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ORIGINAL ARTICLE

Prevalence of candidate single nucleotide polymorphisms on *p53*, *IL-11*, *IL-10*, *VEGF* and *APOE* in patients with repeated implantation failure (RIF) and pregnancy loss (RPL)

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ABSTRACT

Recurrent pregnancy loss (RPL; defined as the loss of three or more consecutive pregnancies) and recurrent implantation failure (RIF; when implantation is not achieved after at least three cycles of IVF) are two of the major challenges that reproductive medicine faces. Some polymorphisms have been identified as possible causes of an increased risk of these diseases. This paper studies the prevalence of the polymorphisms in *p53*, *VEGF*, *IL-10*, *IL-11* and *APOE* in RIF and RPL patients that determines the risk for these pathologies. A total of 255 patients were selected (89 RPL patients, 77 RIF patients and 89 controls) and genotyped for *p53-R72P*; *IL-11-1082-AG*; *VEGF-1154-AG*; *IL-10*; *APOE-R112C*; *APOE-R158C*. Statistically significant differences were found in the prevalence of the E4 isoform (R122-R158) of the *APOE* gene in RPL patients ($p < 0.05$), and in RIF patients, the R72P polymorphism of the *p53* gene and the 1154-AG of the *VEGF* gene showed different distribution ($p < 0.05$). Regarding the *p53* and *IL-11* studied polymorphisms, PP of *p53* gene and GG of *IL-11* are more prevalent in RPL patients without reaching statistical significance. In conclusion, our results suggest patients carrying variants in *p53* and *VEGF* would be at risk of RIF, and those carrying variants in *APOE* gene would suffer RPL.

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Introduction

Recurrent pregnancy loss (RPL) is defined as the loss of three or more consecutive pregnancies after the 12th week. Recently the American Society for Reproductive Medicine (ASRM) has extended this definition considering that the loss of pregnancies not visualized by ultrasound (biochemical pregnancies and/or unknown location pregnancies) should be included in the definition of recurrent pregnancy loss (Kolte et al., 2014). In couples without fertility problems pregnancy loss occurs spontaneously in 15–20% and only become recurrent in 2–3% of them (Rai & Regan, 2006). On the other hand, recurrent implantation failure (RIF) could be considered when implantation is not achieved after at least three cycles of *in vitro* fertilization (IVF) with at least four good quality embryos transferred to a woman under 40 years of age (Coughlan et al., 2014).

Embryo implantation requires the ability of the blastocyst to invade the endometrium and to establish its own vascular network, as well as an adequate maternal immune tolerance to the embryo. A correct trophoblastic invasion is led by an equilibrium between apoptotic and cellular generation processes (Simón, Dominguez, Remohí, & Pellicer, 2001; Smith, 2000). There are a high number of proteins involved in the invasion of the endometrium and the angiogenesis. One of these proteins is p53, a potent tumour suppressor that induces apoptosis and angiogenesis (Savion et al., 2002). Moreover, some studies reveal that p53 is involved in embryo implantation mediated by leukemia inhibition factor (LIF) (Hu, Feng, Teresky, & Levine, 2007). The *p53* gene (17q13) contains 11 exons, and a single nucleotide polymorphism (SNP) in codon 72 comprising the change of a G by C resulting in a proline instead of an arginine. The p53 protein with an arginine in codon 72 is much more efficient in inducing apoptosis, LIF expression and cellular transformation (Pim & Banks, 2004). A decreased expression of genes involved in apoptosis and angiogenesis has been associated with RIF (Smith, 2000).

Another important protein involved in embryo implantation is vascular endothelial growth factor

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(VEGF). VEGF is an angiogenic factor that acts by enhancing and remodelling the vascular growth, as well as by increasing vessel permeability, which is crucial for the embryo implantation and further development of the placenta (Magdoud et al., 2012). Changes in the expression of VEGF in decidual samples in early miscarriages (Vuorela, Carpén, Tulppala, & Halmesmäki, 2000), as well as the committed fertility in VEGF knock-out mice, show the importance of VEGF (Rowe, Wulff, & Fraser, 2003) in embryo implantation and early pregnancy stages. The *VEGF* gene (6q21) contains 8 exons. Several SNPs have been described and associated with an abnormal *VEGF* expression. The SNP located in the promoter region -1154 has a higher population frequency allele (G) which, when substituted by A produces a lower *VEGF* expression (Watson, Webb, Bottomley, & Brenchley, 2000).

The correct embryo implantation and the development of the pregnancy also depend on the maternal tolerance to embryo and fetal tissues. A suitable cytokine production profile will stimulate fetal and placental development (Cha, Sun, & Dey, 2012). Several studies have focused on determining the role of each cytokine during embryo development, their changes during the pregnancy or the effects of an alteration in their levels on implantation and pregnancy. Interleukin 10 (IL-10) is an anti-inflammatory cytokine that plays a key role in maternal fetal tolerance. Decreased levels of IL-10 are associated with pregnancy loss (Wang, Hao, & Lin, 2011). In the IL-10 gene (1q31), the SNP in position -1082A/G controls its expression; A allele produces a decrease in the gene expression. There are other important cytokines for the progress of pregnancy such as Interleukin 11 (IL-11), an inflammatory cytokine with similar functions to LIF. *IL-11* gene is located at 19q13. Different polymorphisms have been identified in the *IL-11* gene that may affect its expression (Shinohara et al., 2001).

Finally, other proteins that are not major players might also affect the correct implantation and development of the pregnancy. For example, apolipoprotein E (APOE) is involved in the transport of lipoproteins, lipid soluble vitamins and cholesterol into the lymphatic system and subsequently into the bloodstream (Mahley, Weisgraber, & Huang, 2009). A deficient protein could produce lipid accumulation in the bloodstream increasing thrombotic risk. The *ApoE* (19q13) gene consists of four exons. It has three polymorphic isoforms: *ApoE* E2 (cys 112, cys 158), *ApoE* E3 (cys 112, arg 158) and *ApoE* E4 (arg 112, arg 158) that produce six different genotypes: E2/E2, E2/E3, E2/E4, E3/E3, E3/E4 and E4/E4. Different genotypes have been associated with reproductive problems mainly RPL (Li, Chen, Wu, & Li, 2014).

In a previous study, it was shown that there is an association between *p53 R72P* polymorphism and RIF and RPL patients (Lledo et al., 2014). We considered to extend the study by obtaining the complete genotype from the same patients for all the possible genetic variants associated with RIF andRPL. The aim of this paper is to study the prevalence of the polymorphisms previously described in *p53, VEGF, IL-10, IL-11* and *APOE* in RIF and RPL patients that determines the risk for these pathologies.

Materials and methods

Study population

RPL and RIF patients were selected according to current definitions, previously discarding any other cause for RPL and RIF in the couple by using ultrasound, hysteroscopy, karyotype, thrombophilia or sperm FISH. All the subjects included in the study gave their informed consent to collection of peripheral blood samples suitable for molecular analysis and this was approved by the Instituto Bernabeu Institutional Review Board (Ref: RL01). Oocyte donors, with no history of RIF or RPL, were used as controls. The DNA samples were obtained from a swab and/or blood. To evaluate the prevalence of SNPs in *p53, VEGF, IL-10, IL-11* and *APOE* genes and their effect in RIF and RPL patients, we designed a retrospective case control study. Two hundred and fifty-five women were selected and genotyped: 89 RPL patients, 77 RIF patients and 89 controls without fertility problems.

Genotyping

Taqman® Life Technologies allelic discrimination real time PCR was used for genotyping (rs1042522 *p53 R72P*; rs1800896 *IL-11 -1082AG*; rs1570360 *VEGF -1154 AG*; rs11668344 *IL-10*; rs429358 *APOE R112C*; rs7412 *APOE R158C*) following the instructions of the manufacturer.

Statistical analysis

The genotyping results were analyzed using SPSS v20.0. To determine significant differences between groups, Pearson's Chi-square was used accepting $p < 0.05$ for significant values and the odds ratio were controlled with binary logistic regression. RIF, RPL and controls were considered as separate groups in order to compare them.

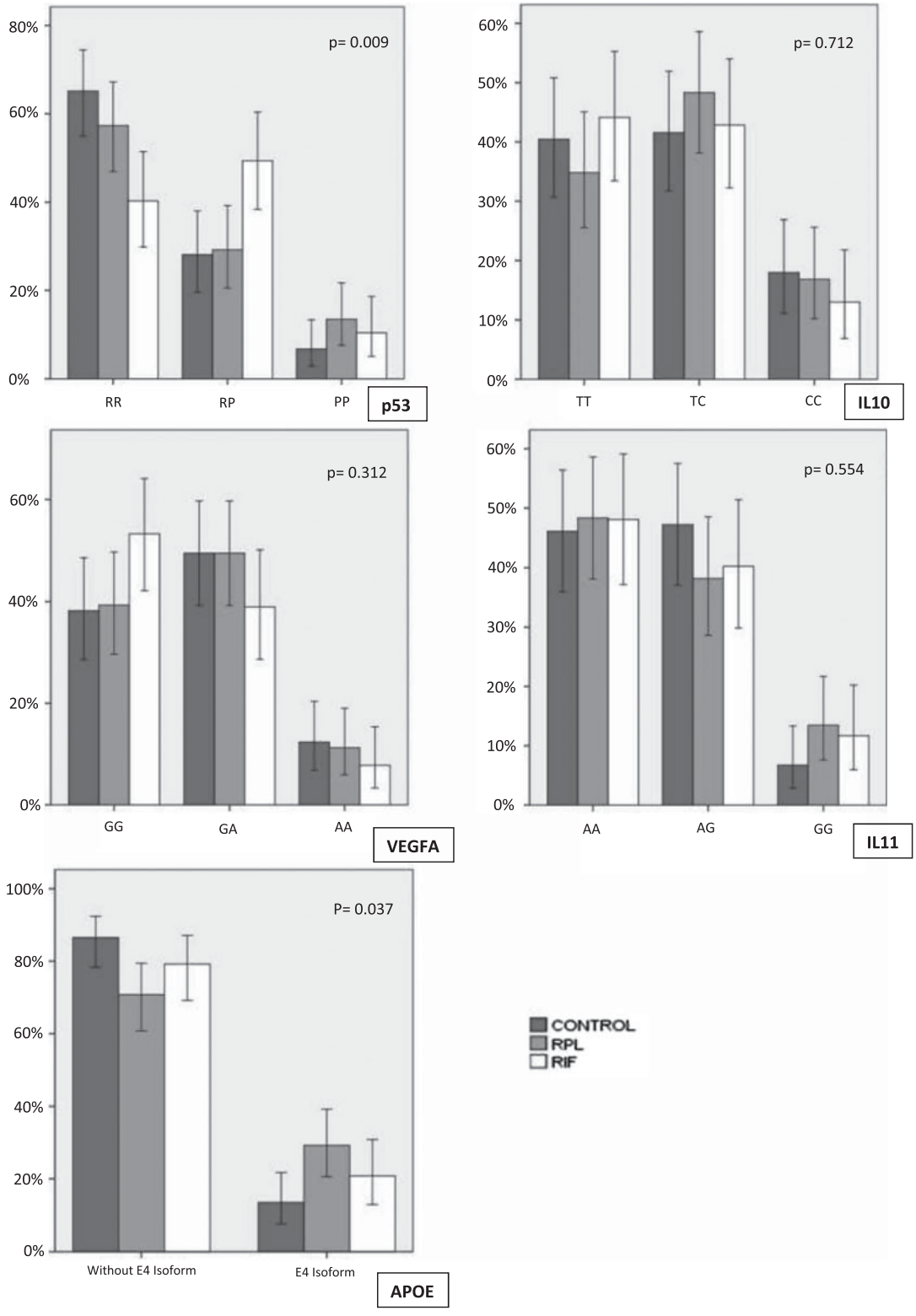

Figure 1. Prevalence distribution of the polymorphisms in *p53*, *IL-10*, *VEGF*, *IL-11*, *APOE* genes.

Results

The allelic frequencies of the different genetic variants studied are shown in Figure 1. Statistically significant differences were found in the prevalence of the R72P polymorphism of *p53* gene and the E4 isoform of *APOE* gene ($p < 0.05$) in RIF and RPL patients. Regarding the studied polymorphism in *VEGF*, *IL-10* and *IL-11* polymorphism showed no differences in the distribution of genotypes.

Table 1. Genotype and allele frequencies for SNPs in *p53*, *IL-11* and *APOE* in RPL patients.

		CONTROL ($n = 89$)	RPL ($n = 89$)	p	OR (95% CI)
p53	RR/RP (%)	93.3	86.5	0.136	2.156 (0.771–60.26)
	PP (%)	6.7	13.5		
APOE	E2E2/E2E3/E3E3 (%)	86.5	70.8	0.010	2.648 (1.238–5.667)
	E4E2/E4E4/E4E3 (%)	13.5	29.2		
IL-11	AA/AG (%)	93.3	86.5	0.136	2.156 (0.771–60.26)
	GG (%)	6.7	13.5		

Table 2. Genotype and allele frequencies for SNPs in *p53* and *VEGF* in RIF patients.

		CONTROL ($n = 89$)	RIF ($n = 77$)	p	OR (95% CI)
p53	RR (%)	65.2	40.3	0.001	2.778 (1.447–5.208)
	RP/PP (%)	34.8	59.7		
VEGF	AA/AG (%)	61.8	46.8	0.048	1.842 (1.002–3.422)
	GG (%)	38.2	53.2		

To investigate the influence of the different allele of each polymorphism we analyzed the pattern of inheritance (dominant vs recessive) for RPL and RIF patients. For RPL patients the E4 variant of the APOE gene is associated with an increased risk of recurrent miscarriage odd ratio 2.648 (CI 95% 1.238–5.667). Moreover, the recessive model of *p53* polymorphism (RR/RP vs PP) and the dominant model of *IL-11* (AA/AG vs GG) showed that the PP genotype for the *p53* gene and the GG for the *IL-11* are increased in RPL patients without reaching statistical significance (Table 1).

For RIF patients, the dominant model of the *p53* polymorphism (RR vs RP/PP) and *VEGF* (GG vs GA/AA) show a different distribution which is associated with an increased risk of implantation failure. The odds ratio of 2.778 (CI 95% 1.477–5.208) is obtained for the *p53* polymorphism and 1.842 (CI 95% 1.002–3.422) for *VEGF* in RIF patients Table 2.

Discussion

Repeated miscarriage and implantation failure are two of the major challenges that reproductive medicine faces due to the multiple factors that can cause them. This requires a multidisciplinary approach for the diagnosis and treatment of these couples. There is a percentage of RPL and RIF patients for which the diagnosis and prognosis from current knowledge is limited. Therefore, the identification of genetic variants that predispose to RIF and RPL opens new areas of research to improve the reproductive success of these patients.

In this paper, we have studied the prevalence of certain genetic polymorphisms in RPL and RIF patients with the aim of identifying potential risk factors. To our knowledge, it is the first study where a genetic profile including different genetic variants for RIF and RPL have been evaluated in the same patient. Our results reveal that the R72P polymorphism in the *p53* gene, the GG in the *IL-11* and the E4 isoform in the *APOE* gene are more prevalent in RPL patients. Regarding RIF patients, in addition to the *p53* polymorphism, we observed a different distribution in the -1154 polymorphism -AA/AG of VEGF gene compared with controls. These results are relevant for the diagnosis of RPL and RIF patients, but they also show that although these two pathologies share some common aspects, there are other totally different factors that make them be treated as separate entities.

Embryo implantation is a complex process that requires a perfect balance between completely opposed mechanisms as apoptotic and cell proliferation processes, as well as inflammatory and anti-inflammatory processes that allow a correct maternal-fetal immunological tolerance and adequate tissue invasion and angiogenesis. In these processes, adequate expression levels of the proteins involved in the different mechanisms, as well as functional proteins, are required, so any genetic variant may alter the embryo implantation and fetal development.

Our data suggest an association between proline variant in position 72 of p53 protein and RPL and RIF. The proline codon at position 72 results in a protein that induces high levels of G1 arrest and therefore a decrease in cell proliferation (Hu, Feng, & Levine, 2009), yielding inadequate trophoblastic growth resulting in RPL or RIF. Likewise, *p53* regulates the LIF expression which is important for proper endometrial receptivity. The proline variant in position 72 has a lower ability to induce the expression of LIF, causing lower implantation rates (Kang et al., 2009).

The correlation observed in our patients with the APOE E4 gene variant agrees with previous results reported in different meta-analysis (J. Li et al., 2014). The increased thrombotic risk produced by the E4 variant as well as a reduction of inflammatory cytokines could explain the aetiology of the relationship between the E4 variant of the *APOE* gene and RPL (Korkmazer, Ustunyurt, Tekin, & Cilingir, 2013). Cytokines, as well as angiogenic factors, play an

important role in modulating the maternal-fetal immune tolerance and the establishment of the vascular network. The genetic variants responsible for modifying the expression of the genes encoding these proteins will affect their own levels and therefore the mechanisms associated with them. In this sense, the *IL-11* polymorphism (Saxena et al., 2015) as well as VEGF polymorphism (Magdoud et al., 2012), would produce insufficient levels of proteins that would cause difficulties for embryo implantation and/or the establishment of pregnancy.

Our results suggest patients carrying variants in *p53* and *VEGF* would be at risk of RIF, and those carrying variants in *APOE* gene would suffer from RPL. To corroborate these results, prospective cohort studies with higher sample size are needed. In conclusion, this study has identified genetic variants associated with RIF and RPL. This information, together with additional markers, could allow the development of diagnostic tests to detect the risk of RIF and RPL and set the prognosis of the cycle before starting the treatment.

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Disclosure statement

No potential conflict of interest was reported by the authors.

References

Cha, J., Sun, X., & Dey, S.K. (2012). Mechanisms of implantation: Strategies for successful pregnancy. *Nature Medicine*, *18*, 1754–1767. doi: 10.1038/nm.3012.

Coughlan, C., Ledger, W., Wang, Q., Liu, F., Demirol, A., Gurgan, T., … Li, T.C. (2014). Recurrent implantation failure: Definition and management. *Reproductive Biomedicine Online*, *28*, 14–38. doi: 10.1016/j.rbmo.2013.08.011.

Hu, W., Feng, Z., Teresky, A.K., & Levine, A.J. (2007). *p53* regulates maternal reproduction through LIF. *Nature*, *450*, 721–724. doi: 10.1038/nature05993.

Hu, W., Feng, Z., & Levine, A.J. (2009). The regulation of human reproduction by *p53* and its pathway. *Cell Cycle*, *8*, 3621–3622. doi: 10.4161/cc.8.22.9938.

Kang, H.-J., Feng, Z., Sun, Y., Atwal, G., Murphy, M.E., Rebbeck, T.R., … Hu, W. (2009). Single-nucleotide polymorphisms in the p53 pathway regulate fertility in humans. *Proceedings of the National Academy of Sciences USA*, *106*, 9761–9766. doi: 10.1073/pnas.0904280106.

Kolte, A.M., van Oppenraaij, R.H., Quenby, S., Farquharson, R.G., Stephenson, M., Goddijn, M., & … Eshre, S.I.G.E.P. (2014). Non-visualized pregnancy losses are prognostically important for unexplained recurrent miscarriage. *Human Reproduction*, *29*, 931–937. doi: 10.1093/humrep/deu042.

Korkmazer, E., Ustunyurt, E., Tekin, B., & Cilingir, O. (2013). Study on potential role of apolipoprotein E in recurrent pregnancy loss. *Experimental and Therapeutic Medicine*, *5*, 1408–1410. doi: 10.3892/etm.2013.997.

Li, J., Chen, Y., Wu, H., & Li, L. (2014). Apolipoprotein E (*Apo E*) gene polymorphisms and recurrent pregnancy loss: A meta-analysis. *Journal of Assisted Reproduction and Genetics*, *31*, 139–148. doi: 10.1007/s10815-013-0128-5.

Lledo, B., Turienzo, A., Ortiz, J.A., Morales, R., Ten, J., Llacer, J., & Bernabeu, R. (2014). Negative effect of P72 polymorphism on *p53* gene in IVF outcome in patients with repeated implantation failure and pregnancy loss. *Journal of Assisted Reproduction and Genetics*, *31*, 169–172. doi: 10.1007/s10815-013-0147-2.

Magdoud, K., Dendana, M., Herbepin, V., Hizem, S., Ben Jazia, K., Messaoudi, S., … Mahjoub, T. (2012). Identification of specific vascular endothelial growth factor susceptible and protective haplotypes associated with recurrent spontaneous miscarriages. *Human Reproduction*, *27*, 1536–1541. doi: 10.1093/humrep/des033.

Mahley, R.W., Weisgraber, K.H., & Huang, Y. (2009). Apolipoprotein E: Structure determines function, from atherosclerosis to Alzheimer's disease to AIDS. *Journal of Lipid Research*, *50*, S183–S188. doi: 10.1194/jlr.R800069-JLR200.

Pim, D., & Banks, L. (2004). p53 polymorphic variants at codon 72 exert different effects on cell cycle progression. *International Journal of Cancer*, *108*, 196–199. doi: 10.1002/ijc.11548.

Rai, R., & Regan, L. (2006). Recurrent miscarriage. *Lancet*, *368*, 601–611. doi: 10.1016/S0140-6736(06)69204-0.

Rowe, A.J., Wulff, C., & Fraser, H.M. (2003). Localization of mRNA for vascular endothelial growth factor (VEGF), angiopoietins and their receptors during the peri-implantation period and early pregnancy in marmosets (*Callithrix jacchus*). *Reproduction*, *126*, 227–238. doi: 10.1530/rep.0.1260227.

Savion, S., Lepsky, E., Orenstein, H., Carp, H., Shepshelovich, J., Torchinsky, A., … Toder, V. (2002). Apoptosis in the uterus of mice with pregnancy loss. *American Journal of Reproductive Immunology*, *47*, 118–127. doi: 10.1034/j.1600-0897.2002.1o027.x.

Saxena, R., Bjonnes, A.C., Georgopoulos, N.A., Koika, V., Panidis, D., & Welt, C.K. (2015). Gene variants associated with age at menopause are also associated with polycystic ovary syndrome, gonadotrophins and ovarian volume. *Human Reproduction*, *30*, 1697–1703. doi: 10.1093/humrep/dev110.

Shinohara, Y., Ezura, Y., Iwasaki, H., Nakazawa, I., Ishida, R., Kodaira, M., … Emi, M. (2001). Linkage disequilibrium and haplotype analysis among ten single-nucleotide polymorphisms of interleukin 11 identified by sequencing of the gene. *Journal of Human Genetics*, *46*, 494–497. doi: 10.1007/s100380170052.

Simón, C., Dominguez, F., Remohí, J., & Pellicer, A. (2001). Embryo effects in human implantation: Embryonic regulation of endometrial molecules in human implantation. *Annals of the New York Academy of Sciences*, *943*, 1–16. doi: 10.1111/j.1749-6632.2001.tb03785.x.

Smith, S.K. (2000). Angiogenesis and implantation. *Human Reproduction*, *15*(Suppl. 6), 59–66. https://www.ncbi.nlm.nih.gov/pubmed/11261484.

Vuorela, P., Carpén, O., Tulppala, M., & Halmesmäki, E. (2000). VEGF, its receptors and the tie receptors in recurrent miscarriage. *Molecular Human Reproduction*, *6*, 276–282. doi: 10.1093/molehr/6.3.276.

Wang, W.J., Hao, C.F., & Lin, Q.D. (2011). Dysregulation of macrophage activation by decidual regulatory T cells in unexplained recurrent miscarriage patients. *Journal of Reproductive Immunology*, *92*, 97–102. doi: 10.1016/j.jri.2011.08.004.

Watson, C.J., Webb, N.J., Bottomley, M.J., & Brenchley, P.E. (2000). Identification of polymorphisms within the vascular endothelial growth factor (*VEGF*) gene: Correlation with variation in VEGF protein production. *Cytokine*, *12*, 1232–1235. doi: 10.1006/cyto.2000.0692.