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Determination of *VEGFR-2* (*KDR*) *-604A>G* polymorphism in recurrent pregnancy loss

LIDIA BOLDEANU[1)], CRISTIAN ADRIAN SILOŞI[2)], VLAD PĂDUREANU[3)], ANDA LORENA DIJMĂRESCU[4)], MARIA MAGDALENA MANOLEA[4)], MARIA CARMEN TABACU[4)], MIHAIL VIRGIL BOLDEANU[5,6)], MIRCEA VASILE POPESCU-DRIGĂ[7)], IOAN SABIN POENARIU[5)], RODICA PĂDUREANU[7)], LILIANA VICTORIA NOVAC[4)], MARIUS BOGDAN NOVAC[8)]

[1)]*Department of Microbiology, University of Medicine and Pharmacy of Craiova, Romania*
[2)]*Department of Surgery, Faculty of Medicine, University of Medicine and Pharmacy of Craiova, Romania*
[3)]*Department of Internal Medicine, University of Medicine and Pharmacy of Craiova, Romania*
[4)]*Department of Obstetrics and Gynecology, University of Medicine and Pharmacy of Craiova, Romania*
[5)]*Department of Immunology, University of Medicine and Pharmacy of Craiova, Romania*
[6)]*Medico Science SRL – Stem Cell Bank Unit, Craiova, Romania*
[7)]*PhD Student, University of Medicine and Pharmacy of Craiova, Romania*
[8)]*Department of Anesthesiology and Intensive Care, University of Medicine and Pharmacy of Craiova, Romania*

Abstract

Background: Placental angiogenesis and vascular adaptation during pregnancy, along with diminished placental trophoblastic vascular endothelial growth factor immunoreactivity, play an important role in the early stages of human pregnancy, being possible causes of recurrent pregnancy loss (RPL). *Aims*: Our focus was directed towards investigating a possible association between vascular endothelial growth factor receptor-2 (kinase insert domain receptor) *VEGFR-2* (*KDR*) *-604A>G* (*rs 2071559*) gene polymorphism and RPL in the study area of Dolj County, Romania. *Patients, Materials and Methods*: In this study, 169 women, diagnosed with RPL, were included. They were hospitalized in the Clinics of Obstetrics and Gynecology, "Filantropia" Municipal Hospital, Craiova, during the following period: October 2009–October 2016. The control group consisted of 145 women. All subjects were genotyped by means of allelic discrimination TaqMan polymerase chain reaction assay with specific probes. *Results*: No statistically significant difference was observed between the RPL patients and the control group, when one genotype was compared to another [in a dominant model, *-604 AG+GG vs. AA*: odds ratio (OR) 1.71, 95% confidence interval (CI) 0.99–2.96, *p*=0.051]. While studying the overall risk of RPL by the genotype frequencies of *KDR* polymorphism between controls and RPL patients, which were stratified according to the number of consecutive pregnancy losses (PLs), the *chi*-square test showed a significant association between the presence of this polymorphism and the increased risk observed in patients with four or more consecutive PLs, to develop RPL (in a dominant model – *G* allele carriers, *KDR -604 AG+GG vs. AA*: OR 1.91, 95% CI 1.03–3.52, *p*=0.037). These results prove that *G* allele carriers have an increased risk of RPL about 1.91-fold higher than those with the *AA* genotype do. Although our results bear limited statistical significance, the study nonetheless represents a step forward in the evaluation of recurrent abortion, which has not yet been explored sufficiently. *Conclusions*: *VEGFR-2* (*KDR*) polymorphism does not influence RPL susceptibility in the study area of Dolj County, Romania. Therefore, further studies, which include a larger sample size, are required in order to clarify the role of *KDR* polymorphism in RPL.

Keywords: recurrent early pregnancy loss, vascular endothelial growth factor receptor-2, polymorphism, genotype.

Introduction

Recurrent pregnancy loss (RPL), also known as recurrent miscarriage is the loss of three consecutive pregnancies, which usually take place before reaching the 20th week mark, starting from the last menstrual period. Epidemiological studies have shown that the incidence of RPL can reach 1–5% of all women of reproductive age [1, 2]. Ford *et al.* (2009) illustrated that the risk of further losses is 30% after two losses and 33% after three losses, compared to women who had no obstetrical history, suggesting the need for studies after two consecutive losses [3].

RPL can be considered a public health problem because it directly compromises the quality of life pertaining to hundreds of women, as demonstrated through its negative effects on physical and mental health. Several different factors have been identified as playing a role in spontaneous abortion, among which of particular note are uterine abnormalities, chromosomal abnormalities, endocrine disorders, thrombophilia, immunohematological disorders, antiphospholipid syndrome, maternal infection and lastly but no less deserving of mention, lifestyle factors. Nevertheless, approximately 50% of RPL is not associated with any of the etiologies described above, and is therefore, considered idiopathic [4].

Angiogenesis is a key component of normal implantation and placentation, the vascular process having an important role to play in the early stages of pregnancy. It is known that embryo implantation and undisturbed pregnancy development is closely correlated with the vasculature of the chorionic villi [5].

In the last two decades, numerous studies have suggested that vascular endothelial growth factor (VEGF) along with nitric oxide synthesis has an important role in angiogenesis mechanisms, both under normal physiological conditions and pathological mechanisms. VEGF is involved in the regulation of angiogenesis in close relation to its receptors, the complex VEGF/VEGF receptor currently being at the forefront of scientific inquiry. VEGF binds to three receptor tyrosine kinases: VEGFR-1, VEGFR-2/kinase insert domain receptor (KDR) and VEGFR-3, which is located on the membrane of endothelial cells. The interaction between VEGF and its KDR receptor is presently considered to be of significant importance [6]. Two studies have communicated in idiopathic RPL a significant association between KDR and a subgroup of women who have had three or more miscarriages [7, 8].

Given the importance of angiogenesis in the early stages of pregnancy and particularly in placental development, it can be hypothesized that women with risk alleles of the *VEGF* gene and/or its KDR receptor may be notably prone to miscarriages. Therefore, our study aims to investigate a possible association between *KDR* polymorphism and the occurrence of idiopathic RPL.

Patients, Materials and Methods

Patients and study protocol

Included in the pilot study were 169 women diagnosed with RPL, having been hospitalized in the Clinics of Obstetrics and Gynecology, "Filantropia" Municipal Hospital, Craiova, Romania, between October 2009 and October 2016. In parallel, we investigated 145 women as control subjects, whom have had a history of at least one birth and no history of miscarriages.

The subjects included in both the control and the pathological group were of Romanian ethnic origin and agreed with the study. Demographics and pathological antecedents for each subject included in the study were obtained from their medical records.

Both large groups were established based on inclusion and exclusion criteria. For the group of patients diagnosed with RPL, the inclusion criterion in the study was the existence of at least three spontaneous abortions in the past. Exclusion criteria from the study for the group of patients with RPL were: cases that were investigated and detected with a definite pathology in previous abortions, patients presenting with acute or chronic infections, endocrine disorders (hyperthyroidism, hypothyroidism, diabetes mellitus, suprarenal pathology), immunological causes (anti-sperm antibodies present, antiphospholipid antibodies present, Rh isommunization), systemic maternal pathology (cardiovascular, pulmonary, renal, hematological), local maternal factors recorded in the anamnesis, following clinical examination (uterine malformations, uterine hypoplasia, uterine deviations, uterine tumors, uterine signs, uterine scars, cervico-ischemic insufficiency), genetic anomalies, or a modified karyotype detected in previous abortions and detected by tissue culture (amnion, embryo) with triplexes, monosomes, tetraploids, mosaicism, translocations, etc., or modified karyotype to one/both spouses, to the cases previously detected.

The inclusion criteria in the study for the control group were: patients without a history of recurrent miscarriage, patients who had at least one normal delivery, completed on a timely basis; the control group consisted of patients who presented for discontinuation at demand of pregnancy. The exclusion criteria from the study for the control group were the same for the group of patients with RPL.

Single-nucleotide polymorphism (SNP) genotyping

Genomic deoxyribonucleic acid (DNA) was purified from the isolated leukocytes from the peripheral blood collected on the anticoagulant from all the patients included in this project, using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's protocol.

All the patients included in this project were genotyped for *KDR -604A>G* (*rs 2071559*) at the University of Medicine and Pharmacy of Craiova, within its Human Genomics Laboratory. Polymorphism genotyping was performed with predesigned TaqMan assays, following the steps provided in the manufacturer's protocol, Applied Biosystems (Foster City, CA, USA): *VEGFR-2* (*KDR*) *-604A>G* (*rs 2071559*). Briefly, genomic DNA was amplified using a TaqMan Pre-Designed SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA), which contains primers and probes specific for each allelic variant.

Real-time polymerase chain reaction (RT-PCR) cycling conditions (RotorGene-Corbett 6200 HRM) were as such: 95ºC for 10 minutes, followed by 50 cycles of 92ºC for 15 seconds and 60ºC for one minute annealing temperature.

Interpretation of the results was achieved with the ViiA™ 7 Software v1.0 and the allelic discrimination option.

Statistical analysis

All polymorphisms have been tested to verify that there is a significant deviation from their respective Hardy–Weinberg balance. The Hardy–Weinberg balance was evaluated by comparing the frequencies of observed and expected genotypes with the χ^2 (*chi*-square) test. A *p*-value of less than 0.05 and *chi*-square greater than 3.84 shows that the polymorphism tested did not respect the Hardy–Weinberg balance. To evaluate possible associations between the *KDR* polymorphism and RPL, we chose odds ratios (ORs) accompanied by a 95% confidence interval (CI). In all analyzed subjects, we used the homozygote genotype as the reference category, being the most common allele in Caucasians. Data processing was performed by statistical analyzes made using statistical indicators applied to subjects studied. For the analysis of the obtained data, we used the 2×2 contingency table for categorical data with a two-tailed *p*-value, determined with the *chi*-square test of association. A two-sided p-value <0.05 was retained to have statistical significance.

Ethical approval

The study was carried out in full compliance with the ethical principles contained in the *Declaration of Human Rights* adopted in Helsinki, in 1975, as revised in 2008. All individual participants voluntarily joined this study

and provided written informed consents. For the purpose of 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, was obtained.

Results

In this pilot study, we have included 314 patients, 169 with RPL and 145 unaffected subjects. 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. The distribution of patients by age group at the time of study showed in both groups that the majority of 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. The two groups of subjects were formed so that there was homogeneity in terms of demographic data, a criterion confirmed by the results obtained for the two groups, presented in Table 1. The *chi*-square test did not denote a difference between the mean age of the two analyzed groups (p=0.09, p>0.05).

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	–
Thrombophilia	–	40 (23.67%)	–
Demographic distribution			
Urban	85 (58.62%)	96 (56.8%)	0.866
Rural	60 (41.38%)	73 (43.2%)	0.836

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

Demographic characteristics of the study group have shown no significant difference in the demographic distribution of the studied cases, with a proportion of 56.8% cases in urban areas and a proportion of 43.2% cases in rural areas, for RPL patients.

Regarding the obstetric antecedents, we compared the number of births in the two groups. The first objective was to compare the number of live births. We have considered the number of live births because this is 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, in the obstetric antecedents, women from the control group have a bigger number of live births, may prove better family planning, prenatal and genetic counseling of couple or may suggest a decrease in the incidence of abortion on demand.

The studied group was evaluated in terms of the number of prior pregnancy losses (PLs). What was subsequently noticed was that the majority of the pregnant women included in the study had at least four PLs, the mean being 3.82 (SD 0.83). In essence, 71 (42.01%) patients had more than four consecutive PLs, while 50 (29.59%) patients had four PLs (Figure 1). Of note, three PLs were observed in the remaining 28.4% (48 patients). The mean gestational age of prior PL was 8.34 weeks (SD 1.73). These findings are consistent with other studies that have been published.

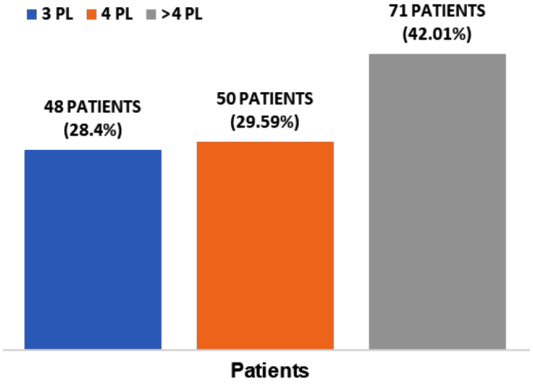

Figure 1 – *Number of prior pregnancy losses (PLs) in studying cases.*

All 314 collected samples from patients with RPL and healthy controls were genotyped. The polymorphism, we studied was in Hardy–Weinberg equilibrium for both RPL and healthy control groups (p>0.05).

Genotype and allele frequencies of *KDR -604A>G* (*rs 2071559*) polymorphism are listed for RPL patients and control groups in Table 2. In both groups, the most common genotype was the *AG* genotype, which has been confirmed by the current literature and international databases [National Center for Biotechnology Information (NCBI)].

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

Polymorphism	RPL patients (*n*=169)	Control	OR (95% CI)	*Chi*-square *p*-value
VEGFR-2 (*KDR*) *-604A>G* (*rs 2071559*)				
Dominant *G* carriers (*AG+GG vs. AA*)	140 (82.84%)	107 (73.79%)	1.71 (0.99 to 2.96)	0.051
AA	29 (17.16%)	38 (26.21%)	Reference	–

RPL: Recurrent pregnancy loss; KDR: Kinase insert domain receptor; VEGFR-2: Vascular endothelial growth factor receptor-2; *n*: No. of cases; OR: Odds ratio; CI: Confidence interval.

The frequency of genotypes of *VEGFR-2* (*KDR*) *-604A>G* (*rs 2071559*) polymorphism in patients for the analyzed group was as follows: with the *AA* genotype, the recurrent abortion rate was 17.16% and in the control group, it was 26.21%; with the *AG* genotype, the recurrent abortion rate was 53.25% and in the control group, it was 48.28%; with the *GG* genotype (homozygous), the recurrent abortion rate was 29.59% and in the control group, it was 25.51%; or in a dominant model – *G* allele carriers, the recurrent abortion rate was 82.84% and in the control group, it was 73.79%. In all these situations, the *chi*-square test showed no significant association between the presence of this polymorphism and patients having an increased risk of developing RPL.

From the comparative analysis of genotypes (by consulting the international databases, we have taken as a reference the genotype – *GG*) and of the statistical

data obtained, it was found that the *VEGFR-2* (*KDR*) *-604A>G* (*rs 2071559*) polymorphism was not associated with an increased risk of RPL.

As shown, no association was established between the presence of this polymorphism and the increased risk for patients to develop RPL when one genotype was compared with another (in a dominant model – *G* allele carriers, *KDR -604 AG+GG vs. AA*: OR 1.71, 95% CI 0.99–2.96, *p*=0.051) (Table 2).

In our statistical analysis, we have been looking to see if there is an association between the number of consecutive PLs and the presence of this polymorphism and whether this risk increases directly in proportion to the number of previously PLs.

We have considered to the verification of this hypothesis because there are studies in the literature that have demonstrated the existence of an association between certain polymorphisms and the number of recurrent spontaneous abortions (RSAs). Thus, Christiansen *et al.* have developed an algorithm for assessing the probability/frequency of embryo/fetal loss according to the number of previous spontaneous abortions and maternal age for the evaluation of recurrent abortion women. The authors have shown that the probability of another spontaneous abortion increases directly in proportion to the number of spontaneous abortions in the past and is inversely proportional to the mother's age [9]. They also indicated that the risk of RSA is much higher in pregnant women who have had previous losses. It was observed that the risk of RSA after two consecutive PLs is 17–25%, while the risk of losing a fourth load after three consecutive losses may be between 25% and 41% [9].

This hypothesis, association of polymorphisms with overall RSA patients (with two or more consecutive losses), which was maintained in the subgroup of patients with only two consecutive PLs and in subgroup with three or more consecutive losses was demonstrated in the case of the factor V Leiden mutation, *VEGF -1154G*/A polymorphism, *KDR -1719A/T* polymorphism, solute carrier family 19, member 1 (*SLC19A1*) polymorphism, micro-ribonucleic acid (*miR*) *-196a2T/C* and *miR -499A/G* [7, 10–13].

In our study, 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 [14, 15].

Respecting these recommendations, we formed three subgroups for the analysis: the first subgroup consisting of all cases of RPL with three or more consecutive PLs, the second subgroup represented by the patients with only three consecutive losses and subgroup with four or more consecutive losses, respectively.

While studying the overall risk of RPL by the genotype frequencies of *KDR* polymorphism between controls and RPL patients, which were stratified according to the number of consecutive PLs, the *chi*-square test showed a significant association between the presence of this polymorphism and the increased risk observed in patients with four or more consecutive PLs, to develop RPL (in a dominant model – *G* allele carriers, *KDR -604 AG+GG vs. AA*: OR 1.91, 95% CI 1.03–3.52, *p*=0.037) (Table 3). These results prove that *G* allele carriers have an increased risk of RPL about 1.91-fold higher than those with the *AA* genotype.

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

Polymorphism	RPL patients PL≥3 (*n*=169)	Control	OR (95% CI)	*Chi*-square *p*-value
VEGFR-2 (*KDR*) *-604A>G* (*rs 2071559*)				
Dominant *G* carriers (*AG+GG vs. AA*)	140 (82.84%)	107 (73.79%)	1.71 (0.99 to 2.96)	0.051
AA	29 (17.16%)	38 (26.21%)	Reference	–
Polymorphism	**RPL patients PL=3 (*n*=48)**	**Control**	**OR (95% CI)**	***Chi*-square *p*-value**
VEGFR-2 (*KDR*) *-604A>G* (*rs 2071559*)				
Dominant *G* carriers (*AG+GG vs. AA*)	38 (79.17%)	107 (73.79%)	1.35 (0.61 to 2.97)	0.454
AA	10 (20.83%)	38 (26.21%)	Reference	–
Polymorphism	**RPL patients PL≥4 (*n*=121)**	**Control**	**OR (95% CI)**	***Chi*-square *p*-value**
VEGFR-2 (*KDR*) *-604A>G* (*rs 2071559*)				
Dominant *G* carriers (*AG+GG vs. AA*)	102 (84.3%)	107 (73.79%)	1.91 (1.03 to 3.52)	*0.037**
AA	19 (15.7%)	38 (26.21%)	Reference	–

RPL: Recurrent pregnancy loss; KDR: Kinase insert domain receptor; PL: Pregnancy loss; VEGFR-2: Vascular endothelial growth factor receptor-2; *n*: No. of cases; OR: Odds ratio; CI: Confidence interval; *Statistically significant *p*-value.

Discussions

We evaluated in this study a possible association between *VEGFR-2* (*KDR*) *-604A>G* (*rs 2071559*) SNP and the occurrence of idiopathic RPL.

Until recently, the established viewpoint was that out of numerous genetic factors, only fetal chromosomal abnormalities have shown evidence associated with increased risk of RPL, representing approximately 50% of the PLs, during the first trimester. The other cases (the remaining 50%) are either of unknown etiology or result from a combination of several etiologies. Among these possible causes that have been investigated include: multifactorial disorders, single gene abnormalities, uniparental disomy, and skewed X chromosome [3, 16].

Increased vascular permeability and angiogenesis is essential for the success of implantation, decidualization and placentation. There have been several studies aimed primarily at changes in the uterus, the expression of several known genetic products which regulate vascular permeability and angiogenesis, including VEGF and its receptors, but without having investigated the angiogenic status of the uterus [17].

VEGF along with its receptors is considered an important growth factor in the regulation of vascular permeability and angiogenesis. Initial studies have indicated

the involvement of VEGF as a vascular permeability factor, subsequently mentioning its role as a strong mitogen for endothelial cells as well as a key regulator of vasculogenesis and uterine angiogenesis during implantation [6]. Six human VEGF isoforms, ranging in length from 121–206 amino acid residues, have been identified. VEGF binds to VEGFR-1 and VEGFR-2 (KDR), the tyrosine kinase receptors found at the endothelial cell membrane surfaces [18, 19]. The VEGF/VEGFR system plays a crucial regulatory role in the process of angiogenesis [20].

Several *VEGF* polymorphisms have been discussed in the impairment of both *VEGF* activity and expression. One particularly common polymorphism (*-1154G/A*) has been reportedly associated with a higher incidence of RSA. However, genetic association studies have, to date, been inconclusive [21].

VEGF and its receptors play an essential role in the development of fetal and placental angiogenesis. Studies in mice showed that the absence of expression of VEGF or any of the two receptors was followed by their intrauterine death as a result of inadequate vascular formation [18].

Studies have reported that increases in VEGF serum levels in umbilical cord blood, taken from patients after premature birth, remain uncertain, the most likely explanation for this being placental VEGF overproduction in response to inflammatory cytokines. This finding additionally adds evidence for the concept of the polygenic etiology of RSA [22].

Studies that have as objectives the interaction between VEGF and its receptors has shown that the interaction between VEGF and KDR is considered to be more important due to the high activity of tyrosine kinase mediating the angiogenic effects. The role of involvement of VEGF–KDR interaction, with statistically significant results, has been highlighted in other areas, such as breast cancer, coronary artery disease, Kawasaki disease, acute pancreatitis and pancreatic cancer [23–26]. Regarding the link between the VEGF–KDR system and idiopathic RPL, there are some data available, obtained from population studies [7].

In the last decade, many studies and meta-analyses have objectively investigated VEGF and its implications in the process of angiogenesis concerning both the pregnancy carried to term, as well as RPL. Thus, four polymorphisms of *VEGF* (*-2578C>A*, *rs 699947*; *-1154G>A*, *rs 1570360*; *-634G>C*, *rs 2010963*; *+936C>T*, *rs 3025039*) were analyzed, which are the most commonly studied loci and considered to have the greatest potential associated with the risk of RPL.

Papazoglou *et al.* reported for the first time in 2005 the association of *VEGF -1154G>A* gene polymorphism with an increased risk of RSA, with the other susceptibility alleles analyzed (*-2578C>A*, *-634G>C*, *+936C>T*) not being associated with RSA [27]. Also, other researchers highlighted the essential role of *VEGF* in trophoblastic cell proliferation, development of embryo vasculature and placental angiogenesis. They observed an increased risk of RSA in women carrying *VEGF -1154G>A* [16, 22, 27–30]. Studies have also reported no associations between the susceptibility alleles of the *VEGF* gene and the increased risk of RSA [31–33].

It can be concluded that the *-1154G>A* polymorphism was the most common allele variant studied for the *VEGF* gene. In addition to *VEGF* gene polymorphisms, VEGF receptor polymorphisms have presented associations between susceptibility alleles of *VEGFR* and the increased risk of RSA.

In our own study, no statistically significant association was detected between the polymorphism of the *KDR* and the RPL when comparing a genotype with other genotypes.

Su *et al.* (2011) conducted a case-control study of Taiwanese-Han women who had at least two consecutive spontaneous losses of pregnancy, which had occurred before the 12th week of pregnancy. The researchers demonstrated for the first time that certain haplotypes of the *KDR* gene are strongly associated with an increased risk of RSA. They analyzed 14 SNPs for *VEGF* and *KDR* genes and three functional SNPs [*rs 1570360* (*-1154G>A*) for VEGF; *rs 1870377* (*Q472H*) and *rs 2305948* (*V2971*) for *KDR*] [7]. The authors hypothesized that allelic variants of *KDR* may contribute to RSA by regulating the *VEGF*/*KDR* – SNP functional signaling *rs 1870377* (*Q472H*) associated with an increased risk of RSA.

Other researchers have observed that two functional SNPs located in *KDR* gene (*rs 1870377*/*Q472H* and *rs 2305948*/*V297I*) as well as one tag SNP in the intron region (*rs 6838752*) were not associated with RSA between patient (Iranian ethnic group) and control individuals [34].

For the *KDR* polymorphisms, some studies conducted on laboratory animals have demonstrated the role of *KDR* in the development of blood vessel networks (an important role in vasculogenesis, angiogenesis and embryonic development, both fetal and placental; along with the *VEGF* gene) [35]. After the binding of VEGF to the KDR, a cascade of signaling pathways, which contribute to endothelial cell migration and proliferation, is activated, thus stimulating angiogenesis [36].

A study published in 2013 by a group of Korean researchers was conducted over a period of 11 years on a large sample of 327 patients who have at least two consecutive PLs (for enrolling patients, the recommendations of the *American Society of Reproductive Medicine* [ASRM] defined recurrent miscarriage by the loss of two or more consecutive clinical pregnancies) [8]. They investigated three polymorphisms of the *KDR* gene (*-604T>C*, *rs 2071559*; *1192G>A*, *rs 2305948*; *1719A>T*, *rs 1870377*). Of the three SNPs, only *KDR -604T>C* polymorphism, *rs 2071559* was associated with an increased risk of RSA. Different forms of allelic variant *604C* (genotypes *-604 TC* and *-604 TC+CC*, genotypes *-604 TC+CC*/*1192 GG*, *-604 TC+CC*/*1719 AA* and *-604 TC+CC*/*1719 TA+TT*; and *CGA* and *CGT* haplotypes of *-604*/*1192*/*1719*, *CG* haplotype of *-604*/*1192* and *CA* and *CT* haplotypes of *-604*/*1719*) had a higher prevalence among women with RSA than in the control group. The *-604T* allelic variant was less common among aborted patients [8]. The authors also obtained an association between the *KDR -604T/C* polymorphism and overall RSA patients (two or more consecutive losses), which was maintained in the two subgroups analyzed, subgroup of patients with only two consecutive PLs and subgroup with three or more consecutive losses, respectively. [8]. This model of association between a polymorphism and

the subgroups mentioned above, was also observed in the case of other polymorphisms analyzed in various studies, such as the factor V Leiden mutation, *VEGF -1154G/A* polymorphism, *KDR -1719A/T* polymorphism, *SLC19A1* polymorphism, *miR -196a2T/C* and *miR -499A/G* [7, 10–13].

In our study, we have followed the recommendations of the *ESHRE* and the *RCOG* guidelines that define recurrent abortion as three or more consecutive PLs [14, 15]. While studying the overall risk of RPL by the genotype frequencies of *KDR* polymorphism between controls and RPL patients, which were stratified according to the number of consecutive PLs, the *chi*-square test showed a significant association between the presence of this polymorphism and the increased risk observed in patients with four or more consecutive PLs, to develop RPL (in a dominant model – *G* allele carriers, *KDR -604 AG+GG vs. AA*: OR 1.91, 95% CI 1.03–3.52, p=0.037). These results prove that *G* allele carriers have an increased risk of RPL about 1.91-fold higher than those with the *AA* genotype. As we can see, we have not obtained that model of association between polymorphisms and the subgroups analyzed, model presented in other studies.

Based on this, there appear to be distinctive results between the two Asian ethnic groups (Korean and Taiwanese) and the Iranian group, which can confirm the hypothesis of two important factors being implicated, the ethnic variation as well as the number of subjects which had been studied.

These data can support the concept according to which, women with RSAs have an increased risk of obstetric complications including fetal abnormalities, dead born babies and neonatal deaths even when pregnancies are ongoing. Also, studies have shown that the chances of having a successful task are roughly the same, after the second and third spontaneous abortion, but then the chances begin to decrease dramatically [9]. It is, of course, quite possible that, after three spontaneous abortions, couples use complex examinations and investigations to try to explain their spontaneous abortions and possibly receive medication or other help, so statistics can be changed.

In most studies, the association between polymorphism and RSA is negative, has not been reproduced in follow-up studies, or has inconsistent and contradictory results. Discrepancies may occur from various causes, such as, possible differences in designing the study (definitions of recurrent abortion groups and control), focus on the maternal element of recurrent abortion instead of focusing on couples or placenta, there was a reduced statistical power determined by the small sample size, or it does not take into account the contribution of lifestyle and environmental factors during pregnancy.

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.

Conclusions

The *VEGFR-2* (*KDR*) *-604A>G* (*rs 2071559*) gene polymorphism does not influence RPL in the study area of Dolj County, Romania. Although the *chi*-square test showed a significant association between the presence of these polymorphism and the increased risk observed in patients with four or more consecutive PLs, to develop RPL. We regard this conclusion as being of limited significance, due to the relatively small sample size of subjects. The results cannot, thus, be extrapolated. Nevertheless, our data does highlight the fact that the *KDR* polymorphism is not associated with the overall risk of RPL, the study being a step forward in the evaluation of aspects based on a number of various different elements already known about the molecular biology of pregnancy, but which in the context of recurrent abortion have not yet been sufficiently explored. Through the results of such research, we could clarify some of the clinical elements and genetic processes regarding this particular pathological aspect of pregnancy, one with major psychosocial implications for couples.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

Cristian Adrian Siloşi, Vlad Pădureanu and Lidia Boldeanu equally contributed to the manuscript.

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Corresponding author
Mihail Virgil Boldeanu, Lecturer, MD, PhD, Department of Immunology, University of Medicine and Pharmacy of Craiova, 66 1 May Avenue, 200628 Craiova, Dolj County, Romania; Phone +40724–515 810, e-mails: laborator.imunologie@umfcv.ro, boldeanumihailvirgil@yahoo.com

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