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Evaluation of *iNOS -2087A>G* polymorphism in recurrent pregnancy loss

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Abstract

Background: Due to its role in angiogenesis, the inducible nitric oxide synthase (*iNOS*) gene promoter polymorphism may have a presumed role in recurrent spontaneous abortions (RSA). It is an intensely studied protein, a biological mediator, a modulator and an effector molecule by implication in numerous physiological processes: vasodilatation, angiogenesis, immunity, tissue remodeling, smooth muscle activity. *Aim*: Our study aims to investigate a possible association between *iNOS -2087A>G* (*rs2297518*) polymorphism and the occurrence of idiopathic recurrent pregnancy loss (RPL). *Patients, Materials and Methods*: In this study, as in the previously published one, 169 women, diagnosed with RPL, in the Clinics of Obstetrics and Gynecology, "Filantropia" Municipal Hospital, Craiova, Romania, were subjected to the analysis, from October 2009 to October 2016. As a control group, we used 145 women. Subjects from both groups were genotyped using specific probes for TaqMan polymerase chain reaction (PCR), allelic discrimination technique. *Results*: We evaluated in this study a possible association between *iNOS -2087A>G* (*rs2297518*) polymorphism and the occurrence of idiopathic RPL. The *chi*-square test showed no significant association between the presence of this polymorphism and the increased risk to develop RPL. When we performed a comparative analysis of the frequency of genotypes and our statistical data, it was observed that this polymorphism, *iNOS -2087A>G* (*rs2297518*), has not been associated with an increased risk of developing RPL. Also, when one genotype was compared with another, we did not obtain any association that would have statistical significance, between the presence of this polymorphism and the increased risk for patients to develop RPL [in dominant – *A* allele carriers, *iNOS 2087 AG+AA vs. GG*: odds ratio (OR) 1.31, 95% confidence interval (CI) 0.83–2.07, *p*=0.24]. Analyzing the overall risk of developing RPL by *iNOS 2087* single-nucleotide polymorphism (SNP) genotype frequencies, between controls and RPL patients (which were stratified by number of consecutive PLs), taking into account the number of consecutive pregnancies, the *chi*-square test showed no association between the presence of this polymorphism and the increased risk for developing RPL in all three subgroups we analyzed (in a dominant model – *A* allele carriers, *iNOS 2087 AG+AA vs. GG*: the first subgroup, OR 1.31, 95% CI 0.83–2.07, *p*=0.24; the second subgroup, OR 1.26, 95% CI 0.76–2.11, *p*=0.37; the three subgroup, OR 1.4, 95% CI 0.77–2.53, *p*=0.272). *Conclusions*: The *iNOS -2087A>G* (*rs2297518*) gene polymorphism does not influence RPL in the study area of Dolj County, Romania.

Keywords: recurrent early pregnancy loss, inducible nitric oxide synthase, polymorphism, genotype.

Introduction

Recurrent pregnancy loss (RPL) is one of the most complex problems of modern medicine and consists of spontaneous interruption of pregnancy if the embryo or fetus is unable to survive independently, generally before the 20th week of gestation. RPL is the most common complication of pregnancy, being a negative experience for both the patient and the physician [1, 2]. It has been noted that the most common complication of human gestation is loss of pregnancy. Most RPLs are unrecognized and may occur before or with the next menstruation. 15–20% of recognized pregnancies can evolve to spontaneous abortion (SA) or extrauterine pregnancies. Population studies have also shown that about 5% of couples trying to get a pregnancy have two consecutive recurrent spontaneous abortions (RSA) and about 1% of couples have three or more consecutive pregnancy losses (PLs) [3].

Nitric oxide (NO) is known to mediate relaxation of smooth muscle vasculature, and the lack of endothelial NO derivatives is associated with vasospasm and vascular infarction. The risk of SA is increased by genetic and environmental factors. Several studies have indicated that there was an association between endothelial NO synthase (eNOS) activity, implantation and maintenance of pregnancy. However, the results of these studies remain controversial [4].

In many women who had three or more SAs, there was no clear reason for this pathology, probably a poor placental villi development in early placentation [5]. It has

been recently discovered that implantation depends on the receptivity of the maternal endometrium, which in turn is influenced by the synergistic action of NO. NO is synthesized by a family of NOS enzymes, in which three isoforms have been identified: neuronal (nNOS, NOS-1), inducible (iNOS, NOS-2) and endothelial (eNOS, NOS-3). Encoding polymorphisms and non-coding eNOS regions have been shown to modify eNOS expression and/or activity and thus result in a reduction in NO synthesis that may predispose patients to hypertension, vasospasm and atherosclerosis, renal failure, pre-eclampsia and RSA [6].

The reactive oxygen species (ROS) and other free radicals can cause oxidative damage to all cell components [proteins, lipids and deoxyribonucleic acid (DNA)] and have been shown to be involved in a number of pathological conditions [7–10]. Unlike calcium-dependent critical regulation of constitutive NOS, iNOS has been described as a non-insensitive calcium element, probably due to its tightly non-covalent interaction with calmodulin (CaM) and Ca^{2+}. iNOS produces large amounts of NO after stimulation, such as proinflammatory cytokines [*e.g.*, interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ)] [11].

The induction of large amounts of iNOS usually occurs in an oxidative environment, and thus the levels of NO increased have the potential to react with superoxide that leads to peroxynitrite formation and causes cellular toxicity. It has been suggested that the pathological generation of NO by increasing iNOS production may decrease the movement of the Fallopian tube and may cause contractions of the uterine muscle fiber and thus affect the transport of the embryo, causing ectopic pregnancy and subsequently eliminating it, causing abortion [11].

Angiogenesis, the appearance of new blood vessels, is required for placental microscopic development. Several candidate genes [epidermal growth factor (*EGF*), hypoxia-inducible factor 1-alpha (*HIF1A*), hypoxia-inducible factor 1-alpha inhibitor (*HIF1AN*), matrix metalloproteinase-2 (*MMP-2*), matrix metalloproteinase-9 (*MMP-9*), *iNOS*, *eNOS*, vascular endothelial growth factor (VEGF)] play a role in angiogenesis. *eNOS* and *iNOS* catalyze the synthesis of NO, which may be proangiogenic [12].

Due to its role in angiogenesis, the *iNOS* gene promoter polymorphism may have a presumed role in RSA. It is an intensely studied protein, a biological mediator, a modulator and an effector molecule by implication in numerous physiological processes: vasodilatation, angiogenesis, immunity, tissue remodeling, smooth muscle activity [13].

Many studies have also reported the roles iNOS and eNOS in regulating normal physiological events during placentation and pregnancy: ovulation, implantation, trophoblast invasion, embryonic development and maintenance of pregnancy [14–16].

Clinical trials of the past decade also aimed to investigate the association between the risk of RSA and the polymorphisms of the *iNOS* and *eNOS* genes.

Aim

Therefore, our study aims to investigate a possible association between *iNOS -2087A>G* (*rs2297518*) polymorphism and the occurrence of idiopathic RPL. This study is part of an extensive research, from which our team has already published an article, in which we have highlighted a significant association between the presence of VEGF receptor-2 (kinase insert domain-containing receptor) *VEGFR-2* (*KDR*) *-604A>G* (*rs2071559*) gene polymorphism and the increased risk observed in patients with four or more consecutive PLs, to develop RPL [17].

Patients, Materials and Methods

Patients and study protocol

In this study, as in the previously published one, 169 women, diagnosed with RPL, in the Clinics of Obstetrics and Gynecology, "Filantropia" Municipal Hospital, Craiova, Romania, were subjected to the analysis, from October 2009 to October 2016. As a control group, we used 145 women, who had at least one birth, but no PL. Subjects from both groups were genotyped using specific probes for TaqMan polymerase chain reaction (PCR), allelic discrimination technique.

From the medical records of the patients, we used the information related to the demographic data and the personal pathological history for each subject included in the study.

For each patient enrolled in this study was prepared an initial evaluation sheet using the same inclusion/exclusion criteria, also used in the study and previously published article [determination of *VEGFR-2 (KDR) -604A>G* polymorphism in RPL].

Single-nucleotide polymorphism (SNP) genotyping

Using peripheral blood collected on the anticoagulant, from all the patients included in this project, we isolated the leukocytes and finally the genomic DNA was purified. The purification kit used was the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), following the steps suggested in the protocol by the manufacturer: the first stage was the lysis of cells and their nuclei (this stage involves the lymphocyte lysis, followed by lysis of leukocytes and their nuclei); the next step was optional and consists in using ribonuclease (RNase) for ribonucleic acid (RNA) digestion (the proteins released from the cells are subsequently removed by salt precipitation, they precipitate the proteins, but maintain high-weight genomic DNA in the solution); in the next step, the DNA was concentrated and desalted by precipitation in isopropanol; the final step involves washing with ethanol, dehydration and rehydration of DNA.

For all patients included in this study, genotyping was performed for *iNOS -2087A>G* (*rs2297518*), in the Laboratory of Human Genomics, University of Medicine and Pharmacy of Craiova. Predesignated TaqMan tests were used, following the steps set out in the manufacturer's protocol, Applied Biosystems (Foster City, CA, USA), *iNOS -2087A>G* (*rs2297518*), which contains primers and probes specific for each allelic variant. As the real-time cycling conditions of the PCR (RT-PCR) (Rotor-Gene Corbett 6200 HRM) were as follows: 95°C for 10 minutes, followed by 50 cycles of 92°C for 15 seconds and 60°C for one minute of annealing temperature. The interpretation of the results was performed with ViiA™ 7 Software v1.0 and with the allelic discrimination option.

Statistical analysis

To verify whether there is a significant deviation, all polymorphisms were tested in terms of their Hardy–Weinberg equilibrium. Hardy–Weinberg equilibrium was verified by comparing the frequencies of observed and expected genotypes with the χ^2 (*chi*-square) test. Thus, in the interpretation, we considered that a *p*-value less than 0.05 and a *chi*-square greater than 3.84 would show that the tested polymorphism did not respect the Hardy–Weinberg equilibrium.

The evaluation of the possible associations between the *iNOS* polymorphism and the RPL was performed using odds ratios (ORs) accompanied by a 95% confidence interval (CI). The reference category, the most common Caucasian allele, used for all subjects analyzed, was the homozygous genotype.

In order to process the data, statistical analyzes were performed using statistical indicators applied to the studied subjects. We analyzed the data obtained, using the 2×2 contingency tables for categorical data with a two-tailed *p*-value, determined by the *chi*-square association test. In order to have a statistical significance, a two-sided *p*-value <0.05 was kept.

Ethical approval

The ethical principles contained in the *Declaration of Human Rights* adopted in Helsinki, in 1975, and revised in 2008, were fully respected in our study. The subjects enrolled, voluntarily participated in this study and completed and signed a written informed consent. We have for this study the Approval of the Committee of Ethics, Academic and Scientific Deontology of the University of Medicine and Pharmacy of Craiova (No. 103/30.09.2013).

Results

We have included in this study 314 patients, 169 with RPL and 145 healthy subjects. Regarding the age of the patients at the time of diagnosis, it was shown that the mean age was 30.79 years [standard deviation (SD) 4.06] in the control group and 30.02 years (SD 4.32) in the studying cases.

Following analysis of patient distribution depending on the age groups at the time of enrollment in the study, it was observed in both groups that most patients with RPL were aged between 25 and 34 years (62.59%) and in the control group the percentage was 59.3% in the same age groups. This range also corresponds to the age range in which women show the highest fertility rate.

In order to have a homogeneity, when we formed the two groups of subjects, the demographic data were taken into account, a criterion confirmed by the results obtained for the two groups, presented in Table 1. The *chi*-square test did not indicate a difference between the mean age of the two groups analyzed (p=0.09 and p>0.05, respectively).

Analyzing the parameter, the residence environment, there was no significant difference in the demographic distribution of the studied cases, with 56.8% cases in urban areas and 43.2% in rural areas, respectively, for RPL patients.

When we have studied the obstetric antecedents' parameter, we have compared the number of births in the two groups. As the primary sub-objective, we have compared the number of live births. This parameter, the number of live births, is considered the element that indicates a good pregnancy completion. We found a highly significant difference (*p*-value for *chi*-square test 0.008, p<0.05) between the control group and RPL patients. The fact that women in the control group have a higher number of live births can be explained as the result of better family planning, may suggest a decrease in incidence of abortion on demand, or may be the result of prenatal and genetic counseling for couples.

Table 1 – ***Demographic characteristics between controls and RPL patients in Romanian women***

Characteristics	Control subjects (*n*=145)	RPL patients (*n*=169)	*p*-value
Age [years] (mean±SD)	30.79±4.06	30.02±4.32	0.09
No. of live births (mean±SD)	1.07±0.79	0.83±0.78	*0.008**
No. of prior PL (mean±SD)	–	3.82±0.83	–
Gestational age of prior PL [weeks] (mean±SD)	–	8.34±1.73	–
Demographic distribution			
Urban	85 (58.62%)	96 (56.8%)	0.866
Rural	60 (41.38%)	73 (43.2%)	0.836
Thrombophilia	–	40 (23.67%)	–

RPL: Recurrent pregnancy loss; *n*: No. of cases; SD: Standard deviation; PL: Pregnancy loss; *Statistically significant *p*-value.

The second sub-objective analyzed was the number of previous PLs. It was noted that the majority of pregnant women included in the study had at least four PLs, the mean being of 3.82 (SD 0.83). Seventy-one (42.01%) patients had more than four consecutive PLs, while 50 (29.59%) patients had four PLs (Figure 1). We noticed that three PL were seen in the rest of 48 (28.4%) patients. The mean gestational age of prior PL was 8.34 weeks (SD 1.73). These results are consistent with those from other published studies.

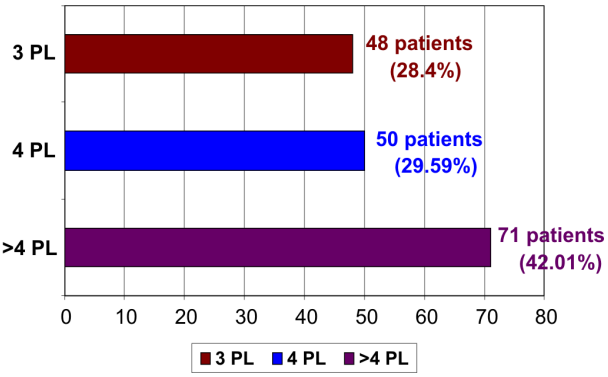

Figure 1 – ***Number of prior pregnancy losses (PLs) in studying cases.***

For all patients included in this substudy, genotyping was performed for *iNOS -2087A>G* (*rs2297518*). The polymorphism we studied was in Hardy–Weinberg equilibrium for both groups (p>0.05).

In Table 2, we exposed for both groups investigated, the frequency of genotype and alleles of the *iNOS -2087A>G* (*rs2297518*) polymorphism. Similar to the data confirmed by the current literature and international databases [*National Center for Biotechnology Information* (NCBI)], for both groups studied, the most frequent genotype was the GG genotype.

Table 2 – *Risk of RPL by the genotype frequency of iNOS polymorphisms between controls and RPL patients*

Polymorphism	RPL patients (*n*=169)	Control (*n*=145)	OR (95% CI)	*Chi*-square test *p*-value
***iNOS -2087A>G* (*rs2297518*)**				
Dominant *A* carriers (*AG+AA vs. GG*)	74 (43.79%)	54 (37.24%)	1.31 (0.83 to 2.07)	0.24
GG genotype	95 (56.21%)	91 (62.76%)	Reference	–

RPL: Recurrent pregnancy loss; iNOS: Inducible nitric oxide synthase; *n*: No. of cases; OR: Odds ratio; CI: Confidence interval.

iNOS -2087A>G (*rs2297518*) polymorphism recorded in the patients in the analyzed group the following frequencies of genotypes: with the *GG* genotype, the recurrent abortion rate was 56.21%, while in the control group, it was 62.76%; or if we analyze in a dominant model – allele carriers, the recurrent abortion rate was 43.79%, and in the control group, it was 37.24%. Applying the *chi*-square test in these situations, the test did not identify the existence of a significant association between the presence of this polymorphism and the patients with an increased risk of performing RPL.

When we performed a comparative analysis of the frequency of genotypes (also using international databases, we took as reference the *GG* genotype) and our statistical data, it was observed that this polymorphism, *iNOS -2087A>G* (*rs2297518*) has not been associated with an increased risk of developing RPL. As can be seen from Table 2, when one genotype was compared with another, we did not obtain any association that would have statistical significance, between the presence of this polymorphism and the increased risk for patients to develop RPL (in dominant – *A* allele carriers, *iNOS 2087 AG+AA vs. GG*: OR 1.31, 95% CI 0.83–2.07, *p*=0.24) (Table 2).

Another objective of our statistical analysis was the hypothesis that there is an association between the presence of polymorphisms and the number of consecutive PLs, as well as whether this risk increases directly in proportion to the previous number of PLs. To date, there are studies that have confirmed this hypothesis, according to which there are significant statistical associations between the presence of various polymorphisms and the overall risk of patients with RSA (with two or more consecutive losses), associations that have been maintained in the subgroup of patients with only two consecutive PLs, respectively in the subgroup of patients with three or more consecutive losses.

These studies analyzed the involvement of the factor V Leiden mutation, of polymorphisms *VEGF -1154G/A*, *KDR -1719A/T*, solute carrier family 19, member 1 (SLC19A1) polymorphism, micro-RNA (*miR*) *-196a2T/C* and *miR -499A/G* [18–22].

As in the previously published article, in which we have analyzed a possible association between *KDR -604A>G* SNP and RPL, and this time, we considered the recommendations of the *European Society of Human Reproduction and Embryology* (ESHRE) and the *Royal College of Obstetricians and Gynaecologists* (RCOG) Guidelines that define recurrent abortion as three or more consecutive PLs [23, 24]. Taking into account these recommendations, we grouped for analysis the persons included in the study into three subgroups: the first subgroup consisting of all cases of RPL with three or more consecutive PLs, the second subgroup represented by patients with only three consecutive losses and the third subgroup with four or more consecutive losses, respectively.

Analyzing the overall risk of developing RPL by *iNOS 2087* SNP genotype frequencies, between controls and RPL patients (which were stratified by number of consecutive PLs), taking into account the number of consecutive pregnancies, the *chi*-square test showed no association between the presence of this polymorphism and the increased risk for developing RPL in all three subgroups we analyzed (in a dominant model – *A* allele carriers, *iNOS 2087 AG+AA vs. GG*: the first subgroup, OR 1.31, 95% CI 0.83–2.07, *p*=0.24; the second subgroup, OR 1.26, 95% CI 0.76–2.11, *p*=0.37; the third subgroup, OR 1.4, 95% CI 0.77–2.53, *p*=0.272) (Table 3).

Table 3 – *Risk of RPL by the genotype frequency of iNOS polymorphism between controls and RPL patients, according to the number of prior PL*

Polymorphism	RPL patients PL≥3 (*n*=169)	Control (*n*=145)	OR (95% CI)	*Chi*-square test *p*-value
***iNOS -2087A>G* (*rs2297518*)**				
Dominant *A* carriers (*AG+AA vs. GG*)	74 (43.79%)	54 (37.24%)	1.31 (0.83 to 2.07)	0.24
GG genotype	95 (56.21%)	91 (62.76%)	Reference	–
Polymorphism	**RPL patients PL=3 (*n*=48)**	**Control (*n*=145)**	**OR (95% CI)**	***Chi*-square test *p*-value**
***iNOS -2087A>G* (*rs2297518*)**				
Dominant *A* carriers (*AG+AA vs. GG*)	45 (42.86%)	54 (37.24%)	1.26 (0.76 to 2.11)	0.37
GG genotype	60 (57.14%)	91 (62.76%)	Reference	–
Polymorphism	**RPL patients PL≥4 (*n*=121)**	**Control (*n*=145)**	**OR (95% CI)**	***Chi*-square test *p*-value**
***iNOS -2087A>G* (*rs2297518*)**				
Dominant *A* carriers (*AG+AA vs. GG*)	29 (45.31%)	54 (37.24%)	1.4 (0.77 to 2.53)	0.272
GG genotype	35 (54.69%)	91 (62.76%)	Reference	–

RPL: Recurrent pregnancy loss; iNOS: Inducible nitric oxide synthase; PL: Pregnancy loss; *n*: No. of cases; OR: Odds ratio; CI: Confidence interval.

Discussions

The *NOS* gene is present on the human chromosome 17q11.2-12. The gene product causes the oxidative deamination of L-arginine amino acids to form NO, which is an important signaling molecule. NO has strong antimicrobial effects, including the ability to inhibit the development of many infectious organisms *in vitro*. The *iNOS* human gene comprises 27 exons, with the transcription start site at exon 2 and the stop codon in exon 27 [25]. Exons 1–13 encode the oxygenase domain and exons 14–27 encode the reductase domain of this protein. Thus,

the iNOS protein is a two-domain catalytic enzyme. Both domains represent different functional parts of the enzyme. The presence of polymorphisms in the *NOS* gene plays an important role in many diseases affecting different populations. The *iNOS* gene has polymorphisms in both coding and regulation. Polymorphisms in the promoter region could affect the level of the gene product, while the polymorphisms in the coding region could alter the activity of the product.

We evaluated in this study a possible association between *iNOS -2087A>G* (*rs2297518*) polymorphism and the occurrence of idiopathic RPL. The *chi*-square test showed no significant association between the presence of this polymorphism and the increased risk to develop RPL.

The most studied polymorphism of the gene is *iNOS Ser608Leu*, which is known to be significantly associated with the risk of gastric cancer in patients with a history of smokers and alcohol users [26], association with gastric atrophy [27], with asthma susceptibility [28]. The *iNOS -2087A>G* (*rs2297518*) SNP analyzed in our study has also been studied in chronic pancreatitis [29], along with *VEGFR-2* (*KDR*) *-604A>G* SNP in pancreatic disorders [30].

Clinical trials of the past decade also aimed to investigate the association between the risk of recurrent abortion and the polymorphisms of the *iNOS* and *eNOS* genes. Thus, Parveen *et al.* analyzed a six SNPs of the *eNOS* gene (*12862A>G*, *12920C>T*, *12932C>T*, *12965 G>T/Glu298Asp*, *13222C>T*, and 27-bp *VNTR* introns) and observed that three SNPs (*12862A>G*, *12965G>T* and 27-bp *VNTR* intron 4) were significantly associated with an increased risk of recurrent miscarriage [6, 31–33]. Also, two studies published in 2015: the first clinical trial confirmed that the *GT* genotype of the *eNOS Glu298Asp* isoform exhibits a 2.3-fold great to do RSA [34]; the second extensive meta-analysis performed by Pereza *et al.* included six studies (1111 patients) and revealed that the *GT* and *TT* genotypes of the *eNOS +894G>T* allele are associated with an increased risk of RSA [35]. These recent studies confirm the hypothesis issued by other researchers [36–39].

Other studies have analyzed the immunohistochemical expression of *iNOS*, in combination with interleukin-33 (IL-33), in serous and mucosal epithelial ovarian tumors, to investigate their prognostic role and significance. It was observed that with the increase of the tumor grade in the malignant mucosal tumors, the expression of *iNOS* was significantly higher, and for IL-33, it was shown that with the increase of the tumor grade, in both the serous and the mucinous malignancies, the expression was significantly higher [40–42].

Conclusions

The *iNOS -2087A>G* (*rs2297518*) gene polymorphism does not influence RPL in the study area of Dolj County, Romania. The *chi*-square test showed no significant association between the presence of this polymorphism and the overall risk of RPL. Our study, as well as other studies conducted in various centers and areas around the world, has limitations related to the retrospective, descriptive nature and data collection – a small sample size. Taking into account that the data collected and processed by us are taken over from the "Filantropia" Municipal Hospital, Craiova, Romania, we tend to believe that the results obtained can be underestimated. Further studies are needed to reproduce our findings in different ethnic groups with a larger sample size.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

Anda Lorena Dijmărescu and Lidia Boldeanu equally contributed to the manuscript.

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