**An Acad Bras Cienc (2020) 92(4): e20200355 DOI 10.1590/0001-3765202020200355**
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

**HEALTH SCIENCES**

# *FAS* gene polymorphisms (rs3740286 and rs4064) were not associated with pre-eclampsia risk

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**Abstract:** Pre-eclampsia results in real risk and significant impact on indicators related to maternal and child health. The only known treatment is delivery of the fetus and placenta. Despite intensive research, the causes of PE remain to be elucidated. It is suggested that pre-eclampsia is caused by a global maternal inflammatory response to a damaged placenta. Besides inflammation, cytotoxic and apoptotic mechanisms are also implicated in the pathogenesis of pre-eclampsia. Considering the importance of apoptosis to pre-eclampsia genesis, the aim of this study was to determine the frequencies of the genotypes for *FAS* gene polymorphisms (rs3740286 and rs4064) and to associate these with pre-eclampsia development. Women with and without pre-eclampsia were investigated. Accordingly, peripheral blood was collected, and DNA extracted, followed by genotyping using Real-time PCR with hydrolysis probe. The results showed no association between genotypes and pre-eclampsia development for both polymorphisms studied ($\chi^2$=3.39; p=.177, for rs3740286 and $\chi^2$=0.119; p=.94 for rs4064). Women with familiar history of pre-eclampsia and primiparity showed more probability to develop the condition, by multiple logistic regression analysis (OR=8.61, CI=3.39-21.86, p<0.0001; OR=6.64. CI=2.94-14.99, p<0.0001, respectively). It seems that *FAS* gene polymorphisms (rs3740286 and rs4064) might not be important candidates for the development of pre-eclampsia.

**Key words:** Apoptosis, Fas Receptor, Polymorphism, Genetic, Pre-Eclampsia**,** Women's Health.

## INTRODUCTION

Pre-eclampsia (PE) results in real risk and significant impact on indicators related to maternal and child health (Ramos et al. 2017). The incidence of PE worldwide is about 3-5% of pregnancies. In Brazil, a systematic review identified an incidence of 1.5% for PE (Abalos et al. 2013). It originates in the placenta and causes variable maternal and fetal problems (Redman & Sargent 2010).

Genetic and environmental factors are involving in its pathogenesis and pathophysiology. The only known treatment is delivery of the fetus and placenta (Romero & Chaiworapongsa 2013). Despite intensive research, the causes of PE remain to be elucidated (Sziller 2018).

There is evidence that PE is a disturbed invasion of the uterine wall by fetal trophoblasts and a breakdown in maternal tolerance to the fetal semiallograft (Sziller 2018). A study suggested that pre-eclampsia is caused by a global maternal inflammatory response to a damaged placenta (Redman & Sargent 2010). Besides inflammation, cytotoxic and apoptotic mechanisms are also implicated in the

pathogenesis of pre-eclampsia (Barakonyi et al. 2014).

Placental apoptosis is a physiological condition seen in all the cell types in normal pregnancies and trophoblasts account for more than half of apoptotic cells. The role of apoptosis in pre-eclampsia is still available to be discussed. In pre-eclampsia, placental hyper fusion and ischemia develop because of insufficient interstitial cytotrophoblast invasion and superficial maternal endovascular invasion. It is, then, shown that hypoxia triggered apoptosis. Thus, increased apoptosis, seen in pre-eclampsia, does not seem to be the reason for trophoblastic invasion but an occasion of pathology developing secondary to it (Dagdelen et al. 2016).

The Fas/Fas ligand (FasL) system represents one of the main apoptotic pathways controlling cell proliferation and tissue remodeling. This system represents one of the major mechanisms through which locally acting cytokines may influence the crucial processes of implantation, trophoblast invasion, placental development, and immune protection (Aschkenazi et al. 2002). In health pregnancies, the binding of trophoblast-associated FasL to Fas-expressing activated maternal T lymphocytes, that invade the trophoblast during implantation, induces apoptosis in Fas-bearing maternal T-cells, allowing fetal trophoblasts to invade the myometrium while escaping immune recognition (Sziller 2018).

Fas/FasL interactions may have an important role in trophoblast-induced endothelial apoptosis (Longtine et al. 2012). It has been hypothesized that the trophoblast associated Fas ligand interacts with Fas on activated T lymphocytes, some of which recognize paternal antigens. The induction of apoptosis in these T cells prevents them from recognizing and destroying cytotrophoblasts invasion into the myometrium. A reduced capacity of cytotrophoblasts to invade the spiral arteries, because of enhanced trophoblast destruction by T cells, results in the development of hypertension and pre-eclampsia (Sziller et al. 2005).

Some evidence supports the hypothesis of maternal immune system involvement in the disease. Invasion of uterine spiral arteries by extravillous trophoblasts in the first trimester of pregnancy results in loss of endothelial and musculoelastic layers. This remodeling is essential for an adequate blood supply to the fetus with a failure to remodel implicated in the etiology of PE. Extravillous trophoblast induction of endothelial apoptosis is a possible mechanism by which the endothelium is removed, and vascular remodeling may occur in uterine spiral arteries. Data using high-resolution confocal immunofluorescence, detection of apoptosis markers and the plasma membrane identify elevated apoptosis of villous cytotrophoblasts in pregnancies complicated by PE compared to normotensive controls (Plaks et al. 2013).

A study showed that median levels of serum soluble FAS were higher in women with Hemolysis, Elevated Liver Enzyme, and Low Platelets (HELLP) syndrome than in healthy ones, suggesting placental and vascular endothelial dysfunction as the possible pathogenesis in HELLP syndrome (Hsu 2012). Also, mean serum soluble Fas levels were significantly higher in preeclamptic than normotensive women (Hsu et al. 2001). As maternal immune cells bear the Fas receptor, soluble Fas could protect these cells from apoptosis initiated by binding with FasL expressed on the trophoblasts. Consequently, the activated immune cells remain at the interface, leading to increased apoptosis in the trophoblasts (Neale & Mor 2005).

Studies developed in different population found a relation between pre-eclampsia development and the A >G substitution at position 670 in the *FAS* gene (Ciarmela et al. 2010, Nasr et al. 2014, Plaks et al. 2013, Raguema et al. 2018, Salimi et al. 2014). This polymorphism would lead to a decrease in Fas production in activated T lymphocytes, altering the immune-privileged site of the trophoblast and, consequently, contributing to an abnormal invasion of cytotrophoblast within the uterine wall, favoring the development of pre-eclampsia.

A variety of molecular and genetic factors are involved in the pathophysiology of pre-eclampsia. Based on the foregoing, it is important to evaluate polymorphisms in genes involved in apoptosis pathways, with polymorphisms in the *FAS* gene being important candidates. Testing for single-nucleotide polymorphisms in maternal genes associated with PE might identify pregnant women with an increased risk for the development of the disease and could be a helpful strategy to prevent morbidity associated to PE.

Therefore, the aim of this study was to associate *FAS*rs3740286 and *FAS*rs4064 polymorphisms and the development of pre-eclampsia in a group of Brazilian women. Elucidation of a relationship between these polymorphisms and pre-eclampsia might be a useful tool in the identification of at-risk pregnant women, thus allowing genetic susceptibility prediction.

## MATERIALS AND METHODS

### Study design and subjects

This is a case-control study, involving 73 unrelated women with pre-eclampsia and 182 women with normal pregnancies. The subjects were recruited between 2008 and 2014, from Gynecology and Obstetrics Clinic of the Hospital das Clínicas at Universidade Federal do Triângulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil. PE diagnose was in accordance with the guidelines from the American College of Obstetrician and Gynecologist Task Force on Hypertension in Pregnancy (American College of Obstetricians and Gynecologists 2013). The control group consisted of women with no history of pre-eclampsia or any other hypertensive episode during pregnancy. Exclusion criteria for both groups included: incomplete medical records, gestational diabetes, chronic hypertension, or other chronic diseases.

### DNA extraction

The mass of red and white blood cells was submitted to osmotic lysis with Tris-EDTA lysis buffer (20:5) consisting of 1M Tris-HCl and 0.5M EDTA, pH 8. The samples were centrifuged at least three times at 13346xg for 15 min at controlled room temperature (approximately 27°C). The DNA was extracted using the phenol-chloroform method (Sambrook et al. 1989). The samples were then resuspended in TE 20:1, the DNA integrity was analyzed by electrophoresis on 1% agarose gel.

### *FAS* gene polymorphisms genotyping

Real-time PCR allelic discrimination was used for genotyping *FAS* rs3740286 (A>G) and rs4064 (G>C) variants, through fluorogenic 5' nuclease assay with Taqman minor groove binder (MGB) probes (Taqman® Life Technologies). The primers and probes for these *FAS* gene polymorphisms were designed using Applied Biosystems (ABI) (Foster City, CA) assay-on-demand services (assay ID: C_27491147_10 and C_12123937_20, respectively). Each reaction consisted of 10 ng DNA, 3.5 µL TaqMan universal master mix (ABI) (2×), 0.3µL primers and probes (10×), and water to a final volume of 10µL. Appropriate negative controls were included in all assays. Real-time PCR was

performed in ABI Step One Plus system (Applied Biosystems). The reaction conditions were 50ºC for 2 minutes, 95°C for 1 min, followed by 50 amplification cycles at 92°C for 15 s and 60°C for 1 min. For each cycle, the software determined the fluorescent signal from the VIC- or FAM-labeled probe, and SDS 2.0 software was used to analyze the fluorescence emitted during the real time PCR and at the end of the PCR (endpoint read).

### Ethical approval

Ethics and Research Committee from Universidade Federal do Triângulo Mineiro (Protocol No. 1115-08) approved the study protocol and both pre-eclampsia cases and control women gave written informed consent to their participation in this project.

### Statistical analysis

The Hardy-Weinberg Equilibrium (HWE) was applied using the $\chi^2$ test with the Haploview 4.2 program. A statistical power of 80% was tested using the G* Power program 3.1.9.2. In addition, a post hoc test with the total sample (n=248 and n=246) was performed, with an effect size of 0.20 and an alpha significance level of 0.05. Genotype and allele frequencies were statistically analyzed using the $\chi^2$ test. To verify the association of risk factors (primiparity, family history of PE and the presence of polymorphic alleles) with PE, the multiple logistic regression model was used through the *Stats Direct* program. Results were presented in Odds Ratio (OR), with a confidence interval (CI) of 95%.

## RESULTS

A total of 255 Brazilian women were included in the study and distributed into two groups. The control group was composed by 182 (71.4%) women with no history of hypertensive episode during pregnancy, the age range was 18-45 years (mean 32.05±7.91), and the number range of pregnancies was 1-7 (mean 2.85±1.39). The other group was the pre-eclampsia one, consisting of 73 (28.6%) pregnant women with pre-eclampsia diagnosed according to the Report of the American National High Blood Pressure Education Program (American College of Obstetricians and Gynecologists 2013); the age range was 18-43 years (mean 26.6±6.5), and the number range of pregnancies was 1-9 (mean 1.918±1.320). It was not possible to genotyping all the individuals of the study, maybe due to technical reasons. There were 255 participants in the study, 248 were genotyped for polymorphism rs3740286, and 246 for rs4064. Regarding the HWE, both control and PE groups are in Hardy-Weinberg Equilibrium for both polymorphisms studied. The p values are 0.0054 and 0.0737 in pre-eclampsia group, 0.22 and 0.01 in control group, for *FAS* rs3740286 and rs4064 gene polymorphisms, respectively.

The frequencies of genotypes AA, AG, GG in the *FAS* gene polymorphism rs3740286 (A>G) in the control and pre-eclampsia groups were analyzed and compared; the distribution is exposed in Table I. No association was observed between genotypes and pre-eclampsia development ($\chi^2$: 3.39; p: 0.18).

The frequencies of genotypes CC, CG, GG, *FAS* gene polymorphism rs4064 (C>G) in the control and pre-eclampsia groups were analyzed and compared; data is organized in table II. It was not found any association between risk for pre-eclampsia occurrence and this polymorphism ($\chi^2$: 0.12; p: 0.94).

After logistic regression analyses (table III), it was found that family history of PE and primiparity were statistically significant (p <0.0001). Women with a family history of PE are eight times more likely to develop the disease

**Table I. *FAS* (rs3740286) genotypes found in women with and without pre-eclampsia.**

| | Phenotype | | | | | | p values |
|---|---|---|---|---|---|---|---|
| | Control Group | | Pre-eclampsia | | Total | | |
| Genotypes | N | % | N | % | N | % | |
| AA | 96 | 69.6 | 42 | 30.4 | 138 | 100 | |
| AG | 65 | 79.3 | 17 | 20.7 | 82 | 100 | **0.18** |
| GG | 18 | 64.3 | 10 | 35.7 | 28 | 100 | |
| Total | 179 | 72.2 | 69 | 27.8 | **248** | 100 | |

than women with no family history. Primiparous women, on the other hand, are five times more likely to develop PE than women with two or more pregnancies. No association with allelic variants (rs3740286 G and rs4064 G) and the risk of PE development in our sample was found.

## DISCUSSION

In this study, we hypothesized that genetic factors are involved in the etiology of PE. Therefore, two single nucleotide polymorphisms in gene related to apoptosis (*FAS* rs3740286 and rs4064) in preeclamptic women were investigated.

Polymorphisms rs3740286 and rs4064 are located on chromosome 10 and they correspond to A>G and C>G alterations, respectively. Our findings revealed that the polymorphisms studied may not play a role in the susceptibility to PE in this sample of Brazilian women.

Pre-eclampsia is a syndrome with unknown etiology. However, some studies have showed the importance of apoptosis to PE genesis (Barakonyi et al. 2014, Huppertz et al. 2006, Romero & Chaiworapongsa 2013). Cytotoxic mechanisms are implicated in the pathogenesis of pre-eclampsia. Different studies showed a significantly higher percentage of decidual cytotoxic cells in severe pre-eclampsia compared with gestational age-matched controls (Quinn et al. 2011, Sasaki et al. 2007). Apoptotic mechanisms are especially important for healthy pregnancy, especially during implantation and placentation. Apoptosis and cytotoxicity play a crucial role during elimination of potentially harmful maternal lymphocytes, contributing to the immunological tolerance against the developing fetus. In pre-eclampsia, at the early pregnancy stage, the invasion of the cytotrophoblast is limited to superficial deciduas (Redman & Sargent 2010).

It was demonstrated that Fas and FasL are highly expressed in invasive extravillous trophoblast cells, which invade the uterine wall and enter spiral arteries, resulting in arterial remodeling (Murakoshi et al. 2003).

Because of all these findings, it is important to evaluate the role of *FAS* gene polymorphisms in the development of PE. To the best of our knowledge, this is the first study of *FAS* gene polymorphisms (rs3740286 and rs4064) in Brazilian women with pre-eclampsia. Other polymorphisms in the same gene were described in the literature.

A>G polymorphisms at position -670 in the *FAS* gene, which reduces the expression of Fas, is associated with pre-eclampsia at < 37 weeks and with IUGR in the corresponding neonates

**Table II. *FAS* (rs4064) genotypes found in women with and without pre-eclampsia.**

| | Phenotype | | | | | | p values |
|---|---|---|---|---|---|---|---|
| | Control Group | | Pre-eclampsia | | Total | | |
| Genotypes | N | % | N | % | N | % | |
| CC | 28 | 71.8 | 11 | 28.2 | 39 | 100 | |
| CG | 63 | 73.3 | 23 | 26.7 | 86 | 100 | **0.94** |
| GG | 86 | 71.1 | 35 | 28.9 | 121 | 100 | |
| Total | 177 | 72.0 | 69 | 28.0 | **246** | 100 | |

(Plaks et al. 2013). Another research supports the hypothesis that the *FAS* 670G is an influencing factor for the development of pre-eclampsia in Italian population (Ciarmela et al. 2010). In southeast Iran, a study showed that *FAS* A-670G is associated with higher risk for PE and there was no association between *FAS Ligand* C-844T polymorphism and PE (Salimi et al. 2014).

Study performed in Cairo, Egypt, confirms an association of the *FAS* 670 A>G gene polymorphism with pre-eclampsia, in which they found the GG/AG genotype in 84% of women with pre-eclampsia versus 60% of normal women (Nasr et al. 2014).

With Iran population, another study showed a significant difference in *FAS* polymorphisms at 1377 G>A, but not at 670 A>G between pre-eclampsia patients and controls, suggesting that the *FAS*-1377G>A polymorphism may have a causative role in the development of PE (Masoumi et al. 2016).

A meta-analysis of tumor necrosis factor-α and FAS/FASL polymorphisms with

risk of pre-eclampsia was performed. The results indicate no significant association between *TNF-α* −850 C/T, −238 A/G and *FASL* −844 C/T polymorphisms and PE risk. The results suggest that FAS −670 A/G polymorphism may be a susceptibility gene for the development of PE (Lin et al. 2019).

This study reinforces data in literature (Mayrink et al. 2018) using multiple logistic regression, showing family history and primiparity as important risk factors for the development of PE. However, the results failed to demonstrate an association of the alleles of the polymorphisms studied with the development of PE.

This study presents some limitations. Among these is our small sample size. Another one was the absence or lack of scientific works on these polymorphisms in PE and other biological conditions, what has made difficult data generalization and comparison. Since the PE patients recruited in our study are all Brazilian, the association between these polymorphisms and other population should also be investigated. Another aspect to be considered is that we did not analyze the severity of PE because of our sample size. Maybe, these polymorphisms are not involved in the development of the disease, but with the severity. It would be interesting to be able to study genetic polymorphisms and gene expression in the placenta. This strategy would allow the association between genotype and phenotype, emphasizing the results obtained. Also, it is possible that other polymorphisms have a main or a major effect in PE susceptibility. However, the main contribution to our study is to highlight the importance of the apoptotic genes in the PE genesis.

**Table III. Assessment of risk factors for PE development by multiple logistic regression analysis.**

| Variable | Odds Ratio | CI (95%) | p value |
|---|---|---|---|
| Family history | 8.61 | 3.39-21.86 | **<0.0001** |
| Primiparity | 6.64 | 2.94-14.99 | **<0.0001** |
| rs3740286 G | 0.67 | 0.33-1.37 | 0.28 |
| rs4064 G | 0.96 | 0.45-2.01 | 0.91 |

***CI confidence interval.**

## CONCLUSION

In conclusion, no difference was found between the polymorphisms studied (*FAS* rs3740286 and rs4064) in pre-eclampsia patients and controls, suggesting that these polymorphisms might not be involved in the PE genesis.

### Acknowledgments

We thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support (446914/2014-2).

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**How to cite**

TANAKA SCSV, ORLANDO JÚNIOR IC, HORTOLANI ACC, CINTRA MTR, BALARIN MAS, DA SILVA SR & PISSETTI CW. 2020. *FAS* gene polymorphisms (rs3740286 and rs4064) were not associated with pre-eclampsia risk. An Acad Bras Cienc 92: e20200355. DOI 10.1590/0001-3765202020200355.

*Manuscript received on March 18, 2020;*
*accepted for publication on September 23, 2020*

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