angiotensin-converting enzyme (ACE) intron 16 insertion/deletion genotype and uteroplacental dysfunction.

Plasma CRP levels (mg/l)				(p/p_{adj})
Total mean (95% CI)	ACE II mean (95% CI)	ACE ID mean (95% CI)	ACE DD mean (95% CI)	
5.5 (5.3–5.8)	5.2 (4.7–5.7)	5.4 (5.1–5.8)	6.0 (5.5–6.5)	$<10^{-4}$ /<0.02
Normal UPC (n=400)	5.2 (4.7–5.7)	5.2 (4.8–5.6)	6.0 (5.6–6.5)	<0.001 / 0.05
Disturbed UPC (n=76)	4.5 (3.8–5.1)	5.7 (5.0–6.4)	8.8 (4.7–12.9)	0.007 / 0.10

P, P-value for nonparametric comparison of CRP levels. Padj, P-value after adjustment for non-independent observations.
CRP, C-reactive protein; ACE, angiotensin converting enzyme; I, insertion polymorphism; D, deletion polymorphism; CI, confidence interval; UPC, uteroplacental circulation.

All statistical analyses were performed using Stata Statistical Software for Macintosh, release 10.1 (StataCorp, USA).

Results

In the study population, the frequencies of the different ACE intron 16 I/D genotypes were: 25.2% for ACE intron 16 II, 49.3% for ACE intron 16 ID, and 25.5% for ACE intron 16 DD. The determined genotype distribution in this cohort was consistent with Hardy-Weinberg equilibrium (p=0.72). Further characteristics of the study population are given in Table 1. Briefly, patients with disturbed uteroplacental circulation (UPC) exhibited a significantly higher BMI when compared with those with normal UPC. Furthermore, fibrinogen levels were significantly higher in case of disturbed UPC (Table 1).

The mean CRP level in the total study population was 5.5 mg/l (Table 2). When comparing the CRP levels depending on the women's ethnicity, there was a highly significant difference (p_{adj}=0.0003). While in women of German origin the mean CRP level was 5.5 mg/l (95% CI: 5.2–5.7), in women of other ethnicities it was considerably higher (6.2 mg/l, 95% CI: 5.7–6.7).

The comparison of CRP levels depending on presence or absence of the ACE intron 16 D allele revealed significantly higher CRP levels in carriers of the ACE intron 16 D allele (p=0.0003). After adjustment for non-independent observations, the difference in CRP levels between ACE D allele carriers and non-carriers was statistically significant (p_{adj}=0.04), as well. Subsequently, we tested, whether CRP levels depend on the number of ACE intron 16 D alleles. The differences in mean plasma CRP levels between the different ACE intron 16 I/D genotypes were moderate (Table 2). However, the statistical assessment indicated a significant increase in CRP levels with ascending numbers of ACE intron 16 D alleles without and with adjustment for non-independent observations ($p<10^{-4}$, $p_{adj}<0.02$) (Table 2).

In patients with disturbed UPC, the mean CRP level tended to be higher (6.1 mg/l, 95% CI: 5.1–7.1) than in those presenting with normal uteroplacental function (5.5 mg/l, 95% CI: 5.2–5.7) (p=0.02, p_{adj}=0.14). When performing the analyses on the association of the ACE intron 16 I/D genotype with CRP levels stratified by the absence or presence of uteroplacental dysfunction, there was only a small increase in mean CRP levels with increasing numbers of ACE intron 16 D alleles in normal UPC (II: 5.2 mg/l, ID: 5.2 mg/l, DD: 6.0 mg/l), while this relation was considerably more pronounced in those patients with disturbed UPC (II: 4.5 mg/l, ID: 5.7 mg/l, DD: 8.8 mg/l) (Table 2). When restricting these analyses on patients with Caucasian origin the results remained essentially unchanged. Mean CRP levels increased with the number of ACE intron 16 D alleles ($p<10^{-3}/p_{adj}<0.04$) and this effect was especially prominent in Caucasian patients with disturbed UPC (II: 4.6 mg/l, ID: 5.7 mg/l, DD: 8.8 mg/l). Mean CRP levels tended to be different between Caucasian patients without (5.4 mg/l, 95%

Table 3. Association of the angiotensin converting enzyme intron 16 insertion/deletion (I/D) genotype with C-reactive protein (CRP) in dependence on uteroplacental dysfunction.

	CRP <10 mg/l versus ≥10 mg/l		
	Total	Uteroplacental circulation	
	OR (95% CI)	Normal OR (95% CI)	Disturbed OR (95% CI)
Carriage of ACE D allele			
Univariate	1.36 (0.98–1.92)	1.11 (0.76–1.64)†	5.34 (1.27–47.6)†
Multivariate	0.86 (0.51–1.45)	0.61 (0.33–1.10)‡	6.06 (1.11–33.1)‡
Trend test			
ACE II	1 (reference)	1 (reference)	1 (reference)
ACE ID	1.23 (0.87–1.73)	0.91 (0.61–1.36)	4.38 (1.08–17.7)
ACE DD	1.61 (1.12–2.32)	1.47 (0.97–2.22)	7.84 (1.98–31.0)

Results of χ^2 testing, multivariate analyses (adjusted for age, weeks of gestation, ethnicity, and body mass index), and trend tests are presented.
†Test of homogeneity between strata defined by normal and disturbed UPC, $p<0.04$, $p_{adj}<0.05$.
‡Test of homogeneity between strata defined by normal and disturbed UPC, $p=0.01$, $p_{adj}=0.03$.
CRP, C-reactive protein; ACE, angiotensin converting enzyme; OR, odds ratio; CI, confidence interval; UPC, uteroplacental circulation.

CI: 5.1–5.7) and with disturbed UPC (6.1 mg/l, 95% CI: 5.0–7.2) ($p=0.006$, $p_{adj}=0.10$).

Carriage of the ACE intron 16 D allele tended to be associated with CRP elevations above twice the normal range (≥10 mg/l, OR: 1.36, 95% CI: 0.98–1.92) (Table 3). When considering the allele-dose, there was a significant trend towards CRP elevations with increasing number of ACE intron 16 D alleles (II: reference; ID: OR: 1.23, 95% CI: 0.87–1.73; DD: OR: 1.61, 95% CI: 1.12–2.32; p for trend <0.009). Interestingly, when stratifying for normal and disturbed UPC, a clear heterogeneity between both strata in respect of the relation of ACE intron 16 I/D genotype and CRP elevations could be found (Table 3). In normal UPC, no association between CRP elevations and either ACE intron 16 D allele carriage or number could be detected in univariate and multivariate analyses. On the other hand, in patients with disturbed UPC there was a significant association of ACE intron 16 D allele carriage (OR: 5.34, 95% CI: 1.27–47.6) and copy number (II: reference; ID: OR: 4.38, 95% CI: 1.08–17.7; DD: OR: 7.84, 95% CI: 1.98–31.0; p for trend=0.004) (Table 3). When restricting these analyses on patients with Caucasian origin the results remained essentially unchanged.

The findings could be confirmed in multivariate analyses adjusting for possible confounders including body mass index and ethnicity. The heterogeneity between both strata defined by normal and disturbed UPC, respectively, in respect of the association of ACE intron 16 I/D genotype and CRP was statistically significant in univariate ($p<0.04$, $p_{adj}<0.05$) as well as multivariate analyses ($p=0.01$, $p_{adj}=0.03$).

Discussion

Based on the interrelationship between inflammation, RAAS, uteroplacental disturbance and preeclampsia we investigated, whether (a) there is a relation between the ACE intron 16 I/D genotype and plasma CRP levels during pregnancy and (b) whether this relation is affected by uteroplacental function. We tested this hypothesis in a cohort of pregnant women. There is evidence that pregnancy itself could be considered as an inflammatory stressor,[23] which translates in elevations of plasma CRP levels even during normal pregnancy.[24] Thus, we tested our hypothesis in a population under slightly proinflammatory conditions. In our cohort, the mean CRP level was 5.5 mg/l, which is in good agreement with the results of a recent study on CRP levels during pregnancy.[23] When comparing patients without and with disturbed UPC there was a trend towards higher CRP levels in patients with disturbed UPC that did not reach statistical significance. Due to the relatively small sample size and the fact that we used a non high-sensitivity CRP quantification technique in patients with only moderate inflammatory stimulation, our results might underestimate the relation between UPC disturbance and CRP levels.

In our study population a highly significant heterogeneity in CRP levels depending on the ethnicity could be recognized ($p_{adj}=0.0003$), a finding, which has been described previously.[23] Due to this heterogeneity our statistical approaches comprised univariate analyses restricted on patients with Caucasian origin as well as multivariate analyses, which were adjusted for ethnicity.

In the present study, we could identify a statistically significant association between the ACE intron 16 I/D genotype and plasma CRP levels. This relation was allele-dose dependent. Most interestingly, this association was significantly restricted on patients presenting with disturbed uteroplacental function as evidenced by high impedance to flow, notching and/or foetal growth restriction (Table 3). The influence of the ACE intron 16 I/D genotype on CRP levels, which we describe, was modest,

which again could be due to the usage of a non-highly sensitive CRP quantification technique presumably resulting in an underestimation of the effect of the ACE intron 16 I/D genotype on the CRP concentration. Furthermore, the results of our study derive from a cohort of pregnant women, thus, it has to be tested, whether our results could be translated to other inflammatory conditions. There are a few other published studies investigating the relation between ACE intron 16 I/D genotype and CRP level. Bahramali and co-workers studied this relation in patients with coronary artery disease and they did not find any evidence of an association between this ACE polymorphism and CRP levels.[25] In another study, this relation was tested in patients suffering from advanced cancers.[26] In this study population a trend towards higher CRP levels in carriers of the ACE intron D allele was found, however, without reaching statistical significance. The absence of a detectable relation between ACE intron 16 I/D genotype and CRP level in these studies could be related to small sample sizes. In respect of the cohort with coronary artery disease another possible explanation could be related to a low pro-inflammatory stimulus in these patients. However, it cannot be excluded that the findings of our study are due to other mechanisms specific for uteroplacental dysfunction.

There are some details, which argue for a true association between ACE intron 16 I/D genotype and CRP levels in patients with disturbed uteroplacental function. Firstly, a considerable influence of the ACE intron 16 I/D genotype on the serum ACE activity has been described, with increasing serum ACE levels with ascending numbers of the ACE intron 16 D allele.[17] Several other groups could replicate these findings.[13] As ACE converts angiotensin I to angiotensin II, which itself exerts proinflammatory effects,[18] the relation of the ACE intron 16 I/D genotype to CRP levels could be mediated by this part of the RAAS pathway. This hypothesis is corroborated by another recent report, which described a significant reduction of plasma CRP levels under the treatment with ACE inhibitors.[27] Secondly, in our study a clear allele-dose dependency of the relation between ACE intron 16 I/D genotype and CRP (Tables 2 and 3) could be identified. This fact argues against a finding mediated by possible uncontrolled confounders. Finally, the statistically significant heterogeneity between women without and with uteroplacental insufficiency argues against an association, which is primarily mediated or biased by pregnancy or pregnancy-related changes, and it is clearly compatible with an involvement of the RAAS and the ACE intron 16 I/D genotype in some aspects of pathogenesis of uteroplacental insufficiency and preeclampsia. However, this assumption as well as the transferability of the relation between the ACE intron 16 I/D genotype and CRP to other inflammatory diseases has yet to be elucidated.

Conflicts of interest

None declared.

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