

Implication of *VEGFR2* in endometriosis: A structural biological and genetic approach

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Abstract. Vascular endothelial growth factor receptor 2 (VEGFR2) is a tyrosine kinase receptor, which exerts its activity by binding to VEGF, a major mediator of angiogenesis. Endometriosis is one of the most common gynecological diseases in women of reproductive age, characterized mainly by pelvic pain and infertility. Among the various hypotheses that have been suggested thus far for the development of this condition, it is considered that it is closely related to angiogenic responses. Polymorphisms of the *VEGFR2* gene have already been shown to be associated with vascular diseases, such as coronary heart disease (CHD). In the present study, the authors sought to clarify the structural/functional consequences of rs2305948, a common single nucleotide polymorphism (SNP) of *VEGFR2* in endometriosis and to investigate the association of this SNP with susceptibility to endometriosis, probably by influencing endothelial cells. This study comprised 162 patients with endometriosis (stages I-IV) hospitalized for endometriosis, diagnosed by laparoscopic intervention and histologically confirmed, and 170 women in the control group from a genetic homogeneous population. Genotyping of the V297I (rs2305948) polymorphism was performed through TaqMan technology. Three-dimensional (3D) homology

modelling was applied for the localization of the polymorphism under study on the VEGFR2 protein. Modelling revealed that the V297I polymorphism may affect the efficiency of trans-autophosphorylation and cell signaling. The results of statistical analysis revealed that there was no significant allelic and genotypic association of the rs2305948 SNP between the disease (stages I-IV) and the control group. Further analysis did not reveal any association of this SNP with an increased susceptibility to endometriosis for stages I and II or III and IV of the disease only. Apart from structural data that suggest that the rs2305948 SNP of *VEGFR2* gene may contribute to the pathogenesis of endometriosis by impairing VEGF signaling and increasing angiogenesis, in the present study, this SNP was not found to be associated with an increased susceptibility to endometriosis.

Introduction

Endometriosis is a complex benign, multifactorial, estrogen-dependent gynecological condition characterized by the growth of endometrial-like tissue outside the uterine cavity and is associated with pelvic pain, dysmenorrhea, intestinal symptoms, dyspareunia and infertility, although a low percentage of patients with the condition may be asymptomatic (1-3). Various genetic and environmental factors are known to influence the susceptibility to endometriosis (4-6) and heritability has been estimated at approximately 50% from twin studies (7). Novel genes are added to the list of endometriosis-associated genes given that novel Genome Wide Association Studies (GWAS) and meta-analyses are still in progress, while the role of epigenetic modifications in the development of endometriosis is being investigated extensively (8). Endometriosis can appear as peritoneal lesions, ovarian endometriotic cysts and deeply infiltrative endometriosis (9). Whilst a high percentage (10-15%) of women of reproductive age suffer from endometriosis (1), the pathogenic mechanisms leading to this disease remain unclear, although several theories have been put forth thus

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far regarding the development of endometriosis. However, there is strong evidence to indicate that the development of endometriosis is highly dependent on angiogenesis, due to its role in the ectopic implantation of endometrial tissue, as well as its increased activity into endometriotic lesions (10-12). Angiogenesis deals with the fundamental process of the formation and growth of new blood vessels from pre-existing ones and represents a biological setting of high importance during development and tissue growth, while it is involved in the pathogenesis of a number of diseases (13,14). Moreover, angiogenesis is considered to play a pivotal role in the pathogenesis of endometriosis, given that it provides a substantial supply of oxygen and essential nutrition required for the maintenance of endometrial tissues (15). Indeed, clinical studies have shown that dense vascularization characterizes all endometriotic lesions (2,10).

There are numerous endogenous regulators of angiogenesis. Among these, a main angiogenic mediator that has attracted much attention is vascular endothelial growth factor (VEGF), which has been suggested to play an important role in the development of endometriosis in combination with its endothelial selective receptor, VEGFR2 (16), also known as kinase insert domain receptor (KDR) (17). VEGF proteins play specific roles in controlling the growth of new blood vessels, while VEGF receptor signal transduction mediates endothelial cell proliferation, migration, organization into functional vessels and the remodeling of the vessel network (18,19). VEGF is produced by endothelial cells, monocytes and fibroblasts in response to hypoxia, which is the major physiological signal for angiogenesis through the activation of its receptor, VEGFR2, which is a member of the tyrosine kinase superfamily (20). A VEGFR2 homodimer is considered to be the main conduit for VEGF signaling (21,22); however, heterodimers involving other VEGF receptors may also transduce a signal (23,24).

Binding of growth factors, such as the VEGF dimer to the extracellular domain of their trans-membrane receptors leads to receptor dimerization, the activation of the intracellular tyrosine kinase domain of the receptor (25) and the initiation of signaling pathways (20). The extracellular region of the receptor is composed of 7 Ig-like domains. Domain deletion experiments have localized the VEGF binding site to domains 2 and 3 (D2 and D3), with D2 seems to play a dominant role (26,27). VEGFR2 D2 alone is necessary and sufficient for high-affinity binding, although D3 enhances affinity (28). By contrast, VEGFR2 constructs lacking domain D3 bind VEGF weakly or not at all; however, domain D2 is also required (26,27). Importantly, both 4th-7th Ig-like extracellular domains play a crucial role in signal transduction. To probe the functional importance of the residues involved in D3-D4 domain interaction interfaces, and to assess their contribution to VEGFR2 dimerization and VEGF binding, a series of alanine mutants have been constructed (29). There is reason to believe that domain 4 is involved in VEGFR2 dimerization, both from analogy with c-Kit (30) (Fig. 1) and from electron microscopy of dimeric VEGFR2:VEGF complexes (31). Four contained mutations on the β -sheet connecting loop, between amino acids 295 and 299 of the D3 domain, which also serve as part of the antibody binding interface, affect the binding affinity of VEGF by a factor of 2 (29).

Notably, it has been shown that angiogenesis differs in benign endometrial polyps and in endometrial cancers and, therefore, it has been proposed that the development of specific angiogenic markers may be important for prognosis of these lesions (32). However, it has been proven that VEGFR2 cannot be used as a prognostic factor in routine diagnostics for the selection of high-risk cases (32). Furthermore, taking into account that angiogenesis is a critical component of normal implantation in the early stages of pregnancy (33), VEGFR2 appears to mediate a significant molecular signaling pathway for endometrial receptivity (34).

Although angiogenesis is crucial for normal growth and development and in protective responses, such as wound healing and inflammation (35), aberrancies in angiogenesis can be observed in various pathological settings that lead to the pathogenesis of various diseases, such as cancer, diabetic retinopathy, rheumatoid arthritis and systemic sclerosis (14,36-38). Considering that the pathogenesis of endometriosis is crucially dependent on angiogenesis that is signaled via VEGF and its receptor, VEGFR2, and taking into account various genetic associations detected between the rs2305948 SNP of *VEGFR2* and endometriosis in different ethnic/racial populations (39-41), this study attempted to examine the potential genetic association between the rs2305948 (V297I) SNP and endometriosis in a homogeneous Greek population. This study also aimed to further elucidate the functional significance of this polymorphism by using a structural biological approach.

Patients and methods

Patient population and study design. In this case control association study, 332 women were enrolled (162 endometriosis patients and 170 controls) from the Department of Obstetrics and Gynecology of Venizeleion Hospital of Heraklion (Crete, Greece). The average age of the Greek endometriosis and control cohorts was 32.25 ± 7.1 and 29.49 ± 6.7 years, respectively. All the women enrolled had undergone surgery in the aforementioned tertiary care centre, while cases were diagnosed surgically (laparotomy or laparoscopy) and biopsies were used in order for the disease to be confirmed histologically. All the members of the control group had given birth to 2-5 children and had no previous medical record of chronic pelvic pain, dysmenorrhea or dyspareunia. Both the cases and controls were unrelated, living in the same urban environment and originated from the same Greek population (Cretan). The stages of endometriosis were defined by using the revised American Fertility Society Classification (42). Accordingly, 87 (53.70%) patients had stage I-II endometriosis and 75 (46.39%) patients had moderate to severe endometriosis (stage III-IV). All the subjects were of self-reported Greek origin. The study was performed in the Section of Molecular Pathology and Human Genetics of the Medical School of Crete, after obtaining the approval of the Research Committee of the Venizeleion General Hospital of Heraklion (ECHR no. 47/773) and was carried out in compliance with the declaration of Helsinki. Written informed consent was obtained from all the patients and control subjects, while the medical records were collected by the clinicians and pathologists of Venizeleion General Hospital, including surgical procedures and findings.

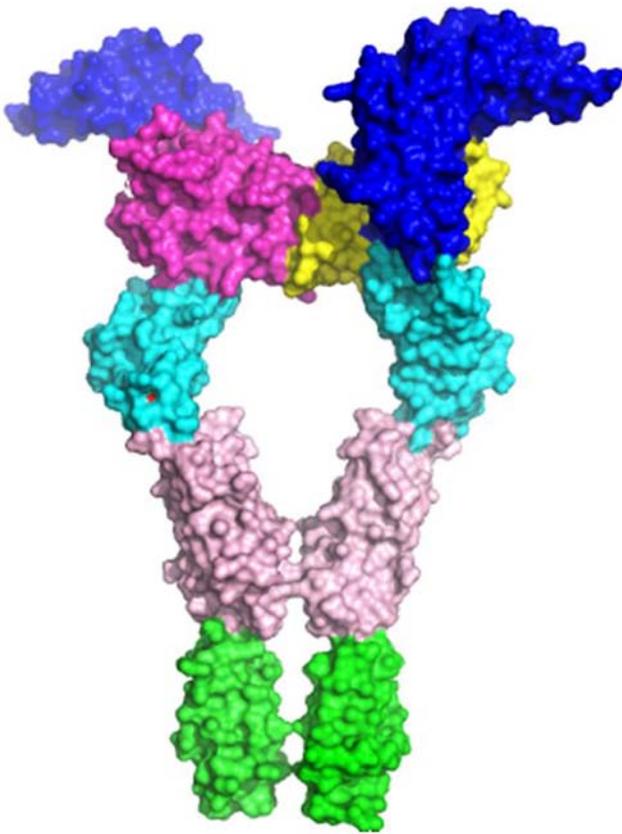


Figure 1. A model of the VEGFR2 receptor built with homology modeling from the homologous Kit structure. The VEGFR2-VEFR dimer complex is shown in surface representation, with the different domains given in different colors. The VEGF dimer is shown in purple-yellow bound on an interface on the D2-D3 VEGFR2 Ig-like domains (blue-cyan). The D3 domain is shown in cyan with the D4 and D5 interacting with their homologs in the dimer in pink and green. The red dot in D3 shows the position of the mutation.

Genetic analysis of the V297I VEGFR2 polymorphism. Whole blood was collected pre-operatively in ethylenediaminetetraacetic acid (EDTA)-containing tubes. Genomic DNA was isolated from peripheral blood leukocytes using a commercial kit (PureLink[®] Genomic DNA Mini kit; Invitrogen; Thermo Fisher Scientific) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until analysis. The rs2305948 1192 C/T (V297I) SNP of the *VEGFR2* gene was genotyped via TaqMan 5' allelic discrimination technology, using a pre-designed SNP genotyping assay provided by Applied Biosystems (TaqMan assay no. C_22271999_20) as previously described in detail (43). Allelic discrimination plots were all reviewed individually for quality and negative controls were also run for this assay. The accuracy of the results was ensured upon the amplification of a random 10% of the total samples. The genotyping success rate was 98% (missingness: 2%).

Construction of VEGFR2 domains' three-dimensional (3D) model. The three dimensional structure of the human VEGFR2 extracellular domain in complex with VEGF or antibodies (PDB codes 2X1W and 3S35) (29,44) as well as the crystal structure of the extracellular domain of Kit (PDB code 2E9W) (30) were downloaded from the Protein Data Bank (<https://www wwpdb.org/>) and used to analyze

the consequences to structure and function of the mutation p.Val297Ile located in the interface between the D3 and D4 Ig-like domains. The mutant was constructed using molecular modeling with the program Maestro (Schrodinger, LLC) which was also used to analyze the conformational changes caused by the mutation. Rotational flexibility on mutated side chains was tested due to the restricted space in the mutation vicinity and the conformation with the least bad contacts was adopted. All figures depicting 3D models were created using the molecular graphics program PyMOL V.2.2 (45).

Statistical analysis. The cases and controls subjects used in the study were unrelated. Statistical analysis was performed using the GraphPad Prism statistical program (GraphPad Software), by applying the additive model. The Chi-squared (χ^2) test, with one or two degrees of freedom or the genetic variants under investigation were evaluated for deviation from the Hardy-Weinberg equilibrium (HWE) by comparing observed and expected genotype frequencies by means of Chi-squared (χ^2) test or Fisher's exact test in the control groups (by using the program named 'Calculate'; Copyright TRG, SR, INMD, 2008). Power calculations were performed by using the CaTS power calculator (46). OR and 95% CI values were calculated using the aforementioned GraphPad Prism statistical program.

Results

Analysis of the structural consequences of V297I VEGFR2 polymorphism. The rs2305948 SNP leads to the substitution of valine (V), a C _{β} branched amino acid residue, to a larger C _{γ} branched hydrophobic residue, isoleucine (I) at position 297 of the protein chain of the VEGFR2 monomer (Fig. 2A and B). Val297 is located close to the D4 interface, on a hairpin loop connecting two β -sheets, forming the Ig-like β -barrel of the D3 Ig-like domain of the extracellular VEGFR2 part (Fig. 3). The D4 domain is responsible for homotypic D4 contacts (47) upon ligand-mediated dimerization of the VEGF receptors (25). This mutation may alter the conformation of the β -sheet connecting loop in the D3 Ig-like domain, by rearranging the domain 3:domain 4 interface of VEGFR2 in such a manner that the receptor can no longer dimerize. As a consequence, the V297I mutation may affect the efficiency of trans-autophosphorylation and cell signaling. Sequence alignment between the D5 domain and the D7 domain of same receptor and KIT D4, known to form homotypic receptor contacts in VEGFR2 and KIT dimerization, respectively, indicate homology between the domains on sequence motifs responsible for mediating homotypic contacts.

VEGFR2 rs2305948 SNP is not associated with endometriosis in a Greek population. In the case of the rs2305948 SNP of the *VEGFR2* gene, no statistically significant difference was found in the frequency of the T allele between the cases and controls (P=0.89, OR=1.073, 95% CI, 0.61-1.88) (Table I). Similarly, no statistically significant difference was observed in the frequencies of the TT and CT genotypes between the cases vs. the controls (P=1, OR=0.960, 95% CI, 0.06-15.5) and P=0.87, OR=1.091, 95% CI, 0.59-2.00, respectively) (Table I).

Notably, in an analysis conducted for endometriosis, no significant association was detected as regards the TT and

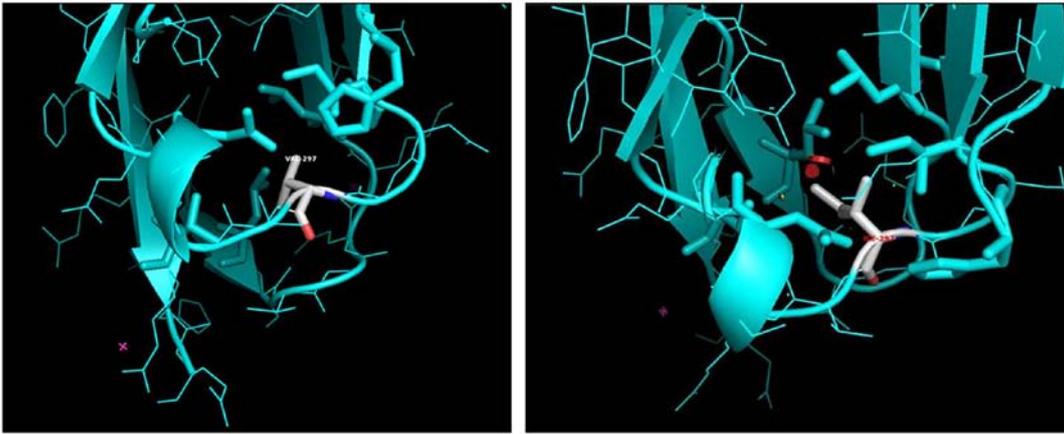


Figure 2. The studied novel mutation V297I in the VEGFR2 structure. The panels illustrate the 3D native structure of the β -sheet interlinking loop in the D2 Ig-like domain (PDB 2X1W) (left panel) and mutant around position #297 (right panel). Val297 (white) is located in the middle position of a long interconnecting loop between the β -sheets in domain D2. The substitution of the small hydrophobic valine with the much larger hydrophobic isoleucine (white) will strongly alter the relative position of the loop, creating interatomic clashes in the D3 domain, weakening the D3 to D4 domain anchoring interactions and the formation of stable dimers. Structures were analyzed, mutant constructed and cartoons rendered using the PyMOL Molecular Graphics System, Version 2.2.

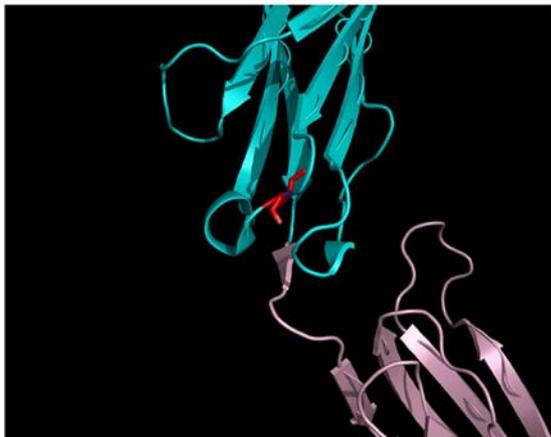


Figure 3. Details of the studied novel mutation V297I in the VEGFR2 structure. The 3D native structure of the β -sheet interlinking loop in the D2 Ig-like domain (PDB 2X1W) is shown. Val297 (purple) is located in the middle position of a long interconnecting loop between the β -sheets in domain D2. The substitution of the small hydrophobic valine with the much larger hydrophobic isoleucine (red) will strongly alter the relative position of the loop, creating interatomic clashes in the D3 domain, weakening the D3 to D4 domain (salmon) anchoring interactions and the formation of stable dimers. Structures were analyzed, mutant constructed and cartoons rendered using The PyMOL Molecular Graphics System, Version 2.2.

CT genotypes of this SNP in patients with stage I/II of the disease and the controls ($P=1$, OR=0.520, 95% CI, 0.03-8.40 and $P=0.85$, OR=1.120, 95% CI, 0.53-2.35, respectively), as depicted in Table II. Furthermore, no evidence for the association with endometriosis cases stratified to stages I and II was found for the T allele ($P=1$, OR=1.026, 95% CI, 0.52-2.00) (Table II).

Similarly, when the patients were stratified to stages III and IV and analyzed, no significant association with endometriosis was detected either at the genotype or allele frequencies of rs2305948. Thus, when genotype CT+TT or allele 'T' frequencies of patients with stage III/IV endometriosis were compared with the controls, no statistically significant difference was observed ($P=1$, OR 1.10, 95% CI, 0.51-2.35 and

$P=0.86$, OR 1.13, 95% CI, 0.55-2.34, respectively), as depicted in Table III. The SNP under investigation did not deviate significantly from the expected Hardy-Weinberg proportion either for cases or for controls (P -value for deviation was 0.20).

Discussion

Aiming for a better understanding of the putative role of VEGFR2 in endometriosis, the authors conducted the present structural biological and genetic study. It was hypothesized that variations of the *VEGFR2* gene may alter the biological function of the encoded protein and, as a consequence, this genetic factor may further influence the endothelial function in women that develop endometriosis. The structural data presented in the current study suggest that the rs2305948 (V297I) polymorphism may be causative for the development of endometriosis due to its effect on the impairment in cell signaling. The structural analysis provided insights into receptor/ligand and dimer formation interactions, which are essential for the understanding of receptor/ligand specificity, and may explain previously published data (25,29,47). The Val297Ile polymorphism may play an important role in D3-D4 Ig-domain interaction, thus affecting the efficiency of dimerization, essential for kinase activation and the cell signaling mechanism.

As previously suggested, the rs2305948 SNP results in a significant increase in the VEGF binding efficiency to VEGFR2 and, as a consequence, an impairment in VEGFR2 function may be associated with vascular dysfunction, abnormal vascular repair and vascular diseases (48). In this study, the authors performed a case-control association analysis in an attempt to reveal, for the first time, an association of the rs2305948 (V297I) *VEGFR2* SNP with an increased susceptibility to endometriosis in a Greek population. It is worth noting that the available literature reports concerning the role of this polymorphism in endometriosis have yielded controversial results. However, this polymorphism was not found to be associated with endometriosis, although the genotype frequencies of rs2305948 from the International HapMap data (49) were

Table I. Genotypes and allele frequencies of the *VEGFR2* rs2305948 SNP analyzed in 162 women with endometriosis and 170 healthy controls.

Genotypes/Alleles	Endometriosis	Controls	P-value	OR (95% CI)
Genotypes	n=162	n=170		
CC	138 (85.18%)	143 (84.12%)	0.87	1.091 (0.59-2.00)
CT	23 (14.20%)	26 (15.29%)		
TT	1 (0.62%)	1 (0.59%)		
Alleles	n=324	n=340		
C	299 (92.28%)	312 (91.76%)	0.89	1.073 (0.61-1.88)
T	25 (7.72%)	28 (8.24%)		

OR, odds ratio; CI, confidence interval.

Table II. Genotype and allele frequency of the *VEGFR2* rs2305948 SNP analyzed in 87 women with endometriosis (stage I and II) and 170 healthy controls.

Genotypes/Alleles	Endometriosis	Controls	P-value	OR (95% CI)
Genotypes	n=87	n=170		
CC	74 (34.48%)	143 (84.12%)	0.85	1.120 (0.53-2.35)
CT	12 (42.53%)	26 (15.29%)		
TT	1 (22.99%)	1 (0.59%)		
Alleles	n=174	n=340		
C	160 (91.95%)	312 (91.76%)	1	1.026 (0.52-2.00)
T	14 (8.06%)	28 (8.24%)		

Table III. Genotype and allele frequency of the *VEGFR2* rs2305948 SNP analyzed in 75 women with endometriosis (stage III and IV) and 170 healthy controls.

Genotypes/Alleles	Endometriosis	Controls	P-value	OR (95% CI)
Genotypes	n=75	n=170		
CC	64 (85.33%)	143 (84.12%)	1	1.10 (0.51-2.35)
CT + TT	11 (14.67%)	27 (15.88%)		
Alleles	n=150	n=340		
C	139 (92.67%)	312 (91.76%)	0.86	1.13 (0.55-2.34)
T	11 (7.33%)	28 (8.24%)		

very similar to those of this study. Of note, similar frequencies of the rs2305948 SNP have been demonstrated in a previous study conducted in the Cretan population (50), aiming to detect a putative association between this SNP and systemic lupus erythematosus (SLE). Importantly, the *VEGFR2* gene locus has not been identified in GWAS for endometriosis susceptibility thus far (6), a finding that is in accordance with the results of the present case-control study.

Previously, it has been reported that a functional polymorphism in the VEGF gene may be associated with the risk of developing endometriosis in women in Northern China, through changes in the transcriptional activity of VEGF (51). Based on the same population, Kang *et al* (39) presented data

suggesting that the 1192C/T polymorphism of rs2305948 of the *VEGFR-2* gene may be associated with endometriosis in women in Northern China Han ethnicity, by conferring protection to women carrying the minor allele 'T'. However, these data have to be examined and evaluated with a critical eye, considering that the control group was recruited based on an ultrasound-based diagnosis only, negative for endometriosis and, as known, this method cannot exclude the occurrence of pelvic endometriosis. Moreover, Cardoso *et al* (41) conducted a study based on a Brazilian population and found that rs2305948 was also protective against the development of endometriosis, with this SNP also reducing cyclical urinary symptoms. *VEGFR-2* is encoded by the *KDR* gene, located

on chromosome 4q11-q12 and consists of 30 exons (27). The SNP under investigation is located in exon 7, within the immunoglobulin-like domain 3, and previous studies have illustrated that it substantially decreases the efficiency of VEGF binding to VEGFR-2 (48,52). Therefore, it is reasonable to speculate that the decreased binding capacity of VEGF appearing as a result of the 1192C/T polymorphism may lead to a decrease the activity of angiogenesis, thus reducing the risk of endometriosis (39). However, a study performed in a population from Belgium (40) presented data demonstrating an association of 1192C/T polymorphism with an increased risk of developing endometriosis. Furthermore, previous studies have demonstrated an association of this SNP with the risk of developing diseases, such as diffuse large B cell lymphoma, colorectal cancer, coronary heart disease and ischemic stroke (53,54). Thus, accumulative data seem to suggest that common genetic variants in the *VEGF* and *VEGFR-2* genes, resulting in alterations regarding the VEGF-VEGFR signaling, may play a role in the molecular pathogenesis of endometriosis.

It is worth noting a limitation of this study. Although this study sample size was sufficient, the statistical power of this study was relatively low due to the very low frequency (globally observed) of the minor allele 'T' of the rs2305948 SNP. However, the authors avoided the inclusion of additional individuals from mainland Greece or immigrants that could reduce the genetic homogeneity of the sample.

To the best of our knowledge, this study is the first to investigate the association of the rs2305948 SNP of the *VEGFR2* gene with endometriosis in the Greek population. Apart from the important role of this SNP in the VEGF/VEGFR2 binding efficiency, its involvement in the development of various diseases and its association with an increased risk of developing endometriosis in different cohorts to date have been well established. However, this study did not succeed in confirming that this polymorphism contributes significantly either to an increased susceptibility for endometriosis or to the severity of this condition. The failure to confirm previous findings may be attributed either to inter-population differences or to interactions between genetic and non-genetic factors. Thus, the results of the present study demonstrate that it is difficult to identify generalizable risk alleles in endometriosis and highlight the importance of conducting comparative studies in different populations in order to determine true risk alleles for this condition.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EE, CM, MM and GNG designed the study and drafted the manuscript; EE, GNG, CM, IM, MM, IK, DAS and MIZ searched the literature; EE, MM, IK, GNG and MIZ analyzed and interpreted the data; MIZ, IM and DAS critically revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee for Human Research of Venizeleio Hospital approved the respective protocol (ECHR no. 47/773). Informed consent was obtained from all the participants.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Managing Editor of the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. All the other authors declare that they have no competing interests.

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