ACE D and Prothrombin 20210a Variants Interact in Increasing Unexplained Early and Recurrent Early Pregnancy-Loss Risk

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Abstract

Introduction: The development of placental vascular network requires a crosstalk between haemostatic and endothelial components. Growing evidence suggests that maternal gene variants may confer an unfavorable genetic burden able to influence pregnancy loss, either in early and late trimester. In this case-control study we analyzed the prevalence of Factor V Leiden, Prothrombin gene mutation and endothelium-related genes polymorphisms in women with history of unexplained fetal loss compared to uneventful pregnancy women.

Materials and Methods: Four hundred and fifty women with history of fetal loss (305 with early and 145 late events) and 300 healthy women were genotyped for inherited thrombophilia and ACE, AGTR1, AGT, eNOS genes polymorphisms.

Results: Inherited thrombophilia and ACED allele were significantly and independently associated with fetal loss and interact in increasing pregnancy failure risk (p = 0.0009). By analyzing genetic markers according to gestational age and recurrence pregnancy failure, we observed that FII20210A was associated with both early (OR = 2.68, p = 0.03) and recurrent early event (OR = 3.34, p = 0.02), whereas ACED allele modulated pregnancy failure risk independently of gestational timing (early: OR = 1.62, p = 0.04; recurrence: OR = 2.17, p = 0.02; late: OR = 3.90 p < 0.0001). An interaction between inherited thrombophilia, and in particular FII20210A, and ACED variant in determining a six-fold (p = 0.005) and a four-fold (p = 0.05) increased risk of early and recurrent early fetal loss, respectively, was found.

Conclusions: ACED allele is able “per se” to modulate the risk of pregnancy failure independently of gestational age, increases the risk of both early and recurrent early pregnancy loss, and confers a four-fold risk of a late event in thrombophilic women.

Keywords: Prothrombin Gene Mutation; Factor V Leiden; ACE; Abortion

Abbreviations: ACE: Angiotensin Converting Enzyme; AGTR1: Angiotensin II Type 1 Receptor; AGT: Angiotensinogen; eNOS: endothelial Nitric Oxide Synthase; RAS: Renin Angiotensin System; FVL: Factor V Leiden; PAI-1: Inhibitor of Plasminogen activator; PC: Protein C; PS: Protein S; AT: Antithrombin

Introduction

Growing evidence suggests that maternal gene variants may confer a prothrombotic burden able to influence unexplained pregnancy loss.

Anormal pregnancy depends on adequate placental circulation; clinical studies have investigated the relationship between inherited thrombophilia and pregnancy loss, often with differing results [1,2]. Recently, a meta-analysis from Sergi et al. evidenced that women carrying inherited thrombophilia, and in particular FVL, may have an increased susceptibility for first trimester recurrent pregnancy loss [2]. It has been speculated that a prothrombotic status may compromise vascular placenta milieu, thus determining micro and/or macro-vascular thrombosis, as well as effects on trophoblast growth and differentiation [3,4].

The development of a placental vascular network requires a crosstalk between haemostatic and endothelial components in the process of implantation, embryo development, and placentation. An endothelial dysfunction related to increased angiotensin II or reduced nitric oxide availability, can lead to impaired placental perfusion, thus compromising oxygen and nutrient supply to the fetus. Functional polymorphisms in genes encoding for renin angiotensin system (RAS) and nitric oxide pathway components, influences angiotensin II and nitric oxide availability [5-13], and clinical studies investigating the relationship between these polymorphisms and pregnancy loss, provided weak and conflicting results [14-18], partly related to different genetic background.

Pregnancy loss may be considered a complex disorder with a polygenic background; therefore, mutations in both hemostasis and endothelium-related genes might cause an unfavorable genetic burden able to induce, either in early and late trimester of pregnancy, a possible pregnancy loss.

Few studies contemporarily investigating inherited thrombophilia and endothelial genes polymorphisms in pregnancy loss are available [19,20], while no data evaluating RAS, endothelial nitric oxide synthase (eNOS) genes polymorphisms, and inherited thrombophilia, according to gestational age and recurrence of obstetric events, are present.

Therefore, we performed this case control study in order to investigate, beyond inherited thrombophilia mutations, the role of endothelium-related genes, such as RAS and eNOS polymorphisms, in women with history of unexplained pregnancy loss according to early and late events, compared with women with uneventful pregnancy.

Materials and Methods

Study population

Recruitment of women referred to Gender Medicine Clinic of the Center for Atherothrombotic Disease, Department of Experimental and Clinical Medicine, Azienda Ospedaliero-Universitaria Careggi, Florence, from 2009 to 2012, is presented in Figure 1. Briefly, we investigated 750 women of whom 450 reported a history of pregnancy loss, a possible pregnancy loss. Recruitment of women referred to Gender Medicine Clinic in order to frame for vascular risk, due to a family history of vascular disorders, thrombophilia and contraception, were considered controls. These women delivered after uneventful
pregnancy, and did not experience vascular disease (Uneventful Pregnancy, UP). Exclusion criteria are reported in Figure 1.

Unexplained early pregnancy loss was defined as loss before 12 weeks' gestation, and late after 12 weeks' gestation according to Royal College Obstetricians and Gynaecologists guidelines [21]; recurrence of pregnancy loss was defined as three or more consecutive pregnancy failures.

Informed written consent for anonymous data analysis was obtained from all women, and the study was approved by the Institutional Review Board of the Azienda Ospedaliero-Universitaria, Careggi, Florence, Italy.

**Genotyping**

Genomic DNA was isolated from peripheral blood leukocytes by using GeneCatcher™ gDNA Blood Kit (Invitrogen) with the aid of automated platform Freedom EVO 150 (Tecan).

All women were analyzed for inherited thrombophilia, RAS and eNOS candidate gene polymorphisms. The eNOS 4a4b polymorphism was evaluated by PCR method, as previously described [22].

Genotyping of ACE gene Insertion/Deletion polymorphism (NT_010783.14:g.20217903_20217904ins5) was performed by TaqMan designed assay, according to Koch et al. [23]. The other polymorphisms [FIV G1691A, rs6025 (NT_004487.19:g.21007691T > A); FII G20210A, rs1799963 (NT_009237.18: g.46701055G > A); ACE -240A > T, rs4291 (NT_010783.14:g.20206205A > T); AGTM235T rs699 (NT_004559.13: g.7047947A > G); AGTR1 1166A > C rs5186 (NT_005612.15:g.54955134A > C); eNOS -786T > C rs2070744 (NG_011992.1: g.12965G > T)] were analyzed by using TaqMan SNP genotyping assay (TaqMan SNP genotyping assay 20X, GTxpress Master Mix, Applied Biosystems) through the real-time TaqMan 7900HT (Applied Biosystems). Genotypes were determined using Applied Biosystems automated Taqman genotyping software SDS 2.3 (Applied Biosystems).

**Statistical analysis**

Statistical analysis was performed by using the SPSS (Statistical Package for Social Sciences, Chicago, USA) software for Windows (Version 11.5).

Age was expressed as median (range), and the categorical variables were expressed as frequencies and percentage.

Tests for conformity with Hardy-Weinberg equilibrium were performed using a standard \( \chi^2 \) test (1 degree of freedom). The \( \chi^2 \)-test was also used to evaluate the differences in genotype distribution and allele frequency between groups investigated.

The association between polymorphisms investigated and fetal loss was assessed by using logistic regression analysis under dominant model of inheritance. The dominant genetic model compares individuals with one or more polymorphic alleles with a baseline group with no polymorphic alleles (e.g. ACE DD+ID vs. II). Smoking habit, BMI > 25Kg/m\(^2\) and familial history of vascular disease were included into the multivariate model. Odds ratio (OR) was used as a measure of effect size. Statistical significance was accepted at \( p \)-value 0.05 (two-sided \( p \)-value). Interaction test used for comparing odds ratio was derived from Altman et al. [24].

Based on our previous observations [25] in Caucasian women, a sample size of at least 290 subjects for each group was deemed sufficient to prove/exclude an association between inherited

**Figure 1:** Flow-chart reported women investigated and excluded from the study
Results

Clinical characteristics of the study population are reported in Table 1. Four hundred and fifty women had history of pregnancy loss, and among them 305 (67.8%) had early (before 12 week of gestation) and 145 (32.2%) had late (between 12-20 week of gestation) events; 158 out of 305 (51.8%) women have had two early fetal losses, and 147 (48.2%) women have experienced more than three consecutive obstetric events, defined recurrent pregnancy losses (Figure 1). A significant higher percentage of women with familial history of cardiovascular disease as well as obstetric complications were observed in patients with respect to that found in controls.

The prevalence of inherited thrombophilia (FV 1691G > A, FII 20210G > A), RAS (ACE ID, ACE-240A > T, AGTR1 1166A > C and AGT M235T) and eNOS (-786 T > C, 894G > T and 4a/4b) rare variants has been investigated in both women with history of fetal loss and uneventful pregnancy women; no deviation from Hardy–Weinberg equilibrium for all polymorphisms investigated was found.

Inherited thrombophilia mutations prevalence was significantly higher in patients than in controls; in particular, FII 20210A mutation was present in 31 out of 450 (6.9%) women with history fetal loss and in 7 out of 300 (2.3%) controls ($p = 0.006$); FV 1691A was present in 35 out of 450 (7.8%) pregnancy loss women and in 12 out of 300 (4.0%) controls ($p = 0.03$) (Table 2). As concerns endothelium-related candidate genes, ACE D allele frequency was significantly higher in pregnancy loss women than in controls (0.58 vs 0.49, $p = 0.0006$) (Table 2); no significant difference was observed when the other polymorphisms in genes encoding for RAS components and eNOS gene have been investigated (data not shown).

At logistic regression analysis showed inherited thrombophilia mutations and ACE D allele were associated with an increased risk of...

<table>
<thead>
<tr>
<th>Variables</th>
<th>FL (n=450)</th>
<th>UP (n=300)</th>
<th>$p$</th>
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<td>35 (21-40)</td>
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<td>BMI &gt;25 Kg/m$^2$, n (%)</td>
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<td>87 (19.3)</td>
<td>51 (17.0)</td>
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<td>Hypertension, n (%)</td>
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<td>2 (0.6%)</td>
<td>0.09</td>
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<td>Dyslipidemia, n (%)</td>
<td>11 (2.4%)</td>
<td>6 (2%)</td>
<td>0.09</td>
</tr>
<tr>
<td>Familial history of cardiovascular disease, n (%)</td>
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<td>30 (10)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Familial history of obstetric complications, n (%)</td>
<td>20 (4.4)</td>
<td>0 (0.0)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
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Figure 1: Univariate (a) and multivariate (b) analysis for inherited thrombophilia and ACE D variant in modulating the risk of pregnancy loss; $^\$ p for interaction test = 0.009.
pregnancy loss also after adjustment for traditional risk factors and familial history of vascular disease (Figure 2a and 2b). A significant interaction between ACE D and FII G20210A variants in increasing pregnancy loss risk was observed (p for interaction = 0.009).

Inherited thrombophilia and endothelium related genes polymorphisms according to history of early, early recurrent and late fetal loss. FII G20210A mutation was significantly higher in women with history of early pregnancy loss, and higher, even if not significantly in women with late pregnancy loss in comparison to that observed in controls. In women with early pregnancy loss, prothrombin gene mutation prevalence was two-fold higher than that observed in women with history of late event (Table 3).

As concerns FV 1691A mutation, a significantly higher prevalence in women with history of early pregnancy loss than that observed in controls was found; a higher, even if not significant prevalence in women with late pregnancy loss was found (Table 3).

Among women with early fetal loss, 147 (48.2%) have experienced more than three events, defined as recurrent early fetal loss, in these women a significantly higher prevalence of FII 20210A and a higher FV 1691A allele frequency than that observed in uneventful pregnancy women was found (Table 3). A significant association between FII 20210A and FV 1691A and both early and recurrent pregnancy failure both at univariate and multivariate analysis was observed, whereas no association between FII 20210A and FV 1691A allele and late fetal loss was found (Table 4).

Concerning endothelium related genes, ACE D allele frequency was similar according to timing of clinical negative events and its recurrence, and significantly higher than that found in controls (Table 3); as regards the other RAS and eNOS polymorphisms, we observed that ACE-240T, eNOS-786C and 4a alleles frequency was higher, even if not significantly, in late pregnancy loss in comparison to that observed in healthy women.

ACE D variant influenced early and recurrent fetal loss risk before and after adjustment for covariates, and was associated with a four-fold increased risk of late negative events (Table 4).

When the contemporary presence of inherited thrombophilia and ACE D allele was investigated, ACE D allele increased the risk of both early and recurrent early pregnancy loss, and in women carrying prothrombin gene mutation, ACE D allele determined a six-fold increased risk of early (p for interaction = 0.005), and a four-fold increased risk of recurrent early fetal loss (p for interaction = 0.05). ACE D allele also conferred a four-fold risk of a late obstetric event in thrombophilic women (Table 4).

Discussion

In the present study we demonstrated that ACE D allele influenced...
"per se" the risk of unexplained pregnancy loss independently of gestational age and recurrence of obstetric events; in particular, ACE D allele increased the risk of both first trimester and recurrence first trimester pregnancy loss in inherited thrombophilic women. This unfavourable genetic profile might affect the vascular milieu, thus determining micro and/or macro-vascular thrombosis [3], insufficient invasion of trophoblast [26], and endothelial dysfunction [17], responsible for placental insufficiency.

To date, a thrombotic event is not the only mechanism for reproductive failure in women with thrombophilic defects [27]: an increased thrombin generation promotes trophoblast apoptosis [4], and it has been demonstrated in early pregnancy failure a premature onset of maternal blood flow into the intervillous space throughout the placenta [28,29], which may contribute to endothelial dysfunction related to increased angiotensin II levels and reactive oxygen species generation.

The human placenta expresses an autonomous RAS, and its correct function is necessary for an uncomplicated pregnancy [30]; really, data from clinical studies evidenced an increase of Angiotensin II levels in placenta mediated pregnancy complications [31,32].

Angiotensin II levels are modulated by ACE, whose plasma levels are associated with ACE D functional allele with a dose dependent effect [6].

In the present study we demonstrated an involvement of ACE D allele in modulating pregnancy loss risk apart from gestational age: we could hypothesize that genetically determined high Angiotensin II concentrations through an impaired hemostatic balance, alteration of vasomotor function, and increased inflammatory and ROS components production, influence the development of feto-placental unit from beginning to the end of pregnancy. We also demonstrated that ACED influenced the risk of recurrence of negative pregnancy events, as previously reported in a recent meta-analysis [14].

Beyond ACEI/D polymorphism we investigated for the first time the role of another ACE gene polymorphism, the -240A>T, in pregnancy loss, and we evidenced its high prevalence in women with late events, thus stressing the role of ACE gene in pregnancy complications risk. Data from literature concerning AGTR1 A>C and AGT M235T polymorphisms are weak and conflicting [33,34]; our findings demonstrated no association between these variants and unexplained pregnancy loss.

Data from two recent meta-analyses, evaluating the association between eNOS gene polymorphisms and recurrence of unexplained pregnancy loss, reported conflicting results [17,18]. Our findings did not demonstrate an involvement of eNOS gene in recurrent pregnancy loss, nevertheless we evidenced a higher; even if not significant, prevalence of eNOS-786C and 4a alleles in women with history of late pregnancy loss. These results may lead us to hypothesize that reduced NO availability by impairing placental perfusion, may compromise oxygen and nutrient supply to the fetus.

In the present study we analyzed the role of inherited thrombophilia in pregnancy loss according to different timing of pregnancy failure, and we observed that IV Leiden and FII 20210A variants were associated with both first trimester and recurrence first trimester pregnancy loss, but not with risk of late fetal loss. These findings are in keeping with those from clinical studies addressed the role of inherited thrombophilia in increasing the risk of unexplained and recurrence of unexplained first trimester pregnancy failure [2,35], but are at variance with data from a recent meta-analysis [1]. These conflicting results might be due to the non-homogeneous timing of pregnancy failure reported in the studies.

Fetal loss in thrombophilic women could be explained by thrombosis of the placental vessels, as well as damage to decidual or chorionic vessels, or reduction of trophoblast invasiveness. Experimental data reported a vascular connection between maternal circulation and placental intervillous space before eight weeks, which progressively changes from a tortuous network to small caliber-delineated channels until at least first trimester [36]. Inherited thrombophilia status, by causing micro-thrombi in the placental vasculature, may affect the feto-placental circulation, and lead to early unexplained pregnancy failure [37]. Moreover, increased fibrin deposition in placenta of aborted fetus may support the thrombotic etiology of early pregnancy failure [37].

Of interest, our results evidenced that ACE gene represents the main actor in modulating pregnancy failure risk; we demonstrated for the first time, that ACE D allele increased risk of early and recurrent early first trimester fetal loss, and conferred a four-fold risk of late obstetric event in thrombophilic women. Therefore, this datum suggests that high Angiotensin II levels, genetically determined [6], by increasing PAI-1 secretion [30] and tissue factor release [39], as well as by reducing nitric oxide production, play a bridging role between a prothrombotic status and endothelial dysfunction.

A limitation of the present study lies in excluding women carrying inherited deficiencies of AT, PC, PS, anticoagulant proteins associated with a prothrombotic phenotype, due to the low prevalence (five out of 750 women, 0.7%) of these inhibitors in our population; moreover, we did not investigated low borderline plasma levels of AT, PC, PS anticoagulant proteins, which are known to be associated with increased risk of venous thromboembolism, and should be considered in the assessment of the individual thrombotic risk [40].

In conclusion, this study is novel in evidencing that ACE D allele, is able "per se" to modulate the risk of pregnancy failure independently of gestational age and recurrence of obstetric event, and is intriguing in demonstrating that this allele beyond increasing the risk of both early and recurrent early pregnancy loss, conferred a four-fold risk of a late obstetric event in thrombophilic women. Although this polymorphism confers a small, but significant increased risk, data from our study support the polygenic nature of unexplained pregnancy failure. Our results might drive to search for "not classic" genetic markers, which beyond traditional inherited thrombophilia, are able to better frame obstetric women risk profile.

References


